

Minireview

Molecular insights on the biosynthesis of antitumour compounds by actinomycetes

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Summary

Natural products are traditionally the main source of drug leads. In particular, many antitumour compounds are either natural products or derived from them. However, the search for novel antitumour drugs active against untreatable tumours, with fewer side-effects or with enhanced therapeutic efficiency, is a priority goal in cancer chemotherapy. Microorganisms, particularly actinomycetes, are prolific producers of bioactive compounds, including antitumour drugs, produced as secondary metabolites. Structural genes involved in the biosynthesis of such compounds are normally clustered together with resistance and regulatory genes, which facilitates the isolation of the gene cluster. The characterization of these clusters has represented, during the last 25 years, a great source of genes for the generation of novel derivatives by using combinatorial biosynthesis approaches: gene inactivation, gene expression, heterologous expression of the clusters or mutasynthesis. In addition, these techniques have been also applied to improve the production yields of natural and novel antitumour compounds. In this review we focus on some representative antitumour compounds produced by actinomycetes covering the genetic approaches used to isolate and validate their biosynthesis gene clusters, which finally led to generating novel derivatives and to improving the production yields.

Introduction

The production of bioactive compounds (antibiotic, antifungal, antitumour, immunosuppressant, antiviral or

antiparasitic agents) from living organisms is mainly supported by microorganisms. From them the most prolific producers are fungi, cyanobacteria, myxobacteria and actinobacteria, mainly actinomycetes. About half of all bioactive microbial metabolites discovered to date are produced by actinomycetes, with the genus *Streptomyces* as the primary bioactive metabolite-producing organism exploited by the pharmaceutical industry (Bérdy, 2005; Newman and Cragg, 2007; Butler, 2008; Olano *et al.*, 2009a). This has stimulated for half a century the study of actinomycetes at all levels: taxonomy, genetics and physiology.

Actinomycetes have large genomes (five to nine megabases) and use 5–10% of their DNA for the production of secondary metabolites (Baltz, 2008); a good number of them are antitumour drugs (Figs 1 and 2). These belong to different structural groups: polyketides such as anthracyclines daunomycin and doxorubicin, both promoting DNA cleavage mediated by DNA topoisomerase II, or aureolic acids mithramycin and chromomycin A₃ that cause DNA-dependent inhibition of RNA synthesis and inhibit topoisomerase I causing DNA cleavage; non-ribosomal peptides such as glycopeptide actinomycin D, which inhibits DNA-directed RNA synthesis by binding to double stranded DNA; mixed polyketide/non-ribosomal peptides as glycopeptide bleomycin, which inhibits DNA synthesis through a metal-dependent oxidative cleavage of DNA; heterocyclic quinones as mitomycin C that cause intra- and inter-strand DNA cross-linking leading to selective inhibition of DNA synthesis; indolocarbazoles such as protein kinases inhibitor staurosporine and topoisomerase I-dependent DNA-damaging agent rebeccamycin, etc. However, even considering the enormous number of natural products discovered to date, there is still a need for generating novel drugs to be used in cancer chemotherapy due to the rapid development of resistance to multiple chemotherapeutic drugs and the high toxicity usually associated with this type of drugs and their undesirable side-effects. In addition, there is a demand for novel antitumour drugs active against untreatable tumours, with fewer side-effects or with greater therapeutic efficiency. To this respect, enormous efforts are under way to discover new drugs from actinomycetes by

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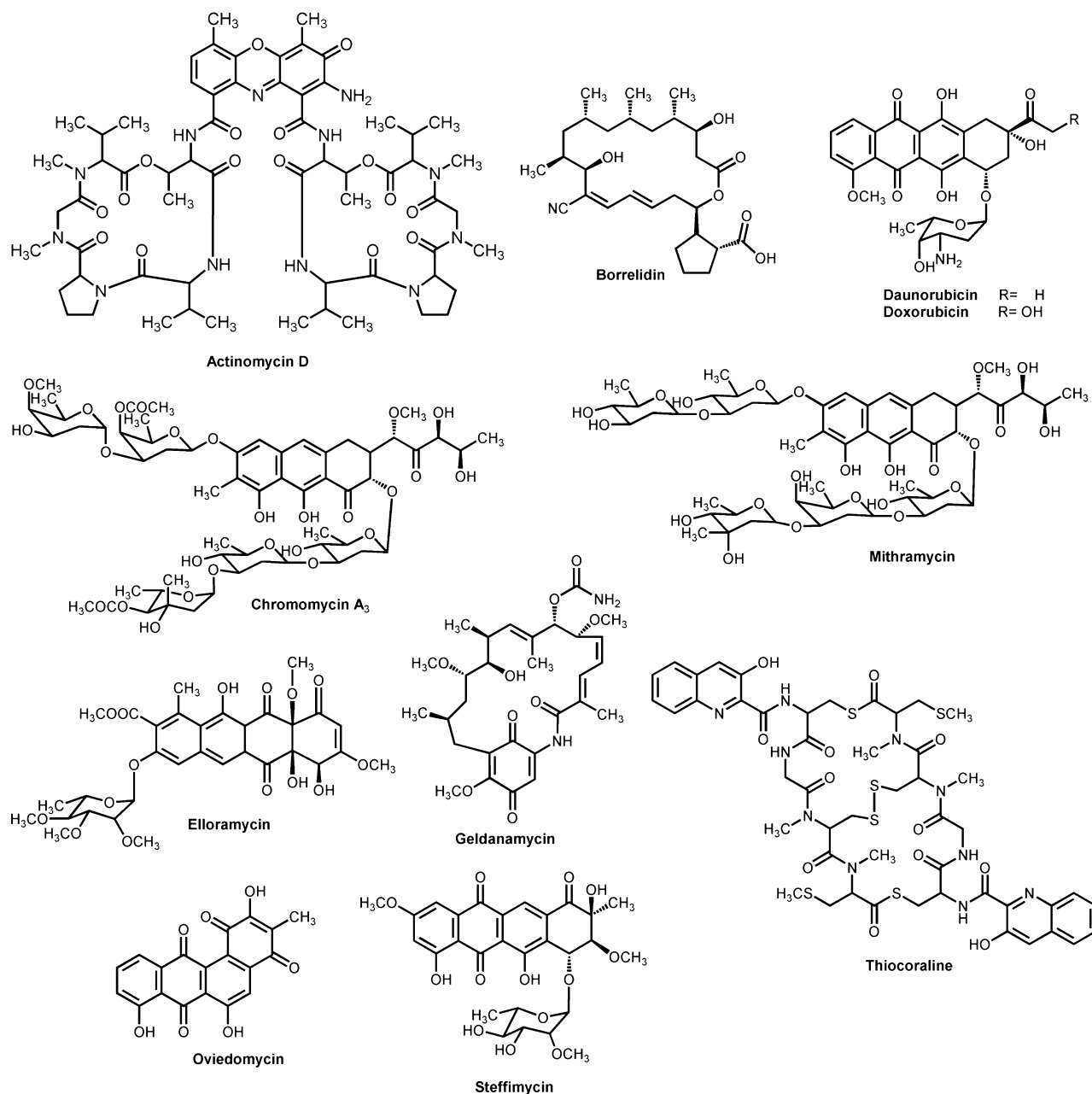


Fig. 1. Structures of some polyketide and non-ribosomal peptide antitumour compounds produced by actinomycetes.

searching uncommon actinomycetes in special ecological niches, mining genomes for cryptic pathways, and applying combinatorial biosynthesis to generate novel secondary metabolites related to existing pharmacophores (Baltz, 2008).

The utilization of combinatorial biosynthesis has become an important alternative approach for developing novel derivatives and for improving production yields of known antitumour compounds (Olano *et al.*, 2008a; 2010). The strategies used in combinatorial biosynthesis (Fig. 3) involve gene inactivation to generate mutant strains that

might accumulate novel products, expression of genes or combination of genes in different heterologous hosts including mutant strains or producers of related compounds, and mutasynthesis (Méndez and Salas, 2003; Floss, 2006; Menzella and Reeves, 2007; Kennedy, 2008; Salas and Méndez, 2009). Obviously, the application of this technology requires the isolation and characterization of the gene cluster involved in the biosynthesis of a particular antitumour compound to unravel its biosynthetic pathway.

