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REVIEW

César Sánchez · Carmen Méndez · José A. Salas

Engineering biosynthetic pathways to generate antitumor indolocarbazole derivatives

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Abstract The indolocarbazole family of natural products is a source of lead compounds with potential therapeutic applications in the treatment of cancer and neurodegenerative disorders. Rebeccamycin and staurosporine are two members of this family, which are produced by different actinomycete strains. Although both compounds display antitumor activity, their distinct structural features determine different modes of action: rebeccamycin targets DNA topoisomerase I, while staurosporine is a protein kinase inhibitor. Here we examine the biosyntheses of rebeccamycin and staurosporine while we summarize our recent work concerning (a) identification and characterization of genes involved in the biosynthesis of indolocarbazoles in actinomycetes, and (b) generation of novel indolocarbazole derivatives in microorganisms by combinatorial biosynthesis.

Keywords Rebeccamycin · Staurosporine · *Streptomyces* · Metabolic engineering · Glycosylation

The indolocarbazole alkaloids and the biogenetically related bisindolylmaleimides constitute an important class of natural products, which have been isolated from actinomycetes, cyanobacteria, slime molds and marine invertebrates [7, 12]. They display a wide range of biological activities, including antibacterial, antifungal, antiviral, hypotensive, antitumor or neuroprotective properties. The antitumor and the neuroprotective activities of indolocarbazoles are the result of one, or several, of the following mechanisms: (a) inhibition of different protein kinases, (b) inhibition of DNA topoisomerases, or (c) direct DNA intercalation [1, 15, 24]. Hundreds of indolocarbazole derivatives have been produced by chemical synthesis or semi-synthesis [7, 12, 24], and several of them have entered clinical trials for the treatment of diverse types of cancer, Parkinson's disease or diabetic retinopathy [3].

Structurally (Fig. 1), the members of this family are characterized by a core consisting of either an "open" bisindolylmaleimide (e.g., arcyriarubin B), or a "closed" indolo[2,3-*a*]carbazole (e.g., tjipanazole F2, rebeccamycin, staurosporine). Most of the latter compounds are in fact derivatives of the indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole ring system, to which a sugar residue is often attached. In the glycosides, the carbohydrate can be attached through a single *N*-glycosidic bond (as in rebecamycin), or it can be joined by two bonds consisting of an *N*-glycosidic bond and an *N*,*O*-ketal (as in staurosporine). Another key structural difference between rebeccamycin and staurosporine resides at the pyrrole moiety, consisting of an imide function in the former or an amide function in the latter (Fig. 1). These differences seem to be essential for target selectivity, since rebeccamycin inhibits topoisomerase I while staurosporine is a protein kinase inhibitor.

As a complementary approach to the methods of organic chemistry, we have recently shown that genetic manipulation of the genes governing indolocarbazole biosynthesis offers a promising alternative to prepare these compounds [28–30]. Here we summarize our findings concerning (a) identification and characterization of genes involved in the biosynthesis of indolocarbazoles in actinomycetes, and (b) generation of novel indolocarbazole derivatives in microorganisms by combinatorial biosynthesis.

Identification of the genes responsible for rebeccamycin biosynthesis

At the onset of our work, little was known about the biochemical or genetic aspects of indolocarbazole biosynthesis. The biosyntheses of staurosporine and rebeccamycin had been studied by feeding isotope-labeled

C. Sánchez · C. Méndez · J. A. Salas (✉)
Departamento de Biología Funcional and Instituto Universitario de Oncología del Principado de Asturias (IUOPA), Universidad de Oviedo, 33006, Oviedo, Spain
E-mail: jasalas@uniovi.es
Tel.: +34-985-103652
Fax: +34-985-103652

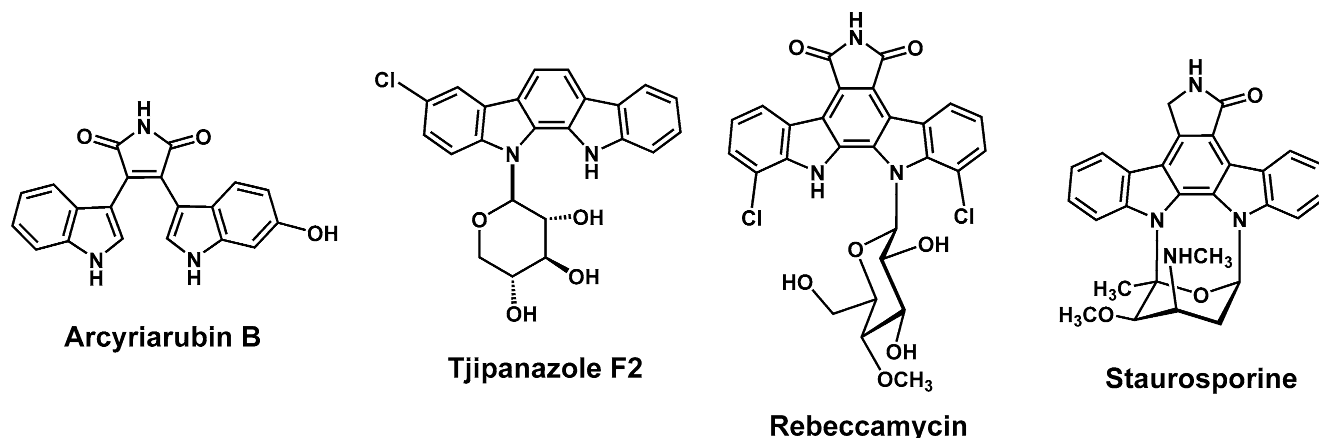

Fig. 1 Some representatives of the indolocarbazole family of natural products

precursors to the actinomycetes *Streptomyces staurosporeus* AM-2282 (staurosporine producer) and *Saccharothrix aerocolonigenes* ATCC39243 (rebeccamycin producer). These results established that the indolocarbazole core was derived from two units of tryptophan (with the carbon skeleton incorporated intact), while the sugar moiety was derived from glucose and methionine [16, 23, 37, 38]. A patent application existed that claimed the cloning of some genes needed for the formation of the staurosporine sugar moiety in a different staurosporine producer, *Streptomyces longisporoflavus* DSM10189 [31]. Also, a gene (*ngt*) encoding an indolocarbazole *N*-glycosyltransferase had been cloned from the rebeccamycin producer *Sac. aerocolonigenes* ATCC39243 [19]. The *ngt* gene, when expressed in either *Streptomyces lividans* or *Streptomyces mobaraensis*, was responsible for the introduction of a D-glucose moiety into indolocarbazoles J-104303 and 6-*N*-methylarcyriaflavin C. These results suggested that *ngt* was probably involved in rebeccamycin biosynthesis, although no definite proof was reported at that time.

Given the precedent that biosynthetic genes for secondary metabolites commonly occur as a cluster in actinomycetes, *ngt* served as the basis for the isolation of the gene cluster responsible for rebeccamycin biosynthesis from *Sac. aerocolonigenes* ATCC39243 (this strain has been re-classified as *Lechevalieria aerocolonigenes* [14], and this name will be used hereafter). In fact, the rebeccamycin biosynthetic locus was independently identified by us [29] and by other laboratories [11, 17, 21] from the same strain. In our approach, we constructed an *L. aerocolonigenes* genomic library in shuttle vector pKC505 [25], and the library was screened using an internal fragment of *ngt* as a probe. The result was the isolation of several overlapping cosmids containing *ngt*. The choice of pKC505 as vector gave us the opportunity to introduce the cosmid clones into an actinomycete host, *Streptomyces albus* J1074 [4]. It was hoped that, if the complete rebeccamycin gene cluster were contained in a single clone, the corresponding strain might become a rebeccamycin producer. Two of the cosmids conferred the ability to produce, in good yields, a compound that was unambiguously identified as rebeccamycin [29]. This result was significant for several reasons. It made possible the first characterization of a complete gene cluster governing the biosynthesis of an indolocarbazole alkaloid. All the biosynthetic genes were isolated on a single fragment, hence facilitating the often-difficult task of defining the boundaries of the cluster. Additionally, the heterologous production of rebeccamycin in good yields indicated that the *L. aerocolonigenes* genes were efficiently expressed, suggesting that *S. albus* could be a good host for engineering indolocarbazole biosynthesis.

The complete DNA sequence of the insert in cosmid 14E8 (one of the cosmids conferring the ability to produce rebeccamycin) was determined, consisting of 25.7 kb [29]. It appeared that the rebeccamycin (*reb*) gene cluster consisted of 11 genes, probably organized in four transcriptional units and spanning 17.6 kb, with *ngt* (renamed as *rebG*) located at one end of the cluster (Fig. 2a). From the sequence analysis and database comparison, we proposed functions for the *reb* genes (Fig. 2) and a hypothetical pathway for rebeccamycin biosynthesis (Fig. 3a). Additionally, heterologous expression of subsets of these genes resulted in the production of three non-chlorinated rebeccamycin derivatives in *S. albus* [29]. The functions proposed for the *reb* genes have been supported on experimental evidence obtained by us and by other laboratories [10, 17, 18, 21, 29, 30, 39], as discussed below.