A large number of antitumour biosynthesis gene clusters have been characterized from producing

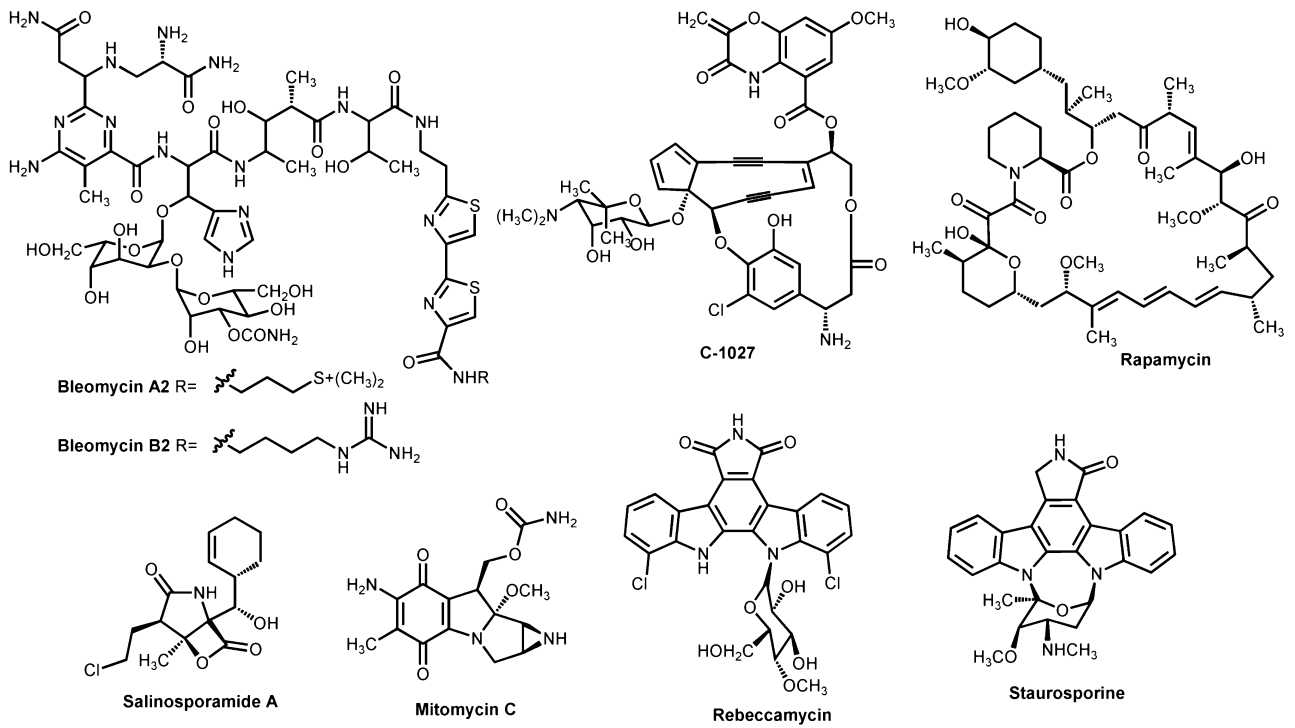


Fig. 2. Structures of some mixed polyketide/non-ribosomal peptide, indolocarbazole and heterocyclic quinone antitumour compounds produced by actinomycetes.

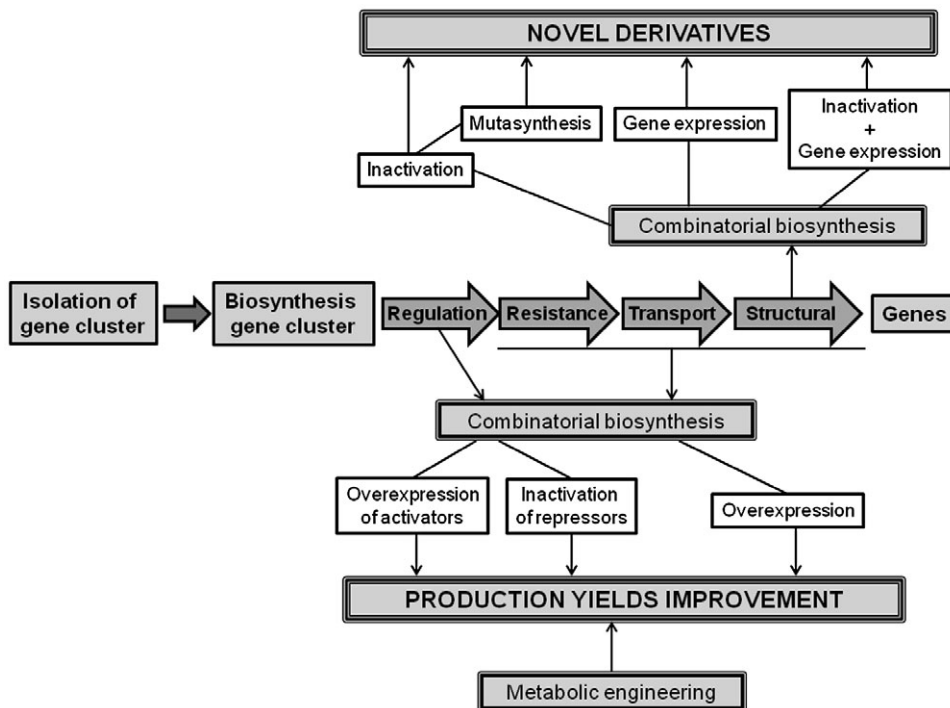


Fig. 3. Strategies used for generating novel antitumour derivatives and improving production yields by combinatorial biosynthesis.

actinomycetes during the last 25 years (Olano *et al.*, 2009b). In this review, we focus on the approaches used for the isolation and validation of secondary metabolite biosynthesis gene clusters from antitumour-producing actinomycetes (Table 1). We will summarize the genetic information that can be gathered from those clusters. We will also address the subsequent use of this information for the development of novel derivatives through combinatorial biosynthesis as well as for the improvement of production titres.

Isolation and characterization of gene clusters

Strategies used for the isolation of the gene clusters covered by this review do not differ from those used for the identification of clusters involved in the biosynthesis of non-antitumour secondary metabolites. Furthermore, in most cases more than one strategy has been applied for the isolation of a single cluster. One of these approaches is based in the fact that structural biosynthetic genes are generally clustered all together with resistance and regulatory genes. This has facilitated the identification of several biosynthetic clusters by cloning resistance genes in an appropriate sensitive host as in the case of these involved in the biosynthesis of bleomycin (Sugiyama *et al.*, 1994a) or mitomycin C (August *et al.*, 1994). In addition, it has been observed that genes from biosynthetically related pathways show cross-hybridization each other. This has prompted the homology-based approach as the most widely method applied for the identification of biosynthesis gene clusters (Table 1). Heterologous DNA probes were used for the isolation of several clusters as for those involved in the biosynthesis of daunorubicin/doxorubicin (Otten *et al.*, 1990), anthracycline-like elloramycin (Decker *et al.*, 1995), serine-threonine kinase mTOR inhibitor rapamycin (Schwecke *et al.*, 1995), mithramycin (Lombó *et al.*, 1996), angucycline oviedomycin (Méndez *et al.*, 2002), chromomycin A₃ (Menéndez *et al.*, 2004a) and angiogenesis inhibitor borrelidin (Olano *et al.*, 2004a). Homologous probes, previously identified or generated by using degenerated oligonucleotides, have also been used for the isolation of actinomycin D (Schauwecker *et al.*, 1998), the DNA-cleaving agent enediyne C-1027 (Liu and Shen, 2000), rebeccamycin (Sánchez *et al.*, 2002), staurosporine (Onaka *et al.*, 2002), the chaperone Hsp90 inhibitor geldanamycin (Rascher *et al.*, 2003) and the DNA polymerase α inhibitor depsipeptide thiocoraline (Lombó *et al.*, 2006) biosynthesis gene clusters. On the other hand, the method for isolating anthracycline steffimycin gene cluster was the direct inactivation of a gene involved in its biosynthesis using a PCR fragment that was amplified from steffimycin producer chromosomal DNA and used to generate a non-producing mutant (Gullón *et al.*, 2006). Finally, genome sequencing

and mining is now a consolidated tool to identify novel biosynthesis gene clusters. Sequencing of *Salinispora tropica* CNB-440 genome was used to locate the 20S proteasome inhibitor salinosporamide A gene cluster (Udway *et al.*, 2007).

Once a gene cluster has been isolated, it is necessary to confirm its involvement in the biosynthesis of the bioactive compound. The validation process is especially important for clusters identified by homology-based approaches and genome mining and can be achieved by several methods that include gene inactivation or the heterologous expression of the cluster. The main objective of gene inactivation is the generation of non-producing mutants as a proof of the involvement of the cluster in the biosynthesis of the compound under study. The expression of gene clusters involves the utilization of genetic tools to mobilize large DNA fragments into a heterologous host to produce the pursued compound. It takes advantage of the availability of a number of *Streptomyces* strains [mainly *S. coelicolor* A3(2), *S. lividans* 66, *S. venezuelae* YJ003 or *S. albus* J1074] to be used as hosts due to the deep knowledge accumulated about their physiology and growth conditions. In addition, some of these strains have been engineered to enhance the expression of heterologous gene clusters. *Streptomyces lividans* was the host used for the expression of elloramycin (Decker *et al.*, 1995; Ramos *et al.*, 2008), staurosporine (Onaka *et al.*, 2002), oviedomycin (Méndez *et al.*, 2002) and thiocoraline (Lombó *et al.*, 2006) gene clusters. *Streptomyces albus* was used for the production of oviedomycin (Méndez *et al.*, 2002), rebeccamycin (Sánchez *et al.*, 2002), staurosporine (Salas *et al.*, 2005), thiocoraline (Lombó *et al.*, 2006) and steffimycin (Gullón *et al.*, 2006). Furthermore, low levels of rebeccamycin production were also achieved in *Escherichia coli* (Hyun *et al.*, 2003). In addition, gene inactivation and heterologous expression of the cluster are also used to establish the cluster boundaries.

Structural genes

Most of the genes in a cluster code for enzymes involved in the biosynthetic process, including the biosynthesis of precursor units, assembling of precursors, generation of other structural elements and tailoring modifications by attachment of the different structural elements or introduction of chemical functionalities (Fig. 4).

Biosynthesis of precursors. Some secondary metabolites are built using, as scaffold elements, precursors not supplied by primary metabolism or, when supplied, an extra amount is required at due time for the production of the secondary metabolite. These elements serve as starter units for the initiation of the pathway or as extender units.