Other laboratories also used *ngt* as the basis for the identification of the *reb* locus, but they followed different approaches. Onaka and co-workers [21] developed a genetic system for *L. aerocolonigenes*, which allowed them to inactivate several *reb* genes by gene-disruption and to identify eight rebeccamycin derivatives from the mutant strains. In particular, these authors identified a key intermediate in the pathway, consisting of 11,11′-dichlorochromopyrrolic acid (Fig. 3a). On the other hand, Hyun and co-workers [11] isolated the

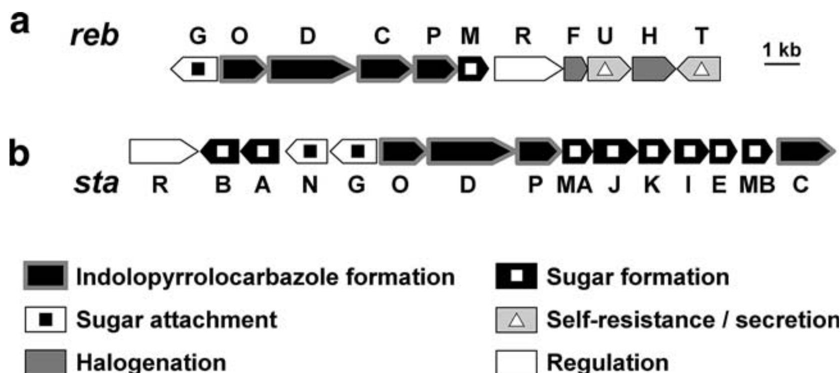

Fig. 2 Gene clusters for biosynthesis of rebeccamycin (**a**) and staurosporine (**b**)

complete gene cluster in a single cosmid clone that conferred the ability to produce rebeccamycin (although at low levels) in a "user-friendly" *E. coli* host.

Dissection of rebeccamycin biosynthesis and generation of derivatives

The availability of the *reb* gene cluster set the stage for elucidation of the molecular basis for indolocarbazole biosynthesis and for the generation of novel indolocarbazole analogs by genetic engineering. Recently, we have been studying indolocarbazole biosynthesis with a focus on the production of novel indolocarbazole compounds in engineered microorganisms, using combinatorial biosynthesis and rational metabolic pathway engineering.

We dissected and reconstituted the entire biosynthetic pathway for rebeccamycin in the *S. albus* host [30]. This was achieved by expressing different combinations of *reb* genes, in a rational way, followed by an analysis of the metabolites being produced by the engineered *S. albus* strains. As a result, we identified 12 rebeccamycin derivatives (compounds **1–12**, Fig. 4a), which supported the functions proposed for individual *reb* genes. The following discussion on rebeccamycin biosynthesis summarizes our own findings, but we also mention, when appropriate, the results obtained by other laboratories.

Tryptophan halogenation

The rebeccamycin pathway starts with chlorination of tryptophan, catalyzed by RebH, an $FADH_2$-dependent halogenase (Fig. 3a). In fact, the functional halogenase consists of a two-component enzyme RebF/RebH, recently purified and characterized, which regioselectively chlorinates L-tryptophan to yield 7-chloro-L-tryptophan [39]. In vitro halogenation by RebH requires the addition of flavin reductase RebF, which catalyzes the NADH-dependent reduction of FAD to provide $FADH_2$ for the halogenase [39]. However, in the *S. albus* host, we could produce rebeccamycin in the absence of *rebF* [30]; this suggests that RebF can be efficiently replaced by other reductases (from the host), as has been shown before for other $FADH_2$-dependent halogenases [33]. The RebH halogenase could not use bisindole intermediates as alternative substrates, since chromopyrrolic acid (CPA) **1** or non-chlorinated aglycone **2** was not chlorinated when fed to an *S. albus* strain expressing *rebH* [30]. However, in the absence of *rebH*, a full pathway for dideschloro-rebeccamycin could be reconstituted, indicating that the rest of the enzymes are able to use non-chlorinated intermediates [21, 29, 30].

Chloride salts, such as NaCl, are the source of chlorine for RebH-catalyzed halogenation [39]. According to this, early experiments showed that a bromo analog of rebeccamycin could be obtained from *L. aerocolonigenes* when grown in a medium supplemented with potassium bromide [13]. In a similar way, we produced brominated versions of rebeccamycin and of six intermediates by replacing chloride with bromide in the fermentation medium of *S. albus* strains expressing *reb* genes (compounds **26–32**, Fig. 4a) [30]. The direct participation of halogenase RebH in the formation of bromotryptophan has been recently confirmed with the purified enzyme [39].

In an effort to increase the number of indolocarbazole derivatives produced, we used two additional genes coding for tryptophan halogenases from different microorganisms: *pyrH* is involved in pyrroindomycin biosynthesis in *S. rugosporus* LL-42D005 [40], and *thal* participates in thienodolin formation in *S. albogriseolus* [34]. PyrH and Thal introduce chlorine at the C-5 and the C-6 positions of tryptophan, respectively, while RebH chlorinate at the C-7 position. Co-expression of *reb* genes together with either *pyrH* or *thal* allowed the production of several new derivatives in *S. albus* (compounds **21–25**, Fig. 4c), indicating that 5- and 6-chlorotryptophan intermediates could be accepted as substrates by rebeccamycin enzymes [30].

Formation of the first bisindole intermediate ("dimerization")

It has been reported that a *rebD*-disrupted mutant of *L. aerocolonigenes* did not produce compounds related to indolocarbazoles, indicating a role for *rebD* in early

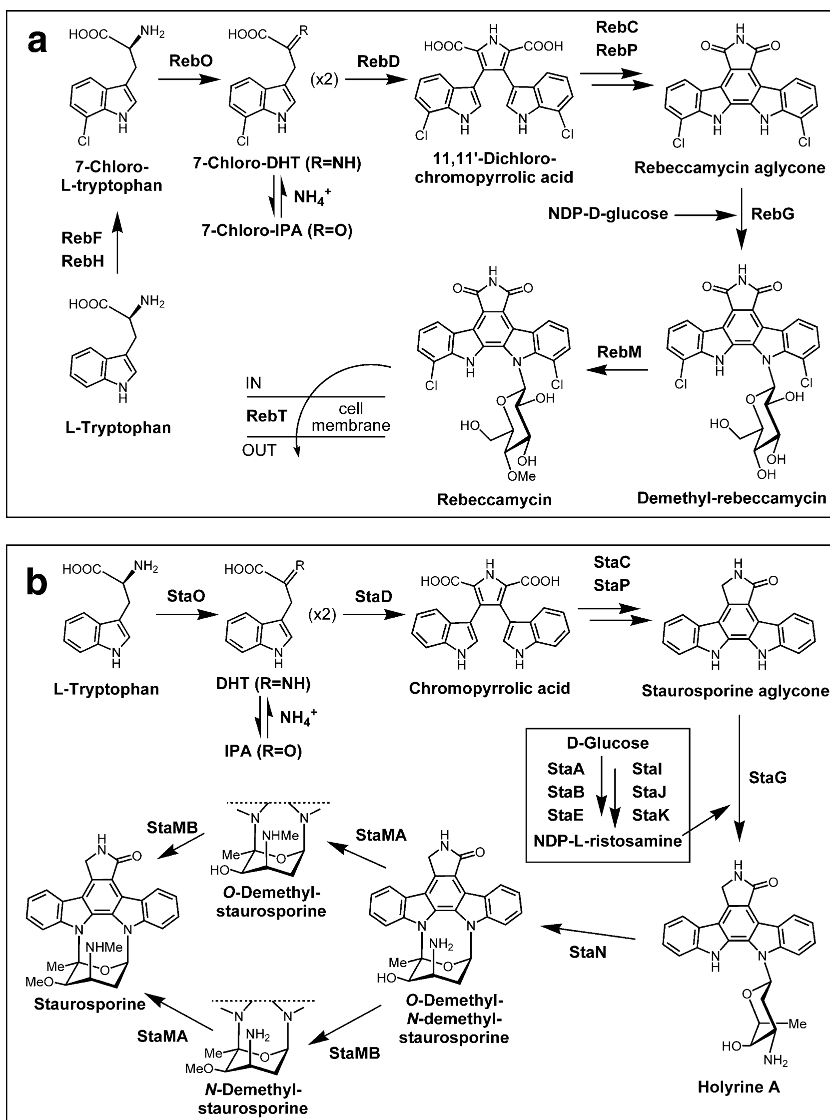

Fig. 3 Updated pathways for the biosynthesis of rebeccamycin (**a**) and staurosporine (**b**). Dehydrotryptophan (DHT) is readily hydrolyzed in solution, existing in equilibrium with indole-3-pyruvic acid (IPA)

steps of the pathway [21]. In fact, the *rebO* and *rebD* genes were both essential for the production of any bisindole intermediates in *S. albus* recombinant strains, and their co-expression yielded the simplest bisindole intermediate that we could identify (compound **1**, Fig. 4a) [30]. This metabolite consisted of CPA, which is a known natural product previously isolated from other microorganisms [6, 9]. The corresponding intermediate en route to rebeccamycin is 11,11′-dichloro-CPA (Fig. 3a), which was produced by a *rebP*-disrupted mutant of *L. aerocolonigenes* [21] and by an *S. albus* strain co-expressing *rebH*, *rebO* and *rebD* (compound **8**, Fig. 4a) [30].

Very recently, the RebO and RebD enzymes have been purified and characterized [10, 17, 18]. RebO is an FAD-dependent L-tryptophan oxidase that converts 7-chloro-L-tryptophan into 7-chloro-dehydrotryptophan, with the production of hydrogen peroxide. RebO shows significant preference for 7-chloro-L-tryptophan over L-tryptophan, further supporting the role of chlorotryptophan as the natural early pathway intermediate [17]. On the other hand, RebD represents the first member in a novel subfamily of heme-containing oxidases. RebD acts as both a catalase and a CPA synthase, converting two molecules of (7-chloro-)dehydrotryptophan into (11,11′-dichloro-)CPA [10, 18]. Both RebO and RebD are dimeric enzymes and require molecular oxygen as a cosubstrate.