Table 1. Some characteristics of gene clusters involved in the biosynthesis of different antitumour compounds by actinomycetes.

Antitumour	Organism	Type	Strategy for cloning	Expressed in	Size (kb) ^a	Genes in the cluster				
						Genes	Reg.	R/T	Struc.	Other
Actinomycin D	<i>S. chrysomallus</i>	NPR	Homology	NE	50	28	4	8	10	6
Bleomycin	<i>S. verticillus</i>	PK/NRP	Resistance	NE	62	28	1	3	16	8
Borrelidin	<i>S. parvulus</i>	PK (I)	Homology	NE	52	20	–	1	19	–
C-1027	<i>S. globisporus</i>	PK/NRP	Homology	NE	73	56	3	4	34	15
Chromomycin A ₃	<i>S. griseus</i>	PK (II)	Homology	NE	43	36	2	3	29	2
Daunorubicin	<i>S. peuceitius</i>	PK (II)	Homology	NE	39	37	3	4	28	2
Doxorubicin										
Elloramycin	<i>S. olivaceus</i>	PK (II)	Homology	<i>S. lividans</i>	17	17	–	1	15	1
Geidanamycin	<i>S. hygrosopicus</i>	PK (I)	Homology	NE	70	23	4	1	15	3
Mithramycin	<i>S. argillaceus</i>	PK (II)	Homology	NE	40	34	2	3	25	4
Mitomycin C	<i>S. lavendulae</i>	HQ	Resistance	NE	55	47	2	4	19	22
Oviedomycin	<i>S. antibioticus</i>	PK (II)	Homology	<i>S. albus</i>	20	17	2	1	13	1
Rapamycin				<i>S. lividans</i>						
Rebeccamycin	<i>S. hygrosopicus</i>	PK/NRP	Homology	NE	107	26	5	3	12	6
	<i>L. aerocolonigenes</i>	IC	Homology	<i>S. albus</i>	18	11	1	2	8	–
				<i>E. coli</i>						
Salinosporamide A	<i>Sa. tropica</i>	PK/NRP	Genome mining	NE	41	30	3	–	17	10
Staurosporine	<i>S. longisporoflavus</i>	IC	Homology	<i>S. lividans</i>	23	15	1	–	14	–
				<i>S. albus</i>						
Steffimycin	<i>S. steffisburgensis</i>	PK (II)	Inactivation	<i>S. albus</i>	27	24	3	2	16	3
Thiocoraline	<i>Micromonospora</i> sp. ML1	NPR	Homology	<i>S. albus</i>	53	26	5	3	18	–

a. Approximate estimation of the size of the gene cluster.

L., *Lechevaleria*; *S.*, *Streptomyces*; *Sa.*, *Salinispora*; PK, polyketide; PK (I), polyketide type I; PK (II), polyketide type II; PK/NRP, mixed polyketide and non-ribosomal peptide; NRP, non-ribosomal peptide; HQ, heterocyclic quinone; IC, indolocarbazole; NE, no heterologous expression of the cluster has been reported; *E.*, *Escherichia*; Reg., Regulatory genes; R/T, Resistance and transport genes; Struc., Structural genes.



Fig. 4. Genetic organization of antitumor biosynthesis gene clusters summarized in Table 1.

Genes belonging to this group are found mainly in polyketide and non-ribosomal peptide biosynthetic pathways (Fig. 4).

Nine genes are present in the borrelidin cluster for the biosynthesis of cyclopentane dicarboxylic acid which is used as starter unit by a type I PKS. This precursor is proposed to derive from tyrosine catabolism through 5-oxo-pent-3-ene-1,2,5-tricarboxylic acid (Olano *et al.*, 2004a). In other cases, such as in the geldanamycin pathway, only one gene (*gdmO*) of those required for the biosynthesis of the type I PKS starter unit 3-amino-5-hydroxybenzoic acid (AHBA) is present in the cluster (Rascher *et al.*, 2003). The remaining genes are located elsewhere in the chromosome as it happens in the ansamitocin biosynthesis gene cluster (Yu *et al.*, 2002). However, in the case of mitomycin C, all genes required

for the biosynthesis of AHBA are located in the cluster (Mao *et al.*, 1999a). The geldanamycin gene cluster contains, in addition, five genes proposed to be involved in the biosynthesis of PKS extender unit methoxy-malonyl-CoA (Rascher *et al.*, 2003). In the case of type II PKS systems, there is no need for a special starter unit since the biosynthesis of these polyketides usually starts with an acetyl-CoA unit. However, an extra supply of the extender malonyl-CoA units is occasionally required. This might be the case of the oviedomycin pathway since genes encoding the three subunits of an acetyl-CoA carboxylase involved in the production of malonyl-CoA are present in the cluster (Lombó *et al.*, 2004a; D. Zabala, pers. comm.).

NRPS systems for the biosynthesis of peptide or mixed polyketide-peptide compounds occasionally require

special starter units synthesized by dedicated enzymes whose genes are present in the cluster. Thiocoraline and actinomycin D starter units, 3-hydroxy-quinaldic acid and 4-methyl-3-hydroxyanthranilic acid, respectively, derive from tryptophan. Both are produced through six enzymatic steps, the corresponding genes being located in their respective gene clusters (Lombó *et al.*, 2006; Keller *et al.*, 2010). Rapamycin PKS uses a shikimate-derived 4,5-dihydroxycyclohex-1-enecarboxylic acid as starter unit, while its NRPS incorporates L-lysine-derived L-pipecolic acid. Two genes of this cluster are involved in the biosynthesis of these precursor units: pteridine-dependent dioxygenase *rapK* and L-lysine cyclodeaminase *rapL* (Molnár *et al.*, 1996; Gregory *et al.*, 2005). In the case of salinosporamide A, there are 13 genes involved in the biosynthesis of two precursors, substrates of the NRPS and PKS systems. On one hand, eight genes are involved in the production of 3-(cyclohex-2-enyl)alanine derived from prephenate, probably originated in the shikimic acid pathway. On the other hand, five genes are required for the production of chloroethylmalonyl-CoA, derived from S-adenosylmethionine (SAM) (Beer and Moore, 2007; Gulder and Moore, 2009).

Inactivation of genes involved in biosynthesis of starter units generally leads to non-producing mutants and has been used for abrogating the production of mitomycin C (Mao *et al.*, 1999b), borrelidin (Olano *et al.*, 2004a), geldanamycin (Gregory *et al.*, 2005) or salinosporamide A (Eustáquio and Moore, 2008). In addition, some of these mutant strains have been used afterwards for mutasynthesis approaches in the generation of novel derivatives.

Biosynthesis of the structural core. A good number (in some cases up to 50%) of genes in each cluster are involved in the assembling of precursor units leading to the formation of the structural core of compounds. PKS and NRPS encoding genes are some examples. The carbon skeleton of polyketides are synthesized 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 harbouring a set of distinct activities (ketosynthase, acyltransferase and acyl-carrier protein domains) 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 enoylreductase) generated during the condensation process, and a thioesterase domain in the last module for polyketide release and cyclization (Fischbach and Walsh, 2006). Most type I PKSs act non-iteratively, thus maintaining the co-linearity rule of 'one extension cycle—one PKS module'. This is the case of PKSs involved in geldanamycin

(Rascher *et al.*, 2003) or rapamycin (Schwecke *et al.*, 1995) biosynthesis. Other type I PKSs are iterative, as those involved in enediyne C-1027 biosynthesis, where a single module performs all cycles of polyketide chain elongation (Liu *et al.*, 2002). There are also type I PKSs that contain both type of modules: non-iterative and iterative, such as borrelidin PKS (Olano *et al.*, 2003). On the other hand, type II PKSs are multienzyme complexes composed by 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 (Shen, 2003). This type of PKS is involved in the biosynthesis of aromatic polyketides such as daunorubicin/doxorubicin, tetracycline, mithramycin, chromomycin A₃, oviedomycin, steffimycin or elloramycin.

NRPSs synthesize non-ribosomal peptides using as monomeric building blocks proteinogenic and non-proteinogenic amino acids, including D-amino acids, and other carboxylic acids. The NRPS assembly lines follow the same chemical logic as PKSs for chain elongation with several domains (in this case adenylation, condensation and peptidyl-carrier protein) organized into modules. Additional domains with epimerization, methyltransferase, reductase or oxidase activity might be also present. These multimodular enzymes are required for the biosynthesis of several antitumour compounds such as thiocoraline (Lombó *et al.*, 2006), actinomycin D (Keller *et al.*, 2010) and mixed polyketide-peptide compounds such as rapamycin (Schwecke *et al.*, 1995), bleomycin (Shen *et al.*, 2002) or salinosporamide A (Udwary *et al.*, 2007). The presence of type I PKSs and/or NRPSs coding genes determine the large genetic extension of the biosynthesis clusters that harbour these systems. Considering that a regular PKS or NRPS module is encoded by approximately 5 kb of DNA, in general the length of the cluster gets bigger as the number of modules grows (Table 1 and Fig. 4).