Formation of the indolopyrrolocarbazole core

For an efficient production of the indolopyrrolocarbazole core **2** in *S. albus*, the co-expression of four genes was needed: those for CPA formation (*rebO*, *rebD*) together with *rebC* and *rebP* [30]. If the *rebH* gene was also included, the rebeccamycin aglycone **10** was obtained. The indolopyrrolocarbazole core (or non-chlorinated rebeccamycin aglycone **2**, Fig. 4a) is identical to arcyriaflavin A, which is a natural product

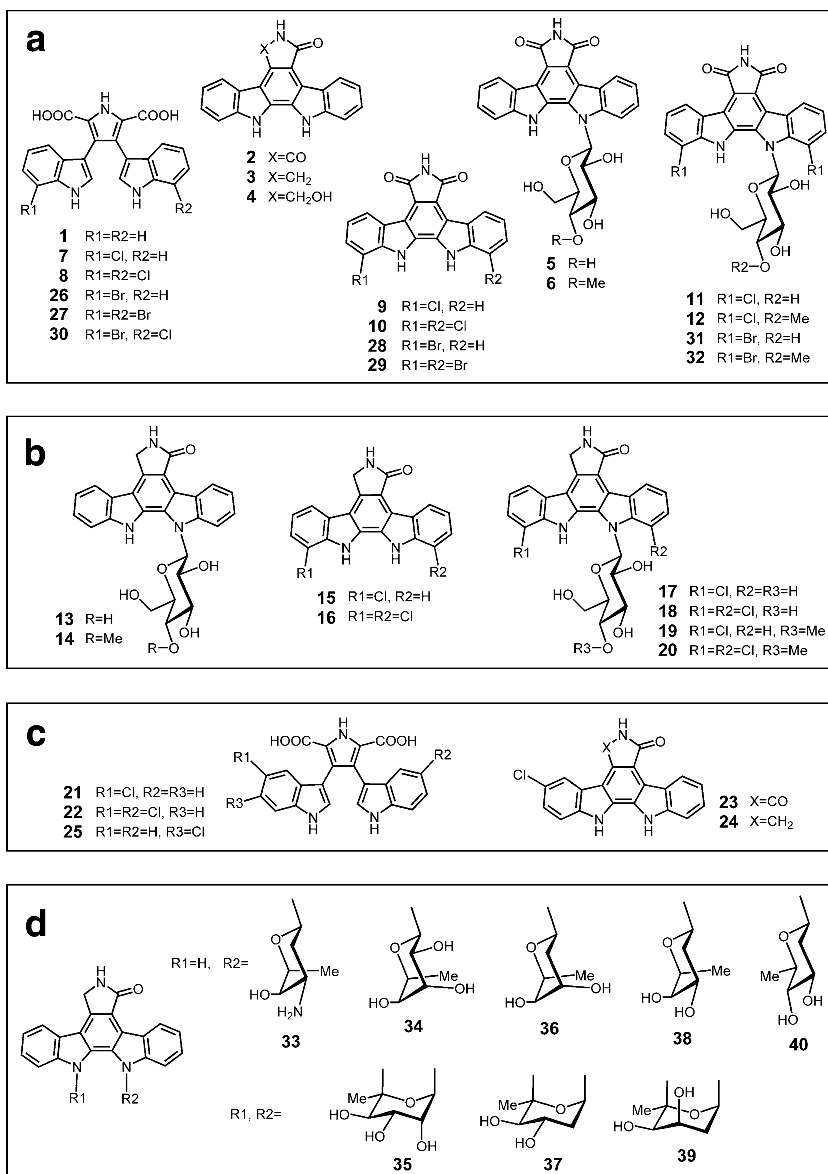

Fig. 4 Indolocarbazole derivatives produced by combinatorial biosynthesis in *Streptomyces albus*. The compounds were obtained by expressing combinations of: **a** only *reb* genes; **b** *reb* genes and *staC*; **c** *reb* genes, *staC*, *pyrH* and *thal*; **d** *reb* genes, *sta* genes and additional sugar biosynthetic genes from different microorganisms

isolated from slime molds and marine invertebrates [8, 32]. Although it was possible to obtain the indolopyrrolocarbazole core in the absence of *rebC* (by the co-expression of *rebO*, *rebD* and *rebP*), the result consisted of a mixture of three derivatives differing only at the C-7 position of the pyrrole: arcyriaflavin (7-oxo), staurosporine aglycone (7-deoxo) and 7-hydroxy-staurosporine aglycone (compounds **2–4**, Fig. 4a) [30]. These results are consistent with those previously obtained with *rebP*- and *rebC*-disrupted mutants of *L. aerocolonigenes*, which accumulated (respectively) 11,11′-dichloro-CPA or a mixture of rebeccamycin derivatives differing at C-7 [21]. These authors also reported that the 7-deoxo or 7-hydroxy derivatives did not seem to be rebeccamycin intermediates, as they were not converted into rebeccamycin in bioconversion experiments. Therefore, it appears that RebP (a cytochrome P450 enzyme) is responsible for the decarboxylative oxidations needed to convert an "open" (dichloro-)CPA into a "closed" indolopyrrolocarbazole. However, the monooxygenase RebC seems to be needed for efficient completion of this reaction and, more interestingly, for the determination of the C-7 oxidation state in the final product.

Sugar attachment and modification

Indolocarbazole glycosylation appears to take place only after the formation of a "closed" planar indolocarbazole ring system, but not on "open" CPA intermediates: no glycosylated CPA was detected either by co-expressing *rebO*, *rebD* and *rebG*, or by feeding CPA to a strain expressing *rebG* [30]. However, efficient production of D-glucosyl-arcyriaflavin (compound **5**, Fig. 4a) was obtained either by co-expressing *rebO*,

rebD, *rebC*, *rebP* and *rebG*, or by feeding arcyriaflavin to a *rebG*-expressing strain [30]. These results agree with those previously obtained with *rebP*- and *rebG*-disrupted mutants of *L. aerocolonigenes*, which accumulated non-glycosylated 11,11′-dichloro-CPA or rebeccamycin aglycone, respectively [21]. Full reconstitution of a pathway for dideschloro-rebeccamycin **6** was achieved by co-expressing *rebO*, *rebD*, *rebC*, *rebP*, *rebG* and *rebM* [30]. Therefore, the *N*-glycosyltransferase RebG is responsible for attachment of a D-glucose to the indolocarbazole aglycone, and the methyltransferase RebM methylates the sugar at the 4-hydroxy position, probably after glycosylation.

When we tried to introduce into *S. albus* some plasmids for constitutive expression of all the genes needed for biosynthesis of either rebeccamycin **12** or demethyl-rebeccamycin **11**, no transformants were obtained [30]. Apparently, the production of any of those compounds seemed to be lethal for the host. This problem was circumvented by including an additional gene, *rebT*, in the plasmids [30]. The *rebT* gene codes for a putative transmembrane transporter, which confers rebeccamycin resistance to the otherwise sensitive *S. albus* host [29], although *rebT* is not essential for rebeccamycin biosynthesis in *L. aerocolonigenes* [21]. Therefore, it appeared that only those derivatives that were both chlorinated and glycosylated resulted toxic for the bacterial host.

Dissection of staurosporine biosynthesis and generation of derivatives

Formation of staurosporine aglycone

As mentioned before, a DNA sequence from *S. longisporoflavus* DSM10189 was available in databases, containing some genes needed for the formation of the staurosporine sugar moiety [31]. On identification of the *reb* genes, we reported that the *S. longisporoflavus* DNA sequence encoded, among other uncharacterized ORFs, three genes showing high homology to *rebD* (uncomplete), *rebC* and *rebP*, respectively [29]. While we were working on the identification of staurosporine biosynthetic genes from *S. longisporoflavus* [28], the complete gene cluster for staurosporine (*sta*) biosynthesis was disclosed by Onaka and co-workers [20, 21] from a different microorganism, *Streptomyces* sp. TP-A0274 (Fig. 2b). Both *sta* gene clusters were essentially identical in the two staurosporine producers [28]. Putative functions were proposed for the *sta* genes, accounting for most of the structural differences between staurosporine and rebeccamycin (Fig. 2). Genes similar to *rebO*, *rebD* and *rebP* were found in *Streptomyces* sp. TP-A0274, but a *rebC* homolog was not reported [20, 21]. However, our own examination of the DNA sequence from *Streptomyces* sp. TP-A0274 revealed (as in the case of *S. longisporoflavus*) that a *rebC* homolog existed, which we named *staC* [30].

Therefore, both *reb* and *sta* gene clusters contained a similar set of genes for indolocarbazole core formation (*rebODCP*, *staODCP*) and, apparently, there was no additional gene in the staurosporine gene cluster that might be specifically responsible for the different oxidation state at the C-7 position of the aglycone. As an initial hypothesis, based on our results obtained for the rebeccamycin genes, we thought that: (a) *staO* and *staD* might be responsible for formation of CPA, which was a plausible intermediate for staurosporine, and (b) *staC* and *staP* might be involved in the conversion of CPA into staurosporine aglycone. To test this hypothesis, we expressed in *S. albus* a series of gene sets including *rebO* and *rebD* (for CPA formation) together with different combinations of *rebC*, *rebP*, *staC* and *staP* (Table 1). For this purpose, *staC* and *staP* were PCR-amplified from DNA of *S. longisporoflavus*. The results showed that *rebP* and *staP* were functionally equivalent, and any of them was sufficient for processing CPA into a mixture of the three indolocarbazoles **2–4**, differing at the C-7 position. When either *rebC* or *staC* was added to these gene combinations, a single product was obtained. Remarkably, the single product consisted of staurosporine aglycone **3** when using *staC*, or non-chlorinated rebeccamycin aglycone **2** (= arcyriaflavin A) when using *rebC* (Table 1).