Heterocyclic quinone mitomycin C and indolocarbazoles staurosporine and rebeccamycin use other types of enzymes to assemble their structural cores. Mitomycin C pathway requires a glycosyltransferase, MitB, and an acyl-CoA ligase, MitE, to condense AHBA and D-glucosamine to generate the mitosane core (Mao *et al.*, 1999a). On the other hand, indolocarbazole cores arcyri-aflavin (the non-chlorinated aglycone for rebeccamycin) and K252c (for staurosporine) are synthesized through the condensation of L-tryptophan moieties after an oxidative deamination and several oxidation and decarboxylation steps (Sánchez *et al.*, 2002; Salas *et al.*, 2005).

Inactivation of genes involved in the assembling of precursor units also leads to non-producing mutants. This has been shown by inactivating C-1027 (Liu *et al.*, 2002), mithramycin (Lombó *et al.*, 1996), oviedomycin (Lombó

et al., 2004a) and borrelidin (Olano *et al.*, 2004a) PKS-encoding genes, thiocoraline (Lombó *et al.*, 2006) NRPS-encoding genes, and mitomycin C *mitB* and *mitE* (Mao *et al.*, 1999a).

Biosynthesis of deoxysugars and other structural elements. Attached to the structural core there are usually other structural elements that, in most cases, correspond to 6-deoxyhexoses derived from D-glucose-1-phosphate. The sugar moieties contribute to the structural biodiversity of compounds and participate in the interaction between the drug and the cellular target (Salas and Méndez, 2007; Méndez *et al.*, 2008). These structures can be composed by monosaccharides (C-10127, daunorubicin/doxorubicin, elloramycin, steffimycin and staurosporine), disaccharides (bleomycin, mithramycin and chromomycin A₃), trisaccharides (mithramycin and chromomycin A₃) and oligosaccharides (landomycin A) (Figs 1 and 2).

The absence of some or all genes involved in the biosynthesis of the sugar from the cluster is the exception rather than the rule. L-rhamnose-containing clusters constitute such an exception. The gene clusters for steffimycin (Gullón *et al.*, 2006) and elloramycin (Ramos *et al.*, 2008) do not contain any L-rhamnose biosynthesis genes. This is a common feature to other clusters containing L-rhamnose in the structures of the bioactive compounds and involved in the biosynthesis of polyketides as spinosad (Waldron *et al.*, 2001) and aranciamycin (Luzhetskyy *et al.*, 2007) or the diterpenoid brasilicardin A (Hayashi *et al.*, 2008). In two cases, spinosyn (Madduri *et al.*, 2001) and elloramycin (Ramos *et al.*, 2008), a rhamnose biosynthesis gene cluster has been identified in other chromosomal location and most probably they use L-rhamnose synthesized by the cell for other cellular functions or structures. The involvement of this rhamnose gene cluster in elloramycin biosynthesis was demonstrated by inactivation of the *rhaB* gene, encoding a 4,6-dehydratase, thus generating a non-producer mutant that accumulated the elloramycin aglycon 8-demethyl-tetracenomycin C (Ramos *et al.*, 2008). In addition, a *Streptomyces peucetius* rhamnose biosynthesis gene cluster has been recently found to be involved in daunorubicin/doxorubicin biosynthesis providing TDP-4-keto-6-deoxy-D-glucose as precursor for the biosynthesis of daunosamine (Singh *et al.*, 2010). Inactivation of genes involved in the production of deoxysugars from daunorubicin and mithramycin clusters has also led to identify non-glycosylated compounds (Lombó *et al.*, 1997; Otten *et al.*, 1997) and derivatives with different patterns of glycosylation (Remsing *et al.*, 2002).

In the case of enediyne C-1025, in addition to a deoxyamino sugar, genes for the biosynthesis of two further structural elements are present in the cluster.

These elements are the β-amino acid (S)-3-chloro-4,5-dihydroxy-β-phenylalanine (derived from L-tyrosine) and benzoxazolinone (derived from chorismate). The biosynthesis of the β-amino acid moiety involves, in addition to other enzymes, different NRPS free standing domains such as an adenylation (SgcC1) and a peptidyl carrier protein (SgcC2) (Liu *et al.*, 2002; Van Lanen *et al.*, 2005; 2008). The inactivation of *sgcA* or *sgcC1* involved in early steps in the biosynthesis of the deoxyamino or β-amino acid moieties led to abolish production of the final compound and no intermediates or derivatives were accumulated (Liu and Shen, 2000; Liu *et al.*, 2002). On the other hand, inactivation of *sgcC*, encoding a phenol hydroxylase catalysing a late step for the biosynthesis of the β-amino acid moiety, led to a new C-1027 derivative (Liu *et al.*, 2002; Van Lanen *et al.*, 2005). These results suggest that C-1027 structural elements are required to stabilize the enediyne polyketide core. The lack of any of these structural elements, including the benzoxazolinone moiety (as will be described in the next section), determine the complete absence of any enediyne derivative.

Tailoring modifications. The introduction of additional chemical modifications into the structural core is performed by tailoring enzymes whose genes are found within the biosynthesis gene cluster. Group transferases such as glycosyl-, methyl-, acyl-, prenyl-, carbamoyl- or aminotransferases belong to this class. Attachment of sugars is carried out by glycosyltransferases, which catalyses the transfer of the sugar from a NDP-activated sugar donor to an acceptor molecule. In most of the cases, attachment of the sugars to the aglycone occurs through O-glycosidic linkages, but C- and N-glycosidic bonds also takes place. Methyltransferases generally use SAM as methyl donor to methylate functional groups either at the compound core or at other structural moieties. Acyltransferases are involved in the attachment of either short (C2–C6) or medium (C8–C12) acyl chains to both amine and alcohol moieties. The acyl transfer process is often one of the latest biosynthetic events representing a key step in generating the final biologically active molecule and, in some cases, it is an important event for self-protection of the producing organism. Prenyltransferases catalyse the transfer of prenyl moieties derived from allylic isoprenyl diphosphates (PP) such as dimethylallyl-PP (C5), geranyl-PP (C10) or farnesyl-PP (C15). Carbamoyltransferases catalyse the attachment of carbamoyl moieties, and aminotransferases incorporate amino groups. On the other hand, enzymes that normally participate in the biosynthesis of structural cores such NRPS can occasionally act as group transferases. This is the case of SgcC5, a NRPS free standing condensation domain, which incorporates β-amino acid (S)-3-chloro-4,5-dihydroxy-β-phenylalanine to the C-1027 polyketide core (Liu *et al.*, 2002).

Other tailoring modifications introduce oxygen-containing functionalities, such as hydroxy, epoxide, aldehyde and keto groups, or modify these moieties by addition or removal of hydrogen atoms. These reactions, catalysed by oxygenases, oxidases, peroxidases, reductases and dehydrogenases, can provide a base for additional modifications such as methyl- amino- or glycosyl-transfer. In addition, the introduction of chloride and bromide in secondary metabolites is frequently performed by halogenating enzymes known as flavin-dependent halogenases.

Tailoring modification genes have also been subjected to inactivation for validating gene clusters. This approach usually generates mutants that accumulate biosynthetic intermediates or novel derivatives. This is the case of inactivation of several group transferases such as glycosyltransferases, methyltransferases, acyltransferases, carbamoyltransferases, aminotransferases or halogenases involved in the biosynthesis of daunorubicin (Otten *et al.*, 1995a), mithramycin (Fernández *et al.*, 1998; Blanco *et al.*, 2000; Nur-e-Alam *et al.*, 2005), chromomycin A₃ (Menéndez *et al.*, 2004b; 2006), borrelidin (Olano *et al.*, 2004b), mitomycin C (Grüschow *et al.*, 2007; Sita-chitta *et al.*, 2007), C-1027 (Liu *et al.*, 2002), geldanamycin (Shin *et al.*, 2008), salinosporamide A (Eustáquio and Moore, 2008) and bleomycin (Galm *et al.*, 2008). However, the inactivation of acyltransferase *sgcD6* involved in the attachment of benzoxazolinolate moiety to C-1027 polyketide core abolished the production of either the final compound or any other related compound (Liu *et al.*, 2002). This, as was mentioned before, points to C-1027 structural elements, deoxyamino sugar, β-amino acid and benzoxazolinolate, to have a role as stabilizing units for the polyketide core integrity. Furthermore, several oxidoreductases have been subjected to inactivation for validating biosynthesis clusters, leading to novel compounds derived from mithramycin (Remsing *et al.*, 2003), chromomycin A₃ (Menéndez *et al.*, 2004a), borrelidin (Olano *et al.*, 2004b), salinosporamide (McGlinchey *et al.*, 2008) or geldanamycin (Shin *et al.*, 2008).

Regulatory genes

The expression of genes from biosynthesis clusters is normally controlled by pathway-specific regulatory genes. They can have either positive (activators) or negative (repressors) effects on gene expression. The number of putative pathway-specific regulatory genes in different clusters range from one in bleomycin, rebeccamycin or staurosporine pathways to five in the rapamycin or thio-coraline pathways (Table 1). However, in some pathways no regulatory genes have been identified, as it is the case of the borrelidin or elloramycin gene clusters.

Approximately half of the pathway-specific activators belong to three groups of transcriptional factors containing DNA binding helix–turn–helix (HTH) signatures: SARP- (seven cases), LuxR- (six cases) and TetR-family (six cases) regulators. The *Streptomyces* Antibiotic Regulatory Protein family (SARP) share sequence similarity with several members of the OmpR family of DNA-binding domains. In particular, regulatory elements of this family are mainly present in aromatic polyketide gene clusters such as those involved in the biosynthesis of daunorubicin (Stutzman-Engwall *et al.*, 1992), mithramycin (Lombó *et al.*, 1999), chromomycin A₃ (Menéndez *et al.*, 2004a) and steffimycin (Gullón *et al.*, 2006). OmpR- and LuxR-like regulators generally act as activators and TetR-family transcriptional regulators generally act as repressors (Ramos *et al.*, 2005), but there are examples of TetR-like positive regulators such as AtrA from *Streptomyces griseus* (Hirano *et al.*, 2008). Only two clusters (bleomycin and C-1027) do not contain any regulatory element of these families (Liu *et al.*, 2002; Shen *et al.*, 2002), while daunorubicin cluster contains one of each (Stutzman-Engwall *et al.*, 1992; Otten *et al.*, 1995b). The regulation of the daunorubicin/doxorubicin production has been studied in detail. Transcriptional regulator DnrO (BirA/TetR-like) binds specifically to the overlapping *dnrN/dnrO* promoter region activating the expression of response regulator DnrN that contains a LuxR-like HTH motif. Then, DnrN activates the transcription of *dnrI* whose product DnrI, a SARP regulator, activates the production of daunorubicin biosynthesis and resistance genes (Madduri and Hutchinson, 1995; Otten *et al.*, 1995b; 2000; Furuya and Hutchinson, 1996; Sheldon *et al.*, 2002). In addition, DnrO has been shown to repress its own expression, effect that could be relieved by the presence of rhodomycin D, one of the first glycosylated intermediates in the daunorubicin/doxorubicin pathway, and enhanced by the final products (Jiang and Hutchinson, 2006; Ajithkumar and Prasad, 2010; Srinivasan *et al.*, 2010). In most cases, inactivation of positive regulatory genes led to completely abolish the biosynthesis of the drug, the exception being *mtrY* of the mithramycin pathway: its inactivation considerably decreased (but did not abolish) mithramycin biosynthesis (Garcia-Bernardo *et al.*, 2000). On the other hand, inactivation of negative regulatory genes led to an increase in the production of the respective secondary metabolite. This has been shown for mitomycin C *mmcW* (Mao *et al.*, 1999a) and chromomycin A₃ *cmmRll* (Menéndez *et al.*, 2007). Some of these regulatory elements, both activators and repressors, might be specifically involved in the control of secondary metabolite resistance/transport systems since they are located next to these elements in several clusters. This is probably the case of regulatory elements *mmcW*, *mtrY* and *cmmRll* from mitomycin C (Mao *et al.*, 1999a), mithramycin (Garcia-Bernardo *et al.*,

2000) and chromomycin A₃ (Menéndez *et al.*, 2007) gene clusters respectively.

Resistance and transport genes

Most antitumour compounds produced by actinomycetes interact with DNA. Therefore, producer microorganisms have developed mechanisms to survive during the biosynthesis of the drug. These resistance mechanisms could be divided into two groups: resistance systems and transport systems dedicated to self-defence and/or extrusion of the final compound or intermediates. Most clusters (except those of salinosporamide A and staurosporine) contain elements of both groups, and only in the case of borrelidin cluster there is not transport system present.

The most extended resistance mechanism is a UvrA-like protein found in daunorubicin/doxorubicin (Lomovskaya *et al.*, 1996), mithramycin (Fernández *et al.*, 1996; Garcia-Bernardo *et al.*, 2000), C-1027 (Liu *et al.*, 2002), chromomycin A₃ (Menéndez *et al.*, 2004a; 2007) and thiocoraline (Lombó *et al.*, 2006) gene clusters. The UvrA protein is an *E. coli* UvrABC complex homologue involved in excision repair of UV-induced DNA lesions (Truglio *et al.*, 2006). This system has been shown to repair DNA damaged by the free oxygen radicals produced from the interaction of anthracyclines to DNA. The UvrA-like encoding genes have been inactivated in different gene clusters showing various effects. In the daunorubicin gene cluster the inactivation of *drrC* could only be obtained in a non-producing mutant (Lomovskaya *et al.*, 1996). Furthermore, the expression of *drrC* is induced by daunorubicin, and DrrC has been shown to bind DNA in the presence of ATP and daunorubicin (Furuya and Hutchinson, 1998). The inactivation of *mtrX* has no apparent effect on mithramycin resistance but the expression of this gene into heterologous host *S. albus* conferred certain level of resistance to mithramycin (Garcia-Bernardo *et al.*, 2000). The inactivation of *cmrX* led to a chromomycin A₃ sensitive strain that, in addition, showed a decreased production of the compound (Menéndez *et al.*, 2007). Moreover, this gene is required to confer a high level of chromomycin A₃ resistance in heterologous hosts. Oxidases have also been reported as resistance mechanisms to mitomycin C and anthracyclines. McrA was shown to be able of re-oxidizing activated mitomycin C before it can initiate the event leading to DNA cross-linking (August *et al.*, 1994; Johnson *et al.*, 1997). Two McrA homologues have been identified in the mitomycin gene cluster: MitR and MmcM. The inactivation of *mitR* and *mmcM* resulted in reduced mitomycin C production in the *mitR* mutant strain while *mmcM* mutant displayed a normal production level (Mao *et al.*, 1999a). By analogy to McrA, DrrD has been proposed to exert a re-oxidizing activity to counteract the effects of the reduced antitumour

drugs daunorubicin and doxorubicin in *S. peuceetius* (Hutchinson, 1997). Furthermore, the bleomycin resistance mechanism involves the inactivation of the drug by an *N*-acetyltransferase (BlmB) (Sugiyama *et al.*, 1994a,b). On the other hand, the expression of *borO*, encoding a borrelidin-resistant threonyl-tRNA synthetase, into *S. albus* was shown to confer resistance to the drug (Olano *et al.*, 2004a).

Regarding the transport/resistance systems, these can be grouped in two different classes. The first corresponds to membrane proteins that mediate the extrusion of secondary metabolites in a coupled exchange with protons or sodium ions. The second are ABC (ATP Binding Cassette) transporter systems that couple the transport of the antibiotic through the cell membrane to ATP hydrolysis. The most widely distributed transport systems among the clusters covered in this review are ABC transporters, which have been found as the unique transporters in daunorubicin/doxorubicin (Guilfoile and Hutchinson, 1991), rapamycin (Schwecke *et al.*, 1995), mithramycin (Fernández *et al.*, 1996), chromomycin A₃ (Menéndez *et al.*, 2004a), thiocoraline (Lombó *et al.*, 2006) and actinomycin D (Keller *et al.*, 2010) biosynthesis gene clusters. In the C-1027 cluster there are genes encoding a Na⁺/H⁺ efflux pump and a membrane protein in addition to the ATP-binding protein of an ABC transporter system (Liu *et al.*, 2002). In some cases the effect of these systems on the resistance/transport of several compounds has been investigated. The daunorubicin ABC transporter composed by the DrrA ATP-binding protein and the DrrB membrane protein has been shown to confer resistance to daunorubicin/doxorubicin when expressed in *S. lividans* (Guilfoile and Hutchinson, 1991). On the other hand, disruption of the *drrAB* genes in *S. peuceetius* resulted in a mutant strain highly sensitive to daunorubicin and with 10-fold decrease in drug production (Srinivasan *et al.*, 2010). On the contrary, inactivation of *mtrA*, encoding the ATP-binding component of mithramycin ABC transporter, is lethal to *Streptomyces argillaceus* and could be only achieved in a non-producing mutant. The expression of *mtrAB* into *S. albus* conferred high level of resistance to mithramycin (Fernández *et al.*, 1996). In the case of chromomycin A₃ ABC transporter, expression of *cmrAB* in *S. albus* conferred only low level of chromomycin A₃ resistance, but it confers a high level of resistance to 4A,4E-*O*-dideacetyl-chromomycin A₃, a biosynthetic intermediate of chromomycin A₃. To achieve a chromomycin A₃ high level of resistance in *S. albus* it was necessary to coexpress the ABC transporter *cmrAB* together with the UvrA-like encoding gene *cmrX*. These results pointed to the participation of the ABC transporter CmrAB in the chromomycin A₃ biosynthesis process by extrusion of an intermediate that might be then acetylated (final tailoring modification) by the membrane-bound CmmA acetyltrans-

ferase, and thus converted into the fully biologically active chromomycin A₃ (Menéndez *et al.*, 2007).

In some cases, there are drug-binding proteins that confer resistance to the compound by drug sequestering or by participating in the transport process as ligand-binding proteins. Genes encoding these drug-binding proteins have been found in actinomycin D (Keller *et al.*, 2010), bleomycin (Sugiyama *et al.*, 1994a; Shen *et al.*, 2002) and mitomycin C (Sheldon *et al.*, 1997; 1999; Mao *et al.*, 1999a) gene clusters. BlmA is a bleomycin-binding protein that confers resistance in *E. coli* by drug sequestering. In the case of mitomycin C Mrd is a resistance protein that binds the drug conferring 30-fold increase resistance when expressed into *E. coli* (Sheldon *et al.*, 1997; 1999). On the other hand, the mitomycin C translocase Mct confers fivefold increase in resistance to mitomycin C in *E. coli*, while disruption of *mct* in *Streptomyces lavendulae* resulted in a strain more sensitive to the drug. Coexpression of *mct* and *mrd* in *E. coli* resulted in a 150-fold increase in resistance (Sheldon *et al.*, 1999).