In conclusion, a monooxygenase RebC or StaC seems to be needed in an indolocarbazole pathway for the determination of the C-7 oxidation state during the conversion of a CPA intermediate into the indolocarbazole core, but each one of the two enzymes determines a different oxidation state in the final product. This finding allowed us to generate a series of 7-deoxo derivatives (“staurosporine-like”) in *S. albus*, by replacing *rebC* with *staC* in our previous gene combinations (compounds **13–20**, Fig. 4b) [30].

Sugar moiety: biosynthesis and double attachment

Using as a probe a fragment of *staD* whose sequence was available at the time [29, 31], we screened an *S. longisporoflavus* genomic library constructed in pKC505.

Table 1 Gene combinations expressed in *S. albus* to yield indolocarbazole cores with different C-7 oxidation state

Gene combination	Compounds produced
rebO + *rebD*	CPA
rebO + *rebD* + *rebC*	CPA
rebO + *rebD* + *staC*	CPA
rebO + *rebD* + *rebP*	AF + K252c + 7-hydroxy-K252c
rebO + *rebD* + *staP*	AF + K252c + 7-hydroxy-K252c
rebO + *rebD* + *rebC* + *rebP*	AF
rebO + *rebD* + *rebC* + *staP*	AF
rebO + *rebD* + *staC* + *rebP*	K252c
rebO + *rebD* + *staC* + *staP*	K252c

CPA Chromopyrrolic acid; *AF* Arcyriaflavin A; *K252c* Staurosporine aglycone

The selected cosmid clones were separately introduced into *S. albus*, and one of them (cos32D1) conferred the ability to produce staurosporine. From this cosmid, we identified and completely sequenced a 4.5-kb fragment containing the *staG* and *staN* genes, encoding a glycosyltransferase and a cytochrome P450 enzyme [28]. Partial sequencing of the *sta* locus confirmed the existence of a gene cluster with a genetic organization identical to that reported for *Streptomyces* sp. TP-A0274 (Fig. 2b).

We reconstituted the complete staurosporine pathway in *S. albus* by co-expressing *reb* and *sta* genes in a two-plasmid system [28]. The first plasmid (''aglycone plasmid'') contained genes required for aglycone formation (*rebO*, *rebD*, *staC*, *rebP*), together with two genes that were the likely candidates for the double attachment of the sugar moiety: *staG* (coding for a glycosyltransferase) and *staN* (coding for a cytochrome P450 enzyme). The second plasmid (''sugar plasmid'') included a 6.9 kb fragment from the chromosome of *S. longisporoflavus* harboring a group of genes proposed to be involved in the biosynthesis of the sugar moiety. This DNA fragment contained the following genes: *staMA* (*N*-methyltransferase), *staJ* (2,3-dehydratase), *staK* (4-ketoreductase), *staI* (3-aminotransferase), *staE* (3,5-epimerase) and *staMB* (4-*O*-methyltransferase) (Fig. 2b). When both plasmids were introduced into *S. albus*, the resulting strain produced staurosporine [28]. This result indicated that the *S. albus* host was providing the first two enzymatic activities required for deoxysugar biosynthesis, as the *staA* and *staB* genes (Fig. 2) were not included in the plasmids.

Then we tested the effect of independently removing either *staG* or *staN* from the ''aglycone plasmid''. When the glycosyltransferase *staG* gene was removed, no glycosylated product could be detected in *S. albus*. On the other hand, removing the *staN* gene resulted in the production of a glycosylated product consisting of holyrine A, a compound previously isolated as a minor product from a staurosporine-producing marine actinomycete (compound **33**, Fig. 4d) [36]. In holyrine A, an L-ristosamine sugar moiety is singly attached through an *N*-glycosidic bond to the aglycone. Holyrine A differs from staurosporine not only in the lacking of one of the sugar-aglycone linkages, but also in the absence of the two sugar methylations (despite the presence of both methyltransferase genes *staMA* and *staMB* in the ''sugar plasmid''). These results suggested that: (a) glycosyltransferase StaG formed an *N*-glycosidic bond between the aglycone and L-ristosamine, (b) the cytochrome P450 enzyme (StaN) was involved in establishing a non-glycosidic linkage between sugar and aglycone, (c) the non-glycosidic linkage was formed only after the glycosydic bond had been made, and (d) both sugar methylations were late steps in staurosporine biosynthesis, taking place only after the second linkage had been established [28]. Similar conclusions were recently drawn from experiments made with a *staN*-disrupted mutant of *Streptomyces* sp. TP-A0274 [22].