Other genes

To this group belong genes to which no specific function has been ascribed. This is the case of several genes from the mitomycin C biosynthesis cluster (*mitI*, *mmcA*, *mmcB* and *mmcP*). Its inactivation led to non-producer mutants thus confirming their involvement in the biosynthesis of mitomycin C (Mao *et al.*, 1999a). Another example is *cagA* found in C-1027 biosynthesis cluster (Liu *et al.*, 2002). C-1027 is a chromoprotein antibiotic composed of a chromophore (Fig. 2) and an apoprotein encoded by *cagA* (Liu and Shen, 2000). CagA is required for the antibiotic activity of C-1027 due to the extreme lability of the chromophore. However, inactivation of *cagA* has no effect on the biosynthesis of the chromophore itself (Cui *et al.*, 2009).

Biotechnology processes using combinatorial biosynthesis and metabolic engineering

Once a biosynthesis gene cluster has been isolated and characterized, the available genetic information can be used for the generation of novel derivatives or to improve the production of both the native natural product and the novel compounds generated (Fig. 3).

Generation of novel derivatives

The non-producer mutant strains generated by inactivating genes involved in the generation of starter units can be used for precursor-directed biosynthesis of novel compounds. This approach, known as mutational biosynthesis or mutasynthesis, couples the utilization of chemical syn-

thesis with molecular biology (Kennedy, 2008). Several non-natural compounds derived from different metabolites such as rapamycin, borrelidin or salinosporamide have been generated following this method (Fig. 5). BC210 is a mutasynthetic derivative of rapamycin generated by feeding cyclohexane carboxylic acid to *Streptomyces hygroscopicus* mutant MG2-10. This compound was found to be a better inhibitor of mTOR kinase activity and to show anticancer activity both *in vivo* and *in vitro* (Gregory *et al.*, 2005). In addition, BC210 is not a substrate of the multidrug resistance efflux pump P-glycoprotein, has good oral bioavailability and has a highly effective penetration of the blood–brain barrier, which makes this compound a potential therapeutic drug for the treatment of brain tumours (Zhang and Wilkinson, 2007). Six borrelidin derivatives were generated by mutasynthesis using different dicarboxylic acids as starter units (Moss *et al.*, 2006). One of these derivatives, containing a carboxyl-cyclobutane instead of a carboxyl-cyclopentane moiety, was found to be 15-fold less cytotoxic than borrelidin but sixfold more antiangiogenic (Wilkinson *et al.*, 2006). Mutasynthesis has been used, in addition, to generate fluorosalinosporamide, a salinosporamide A analogue produced by a *Sa. tropica* chlorinase *salL* mutant that was supplemented with synthetic 5'-fluoro-5'-deoxyadenosine. This compound is the most potent salinoporamide analogue showing high proteasome binding properties in which the fluoro group significantly extends its residence time on the proteasome (Eustáquio and Moore, 2008).

Genetic manipulation of genes encoding condensing enzymes has been a successful approach to generate novel compounds with modifications in the structural core from different natural products. As an example, acyltransferase substitution in geldanamycin PKS module 7 (GdmA3) by the rapamycin equivalent from module 2 led to the production of several novel geldanamycin derivatives. In particular, compound KOSN1559 (Fig. 5) showed fourfold higher binding affinity towards Hsp90, a chaperone overproduced in several types of human cancers and new target for cancer therapy (Patel *et al.*, 2004). Occasionally, modifications in the structural core can be obtained by targeting genes involved in tailoring modification. This is the case of insertional inactivation of mithramycin *mtmW*, encoding a ketoreductase involved in side-chain modification, which led to the production of several new mithramycin derivatives (Remsing *et al.*, 2003). Among them, mithramycin SK (Fig. 5) showed an antitumour activity ninefold higher than that of mithramycin (Remsing *et al.*, 2003), being especially effective against colon carcinoma cells (Bataller *et al.*, 2008; 2010).

Targeting genes involved in the generation of structural elements attached to the core structure, including those tailoring modification, is the most productive approach to

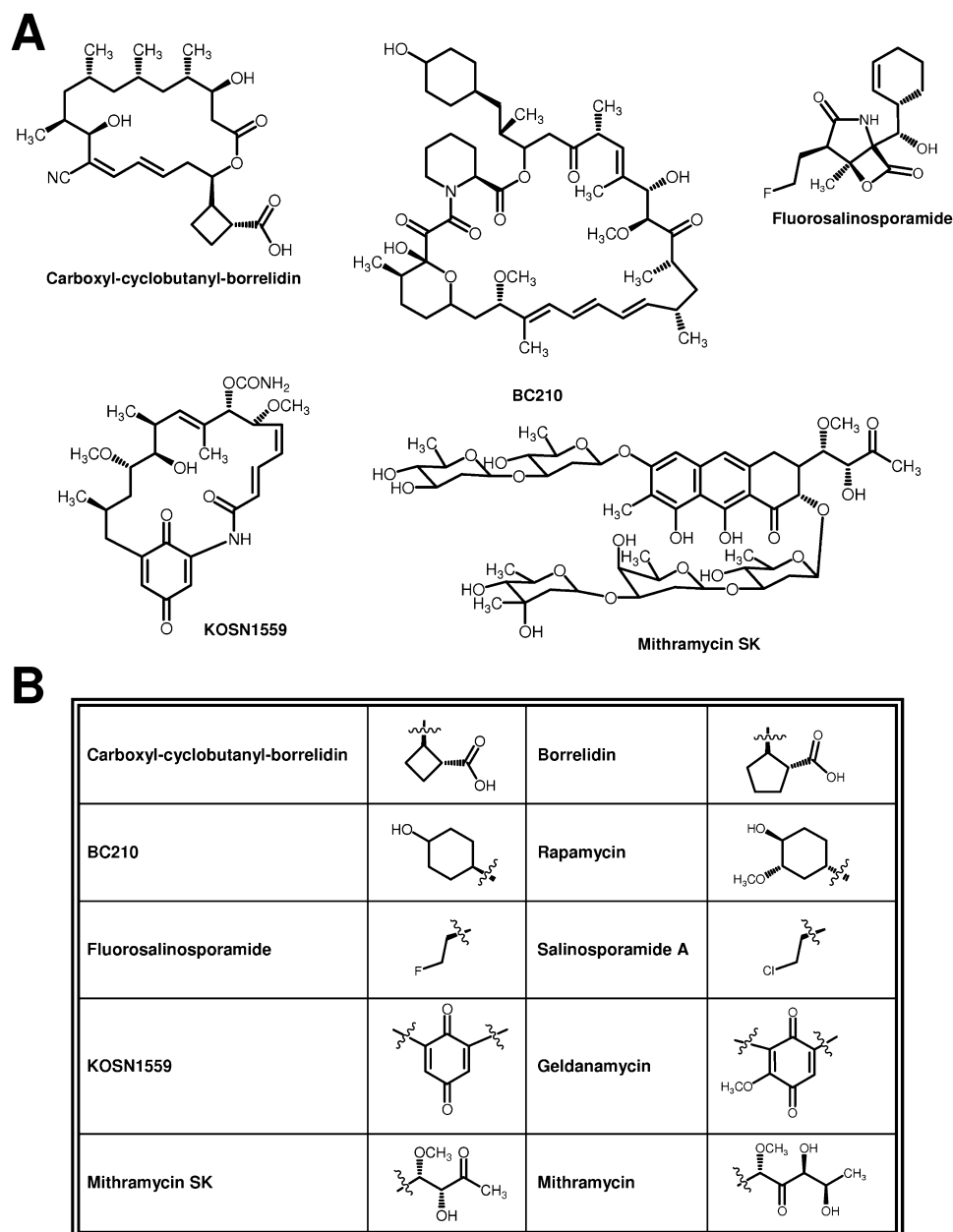


Fig. 5. A. Structures of novel antitumor derivatives generated by mutasynthesis or modification of the structural core. B. Altered features and their comparison with the unmodified compound.

generate novel derivatives. Examples are the production of 22-dehydroxy-C-1027 and 3-morpholino-propylamine-decarbonyl-bleomycin (Fig. 6). The first one was generated by inactivating *Streptomyces globisporus* FAD-dependent monooxygenase *sgcC* (Liu *et al.*, 2002). The second compound was produced by inactivating *Streptomyces verticillus* carbamoyltransferase *blmD* (Galm *et al.*, 2008). In addition, the expression of different gene combinations in a heterologous host can bypass the eventuality of working with actinomycetes refractory to genetic manipulation. This was the method used to obtain several

oviedomycin derivatives in *S. albus* by combining type II PKS and cyclase genes together with those of three FAD-dependent oxygenase genes from its own cluster. Three of these derivatives: prejadomycin-2-carboxylate, prejadomycin and 4a-12b-dehydro-UWM6 (Fig. 6), showed significant improved antitumor activity (Lombó *et al.*, 2009).

The combination of targeting gene disruption and expression of genes or combinations of genes has been widely used to modify the glycosylation pattern of different antitumor compounds (Figs 6 and 7). The important

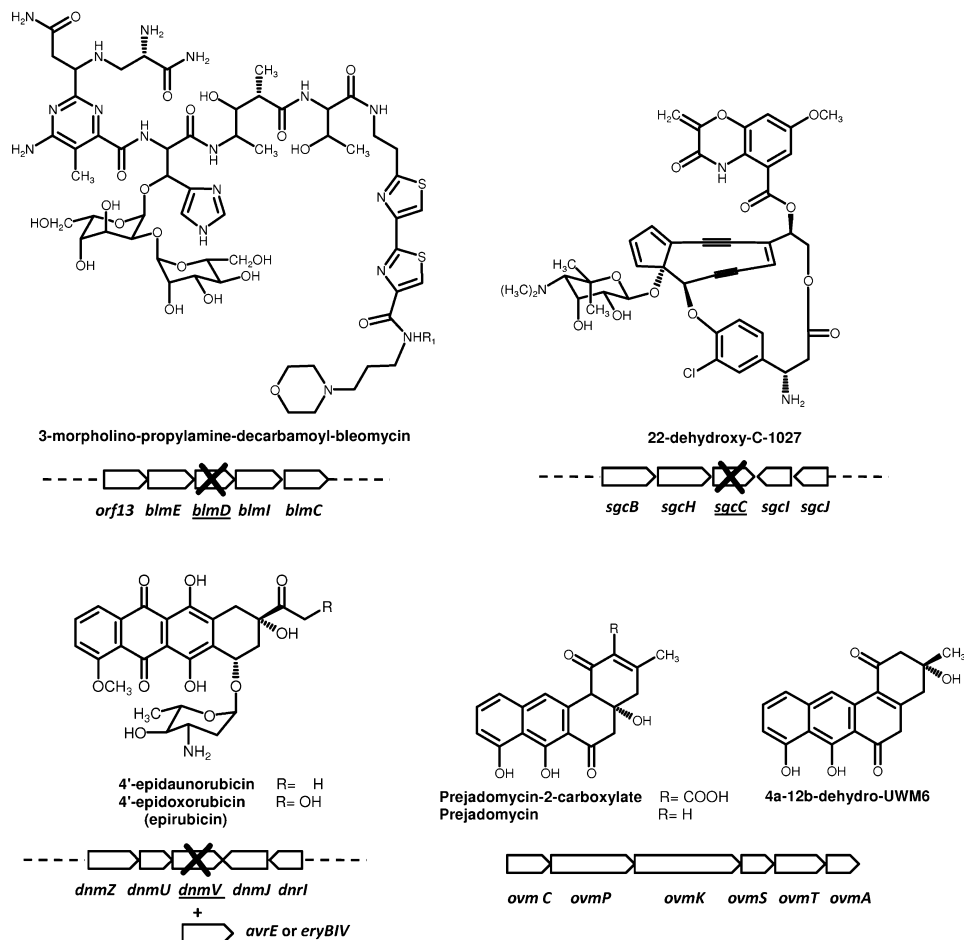


Fig. 6. Structures of novel antitumour derivatives generated by tailoring modification showing the genes inactivated (crossed) or expressed for the generation of the novel compound.

antitumour drug 4'-epidoxorubicin (epirubicin), traditionally obtained semisynthetically, along with its precursor 4'-epidaunorubicin, was generated using a genetically engineered *S. peuceitius* strain defective in the daunomamine biosynthesis gene *dnmV*. This strain was used to express the avermectin biosynthetic gene *avrE* from *Streptomyces avermitilis* or the erythromycin biosynthetic gene *eryBIV* from *Saccharopolyspora erythraea*, which led to 4'-epianthracyclines (Madduri *et al.*, 1998) (Fig. 6). A number of anthracyclines with modification in the glycosylation pattern have been obtained by expressing the steffimycin gene cluster in *S. albus* together with plasmids directing the biosynthesis of different deoxysugars. Three of these compounds showed higher antitumour activity than the parental natural product. In particular, D-digitoxosyl-8-demethoxy-10-deoxysteffimycin (Fig. 7) was found to be 24-fold more active than steffimycin when tested against different human tumour cell lines (Olano *et al.*, 2008b). Analogues with improved activity of other aromatic compounds such as mithramycin and elloramycin have been also obtained by modification of the sugar

moieties. The expression of genes involved in the biosynthesis of different deoxysugars in *S. argillaceus* led to 11 new mithramycin derivatives with altered glycosylation patterns (Baig *et al.*, 2008; Pérez *et al.*, 2008). Two of these, demycarosyl-3D-β-D-digitoxosyl-mithramycin and deoliosyl-3C-β-D-mycarosyl-mithramycin, showed improved activity against an oestrogen receptor-positive human breast cancer cell line. In addition, demycarosyl-3D-β-D-digitoxosyl-mithramycin was highly active against an oestrogen receptor-negative human breast cancer cell line that is not strongly inhibited by mithramycin (Baig *et al.*, 2008). On the other hand, coexpression of elloramycin gene cluster in *S. albus* with plasmids directing the biosynthesis of different deoxysugars has led to the isolation of new elloramycin (R₁=CH₃) and 8-demethyl-tetracenomycin C (R₁=H) derivatives (Fig. 7) with different sugar moieties (Fischer *et al.*, 2002; Rodríguez *et al.*, 2002; Lombó *et al.*, 2004b; Pérez *et al.*, 2005; 2006). From these compounds, L-mycarosyl-elloramycin showed improved activity against three cancer cell lines (Lombó *et al.*, 2004b). The modification of glycosylation patterns

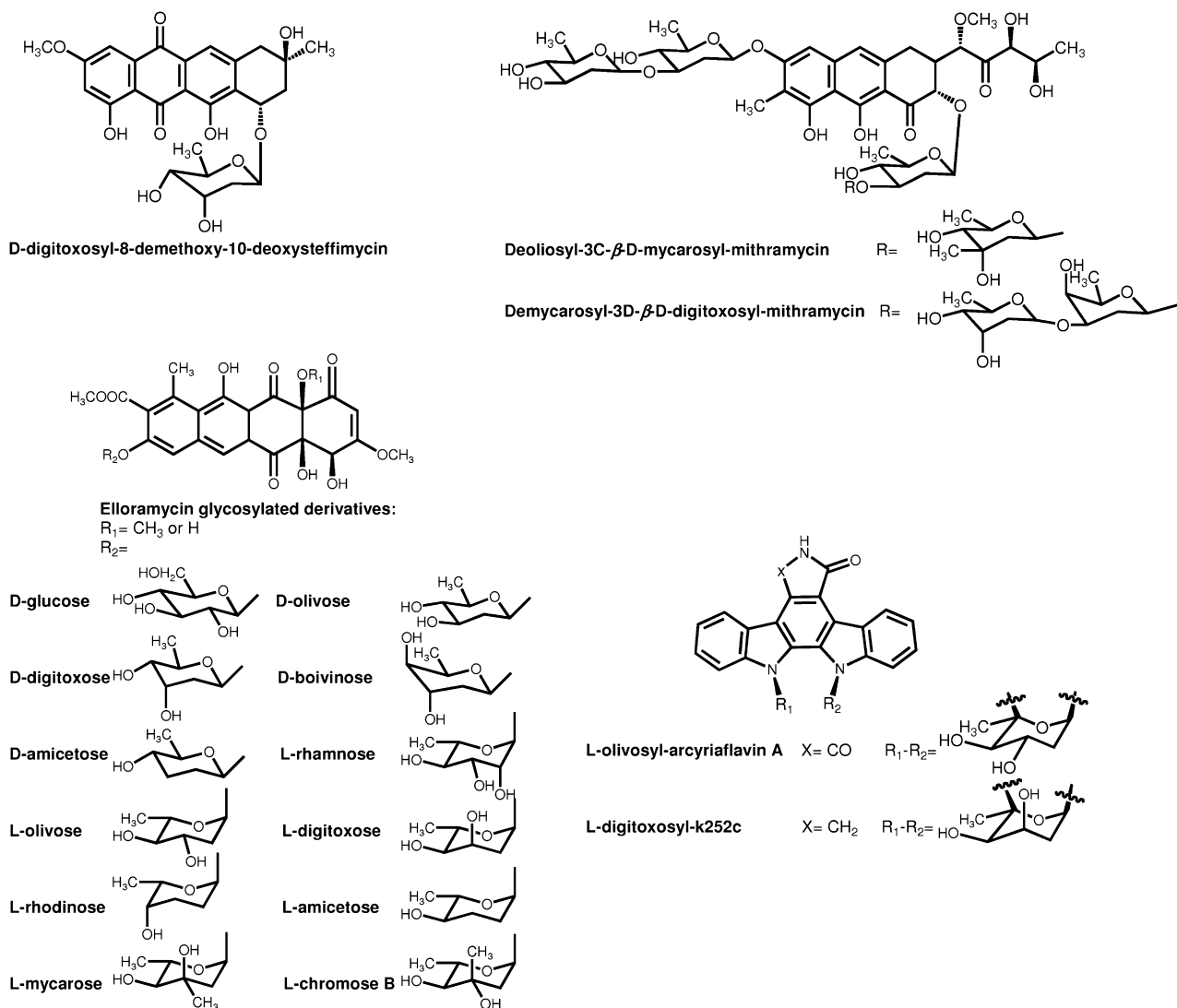


Fig. 7. Structures of novel antitumour derivatives with an altered glycosylation pattern by using plasmids directing the biosynthesis of different deoxysugars.

has not only been applied to polyketide natural products. Several indolocarbazole derivatives were generated by using this approach (Salas *et al.*, 2005). Rebeccamycin, a DNA topoisomerase I inhibitor, and staurosporine, a promiscuous kinase inhibitor, contain a sugar moiety (Fig. 2) attached through a single *N*-glycosidic bond (rebeccamycin) or by two *N*-glycosidic bonds (staurosporine). The coexpression of rebeccamycin and staurosporine biosynthesis genes together with plasmids directing the biosynthesis of deoxysugars in *S. albus* has allowed the generation of novel derivatives from both compounds (Fig. 7). Some of these analogues were found highly active against specific kinases. L-olivosyl-arcyriaflavin A is a potent inhibitor of Ikk β , kinase that prevents activation of the NF- κ B pathway and thus has potential application on inflammation and cancer. On the other hand,

L-digitoxosyl-k252c is a subnanomolar JAK2 inhibitor, kinase with potential application in myeloproliferative disorders (Sánchez *et al.*, 2009).