Our conclusions were additionally supported by feeding holyrine A to a series of *S. albus* strains expressing different combinations of *staN*, *staMA* and *staMB* [28]. When all three genes were co-expressed, holyrine A was converted into staurosporine, supporting its role as an intermediate. Independent removal of either *staMA*, *staMB*, or both resulted in the production of *N*-demethyl-, *O*-demethyl-, or *N*-demethyl-*O*-demethyl-staurosporine, indicating that each one of the methylation steps can occur in the absence of the other. However, the single deletion of *staN* resulted in no bioconversion, further supporting that both methylations are late steps in the pathway [28]. Concerning the *O*-methylation event, it has been previously reported that *O*-demethyl-staurosporine could be converted in vitro into staurosporine by using cell-free extracts of *S. longisporoflavus*, and this methyltransferase activity was partially purified and characterized [35].

In summary, staurosporine biosynthesis can be depicted as follows (Fig. 3b). The formation of the aglycone seems to be performed by a set of four enzymes (StaO, StaD, StaC, StaP) with activities similar to those of their homologs in the rebeccamycin pathway. Very recently, the formation of CPA by StaD has been confirmed [2]. However, lacking a halogenase, all the intermediates in the staurosporine pathway are nonchlorinated. Another key difference between the pathways for the two aglycones resides in the activities of monooxygenases StaC and RebC, which determine different oxidation states at the C-7 position of the aglycone. The genesis of the sugar moiety in staurosporine is much more complex than that of rebeccamycin. In the rebeccamycin pathway, a common sugar (D-glucose) is attached to the aglycone through a single *N*-glycosidic bond and then suffers an *O*-methylation. For staurosporine biosynthesis, six enzymes are apparently required for the formation of an L-ristosamine deoxysugar prior to its attachment to the aglycone (Fig. 3b). Moreover, sugar attachment involves the participation of a glycosyltransferase (StaG) to form an *N*-glycosidic bond, followed by the action of a cytochrome P450 enzyme (StaN) to establish a second C–N bond between sugar and aglycone. Finally, the sugar moiety is methylated by methyltransferases StaMA and StaMB to yield staurosporine.

Generation of derivatives with variations at the sugar moiety

In order to test if the StaG and StaN enzymes were capable of attaching novel sugars to the staurosporine aglycone, we used several modified versions of our two-plasmid system [28]. While keeping the ''aglycone plasmid'' unaltered, we replaced the ''sugar plasmid'' with any of four other plasmids, each one directing the biosynthesis of a different deoxysugar: pRHAM (L-rhamnose), pLN2 (L-olivose), pLNBIV (L-digitoxose) and pLNR (D-olivose). These plasmids have been

successfully used before for the modification of other natural products [5, 26, 27]. Using the two-plasmid system in *S. albus*, we produced a new series of glycosylated derivatives consisting of staurosporine aglycone attached to any of the four deoxysugars (compounds **34–40**, Fig. 4d). Each one of those strains producing L-sugars yielded a mixture of two glycosylated compounds, with the L-sugar attached to the aglycone through one or two linkages, respectively (e.g., compounds **34** and **35**). On the other side, the strain producing D-olivose yielded a single glycosylated compound **40**, with the sugar moiety attached only through a glycosidic bond [28]. In other words, while glycosyltransferase StaG was able of attaching any one of the four tested deoxysugars to the staurosporine aglycone, the cytochrome P450 enzyme (StaN) established the second linkage only if the carbohydrate was an L-sugar.

Concluding remarks

The indolocarbazole family of natural products is a source of lead compounds with potential therapeutic applications in the treatment of cancer and neurodegenerative disorders. As an addition to chemical synthesis, the introduction of biological processes might help in the production of indolocarbazole derivatives in a cost-effective way and with lower environmental problems. The identification of the genes responsible for indolocarbazole biosynthesis provided the necessary "toolkit" for such purpose. By making use of this toolkit, 40 indolocarbazole derivatives (or its precursors) have already been produced, in a rational way, through the expression of selected genes in a convenient bacterial host.

Indolocarbazole biosynthetic pathways appear to possess two features that make them promising targets for combinatorial biosynthesis and rational metabolic pathway engineering: (a) indolocarbazole pathways are relatively simple (as compared to pathways for other natural products such as complex polyketides or nonribosomal peptides), and (b) at least some of the indolocarbazole biosynthetic enzymes seem to have useful degrees of substrate flexibility, as they are able to process different intermediates to yield novel derivatives in vivo. Current progress in the characterization of key enzymes opens the way, additionally, for in vitro methods (e.g., chemoenzymatic) to produce and/or modify indolocarbazoles.

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