Improving production yields

Current methods to increase the productivity of industrial microorganisms are classical random mutagenesis and optimization of large-scale industrial fermentations. Metabolic engineering can help, in addition, to improve the production of bioactive metabolites (Fig. 3). Overproduction of a particular compound can be achieved by engineering different aspects of cellular metabolism at the level of primary metabolism such as precursor supply or ribosome engineering. The production of bioactive metabolites can also be improved by engineering genes

from their specific biosynthesis clusters: pathway-specific regulators, structural and self-resistance genes. In addition, the expression of heterologous genes or the expression of the gene cluster in a heterologous host can be used to enhance production yields.

At the level of primary metabolism, it has been observed that overexpression of SAM synthetase results in higher secondary metabolite yields. The characterization of two *S. peuceitius* SAM synthetase genes, *metK1-sp* and *metK2-sp*, and their independent overexpression in the same organism resulted in 2.1- and 1.4-fold increase of doxorubicin production (Oh *et al.*, 2010). Ribosome engineering has been used as a rational approach to enhance antibiotic production in different *Streptomyces* spp. by selecting mutants resistant to several antibiotics. In particular, ribosomes containing a mutation in ribosomal protein S12 gene, *rpsL*, which confers resistance to streptomycin, led to high increases in secondary metabolite production due to a high level of protein synthesis and ppGpp accumulation (Wang *et al.*, 2008). Actinomycin D production in *Streptomyces antibioticus* has been enhanced 5.2-fold by this method (Hosoya *et al.*, 1998). On the other hand, transport/resistance systems encoded in the biosynthesis gene cluster have been observed to be involved not only in self-protection but also in the export of biologically active intermediates (Menéndez *et al.*, 2007). The production of doxorubicin by *S. peuceitius* has been enhanced by overexpressing the resistance genes *drrA*, *drrB* and *drrC* in different combinations. The expression of *drrAB* led to a 2.2-fold increase in doxorubicin yields while the *drrC* led to a 5.1-fold increase. Surprisingly, only a 2.4-fold increase was obtained by expressing the three genes together (Malla *et al.*, 2010a). Consequently, increasing self-resistance levels in producing organisms can be also used as an approach for increasing production yields.

Engineering pathway-specific regulators is a useful method to bypass the imposed constraints to the expression of biosynthetic and transport/regulatory genes thus deregulating the production of secondary metabolites. Overexpression of pathway-specific activators under the control of constitutive promoters and using multicopy plasmids has been used to improve the production of mithramycin (Lombó *et al.*, 1999; Garcia-Bernardo *et al.*, 2000), daunorubicin (Stutzman-Engwall *et al.*, 1992; Otten *et al.*, 1995b), doxorubicin (Parajuli *et al.*, 2005; Malla *et al.*, 2010b), rapamycin (Kuscer *et al.*, 2007) and C-1025 (Wang *et al.*, 2009). Furthermore, inactivation of pathway-specific repressors can also lead to overproduction of secondary metabolites. This is the case of mitomycin C (Mao *et al.*, 1999a) and chromomycin A₃ (Menéndez *et al.*, 2007) that were overproduced when pathway-specific transcriptional repressors *mmcW* and *cmmRll* were inactivated in their respective producer strains.

Biosynthesis structural genes can also be used to improve secondary metabolite production. Overexpression of *sgcA1*, encoding a NDP-glucose synthase, resulted in a twofold improvement for enediyne C-1027 production, and up to fourfold if *cagA* coding for C-1027 apoprotein was coexpressed (Murrell *et al.*, 2004). On the other hand, the expression of *cagA* alone, either as an extra copy under a constitutive promoter or as multiple copies of the gene under its native promoter, also resulted in a significant increase in enediyne production (Cui *et al.*, 2009). The production of doxorubicin has also been improved by expressing genes involved in the biosynthesis and attachment of deoxyamino sugar daunosamine. By expressing the daunorubicin glycosyltransferase genes *dnrS/dnrQ* together with glucose-1-phosphate thymidyltransferase *desIII* and TDP-D-glucose-4,6-dehydratase *desIV* from *S. venezuelae* involved in the formation of the intermediate TDP-4-keto-6-deoxy-D-glucose a 5.6-fold increase in doxorubicin yield was obtained (Malla *et al.*, 2009). The utilization of heterologous genes has also allowed improving 4'-epidaunorubicin yields in *Streptomyces coeruleorubidus*. In this case, by increasing threefold the *aveBIV* (formerly *avrE*) copy number, 4'-epidaunorubicin yields increased in the same proportion (Shao *et al.*, 2010). The expression of heterologous genes in mutant strains can also be used to bypass the utilization of mutasynthesis approaches for the generation of certain derivatives. The production of fluorosalinosporamide, generated by mutasynthesis, has been induced in *Sa. tropica* by replacing the chlorinase gene *salL* with the fluorinase gene *flA* from *Streptomyces cattleya* (Eustáquio *et al.*, 2010).

Another approach for improving metabolite production is to remove genes involved in the generation of undesired compounds thus enhancing the yields of the desired one. Following this philosophy, inactivation of *S. peuceitius* *dnrX* and *dnrH* improved 3- and 8.5-fold the biosynthesis of doxorubicin and daunorubicin respectively (Scotti and Hutchinson, 1996; Lomovskaya *et al.*, 1998). These genes are involved in the transformation of daunorubicin and doxorubicin into their polyglycosylated forms known as baumycins. In addition, disruption of *dnrU*, involved in the transformation of daunorubicin into 13-dihydrodaunorubicin, has a similar effect on doxorubicin production since the production of an undesired compound is abolished (Lomovskaya *et al.*, 1999). The combination of these beneficial gene disruptions further improved doxorubicin yield, up to sevenfold in the *dnrU/dnrX* double mutant and to 26-fold in the *dnrU/dnrX/dnrH* triple mutant. Furthermore, an additional 1.3- to 2.8-fold increase was obtained by overexpressing in the double or triple mutants mentioned above *dnrV* and *doxA* genes involved in late steps during doxorubicin biosynthesis (Lomovskaya *et al.*, 1999). Using the same approach,

overexpression of *dnmT*, involved in the biosynthesis of daunorubicin deoxysugar L-daunosamine, in the *dnrH* mutant led to improve daunorubicin production (Scotti and Hutchinson, 1996). The production of 4'-epidaunorubicin in *S. peucetius dnmV::avrE* was enhanced also by inactivating *dnrH* and overexpressing *dnmT* (Madduri *et al.*, 1998).

Occasionally, it is important to narrow the substrate flexibility of an enzyme to make it more efficient for the generation of certain derivatives. This objective can be accomplished by site-directed mutagenesis targeting specific residues in the substrate binding region. Using this approach, elloramycin glycosyltransferase ElmGT that possesses remarkable donor substrate flexibility has been engineered to enhance the production of different 8-demethyl-tetracenomycin C derivatives. As mentioned above ElmGT has been shown to transfer at least 11 different sugars, including both L- and D-isomeric forms, to its aglycon. Site-directed mutagenesis in certain residues located in the $\alpha/\beta/\alpha$ motif within the ElmGT nucleoside diphosphate-sugar binding region has led to obtain several versions of the enzyme with improved ability to transfer particular deoxysugars: D-boivinose 1.5-fold, L-rhamnose twofold and L-olivose up to sixfold (Ramos *et al.*, 2009).

Finally, in some cases production titres can be enhanced by expressing the biosynthesis gene cluster in a heterologous host. This was the case of the indolocarbazole rebeccamycin produced by *Lechevaleria aereocolonigenes*. The expression of the cluster in *S. albus* led to production levels several folds higher than those of the original strain (Sánchez *et al.*, 2002).

Future perspectives

A great effort has been made in the last decades for the discovery of new natural products, antitumour drugs included. However, many of the novel compounds identified usually present some undesired biological and chemical properties, i.e. unspecific cytotoxicity, side-effects, no effect on resistant strains or lack of solubility for oral or parenteral administration, which represent a step back in the expectations for their commercial use. The development of recombinant DNA technology during the second part of the last century has set up the foundations for unravelling the information encoded in microbial genomes and to access the information regarding the biosynthesis of natural products. All the efforts executed for the isolation, characterization and manipulation of biosynthesis gene clusters have crystallized in the generation of a large number of analogues of bioactive compounds. Some of these compounds show improved biological properties, which make them lead compounds for their application in clinic. In addition, these gene clusters contain elements

that can be used to increase the production of both natural and engineered products. Keeping on with combinatorial biosynthesis approaches will provide increasing numbers of safer and more specific compounds that might enhance the arsenal of antitumour drugs available for chemotherapy.

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