Combinatorial biosynthesis of antitumor indolocarbazole compounds

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Rebeccamycin and staurosporine are natural products with antitumor properties, which belong to the family of indolocarbazole alkaloids. An intense effort currently exists for the generation of indolocarbazole derivatives for the treatment of several diseases, including cancer and neurodegenerative disorders. Here, we report a biological process based on combinatorial biosynthesis for the production of indolocarbazole compounds (or their precursors) in engineered microorganisms as a complementary approach to chemical synthesis. We have dissected and reconstituted the entire biosynthetic pathway for rebeccamycin in a convenient actinomycete host, *Streptomyces albus*. This task was achieved by coexpressing different combinations of genes isolated from the rebeccamycin-producing microorganism. Also, a gene (*staC*) was identified in staurosporine-producing microbes and was shown to have a key role to differentiate the biosynthetic pathways for the two indolocarbazoles. Last, incorporation of the *pyrH* and *thal* genes, encoding halogenases from different microorganisms, resulted in production of derivatives with chlorine atoms at novel positions. We produced >30 different compounds by using the recombinant strains generated in this work.

cancer

Indolocarbazole alkaloids constitute a group of natural products that have attracted great attention because of their original structural features and potential therapeutic applications (1). Most of them are characterized by possessing an indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole core with a sugar attached (Fig. 1). Various biological activities have been reported for indolocarbazoles, but the greatest interest is focused on compounds that possess antitumor and neuroprotective properties (2–4). These activities may be due to different mechanisms of action, including DNA intercalation, inhibition of DNA topoisomerases, and inhibition of protein kinases. Great efforts are made to generate indolocarbazole derivatives with improved properties for the treatment of cancer, neurodegenerative disorders, and diabetes-associated pathologies, and several analogs have entered clinical trials (2–7).

Studies on the biosynthesis of rebeccamycin and staurosporine in the producing microorganisms have shown that the indolocarbazole core is formed by decarboxylative fusion of two tryptophan-derived units, whereas the sugar moiety is derived from glucose (8, 9). Recently, we cloned and characterized the rebeccamycin biosynthetic gene cluster from the actinomycete *Lechevalieria aerocolonigenes* (formerly *Saccharotrix aerocolonigenes*) (10). Expression of the entire gene cluster and of different subsets of genes in a heterologous host yielded rebeccamycin and three biosynthetic intermediates (10). The same cluster was later isolated by other researchers (11, 12) and expressed at a low level in *Escherichia coli* (12), and different insertional inactivation mutants were generated in the producer organism (11). The entire staurosporine gene cluster has been isolated from *Streptomyces* sp. TP-A0274 (13), although a previous patent application reported the identification of some genes involved in biosynthesis of the staurosporine sugar moiety in *Streptomyces longisporoflavus* (14).

Combinatorial biosynthesis is a recent addition to the metabolic engineering toolbox by which genes responsible for individual metabolic reactions from different organisms are combined to generate metabolic pathways to biosynthesize products that were previously inaccessible or difficult to obtain (15–20). Here, we report the use of a combinatorial biosynthesis approach for dissection and reconstitution of the entire rebeccamycin pathway. This combinatorial approach has also been extended to create metabolic pathways by coexpression of rebeccamycin genes with selected genes from other microorganisms to produce >30 indolocarbazole derivatives in an actinomycete host.

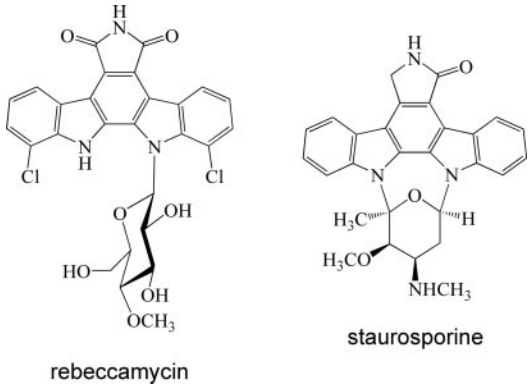

Fig. 1. Structures of rebeccamycin and staurosporine.

Methods

Bacterial Strains, Culture Conditions, and Vectors. *L. aerocolonigenes* ATCC39243, *S. longisporoflavus* DSM10189, *Streptomyces albus* J1074 (21), and *E. coli* XL1-Blue (22) were used in this work. Plasmids pEM4 (23), pWHM3 (24), and pKC796 (24) have been described. Vector pUWL201 was obtained from U. Wehmeier and W. Piepersberg (Bergische Universitaet, Wuppertal, Germany). Plasmids pHI1536 and pSZ1050, containing a fragment of genomic DNA from *Streptomyces albogriseolus* and the *pyrH* gene from *Streptomyces rugosporus*, respectively, were provided by K.-H. van Pée (Institut für Biochemie, Technischen Universität, Dresden, Germany). For indolocarbazole production, *S. albus* strains were cultured by using R5A medium (described as "modified R5 medium" in ref. 25) as described in *Supporting Methods*, which is published as supporting information on the PNAS web site.

DNA Manipulation and Construction of Plasmids. DNA manipulations and transformation of *S. albus* protoplasts followed standard procedures (22, 24). Individual genes from rebeccamycin and staurosporine gene clusters were isolated by PCR using total DNA from the corresponding organism and primers indicated in Table 3, which is published as supporting information on the PNAS web site. The *thal* gene was amplified by PCR from pHI1536 using primers shown in the same table. The *pyrH* gene was excised from pSZ1050

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as a *Spe*I fragment. We also used an additional version of the *rebH* gene, including a downstream putative transcriptional terminator and a DNA fragment containing the four genes *rebO*, *rebD*, *rebC*, and *rebP* with their natural translational-coupling organization (see *Supporting Methods*). The isolated genes were spliced together (in tandem) by using restriction sites incorporated into the PCR primers. We used pWHM3, pEM4, and pUWL201 as replicative shuttle vectors, and we used pKC796 as an integrative shuttle vector. Plasmids derived from pWHM3 and pKC796 required the addition of the promoter *ermE**p, which was obtained as a *Hin*dIII–*Xba*I fragment from pEM4.

HPLC–MS Analysis. HPLC–MS analyses were performed with an Alliance chromatographic module coupled to a 2996 photodiode array detector and a ZQ4000 mass spectrometer (Waters, Micromass). We used a Symmetry C18 column (2.1 × 150 mm, Waters), and acetonitrile and 1% formic acid in water were used as solvents. Elution started with 10% acetonitrile for 4 min, followed by a linear gradient up to 88% acetonitrile at 30 min and a final isocratic hold with 100% acetonitrile for 5 min at a flow rate of 0.25 ml/min. Mass analysis was done by electrospray ionization in the positive mode, with a capillary voltage of 3 kV and cone voltages of 20, 60, and 100 V.

Purification of Compounds and Structure Elucidation. The following compounds were purified from *S. albus* harboring the plasmids mentioned in brackets: chromopyrrolic acid **1** (pCS014), K-252c or staurosporine aglycone **3** (pCS039), 11-chlorochromopyrrolic acid **7** (pCS018), 1-chloroarcyriaflavin **9** (pCS020), 9-chlorochromopyrrolic acid **21** (pCS049) and 3-chloroarcyriaflavin **23** (pCS037). For a detailed description of the purification process, see *Supporting Methods*.

Five indolocarbazole derivatives were characterized by HRMS and by ^{1}H and ^{13}C NMR spectroscopy for the confirmation of the expected structures: **1**, **3**, **7**, **21**, and **23**. The newly isolated compounds **1** and **3** showed identical NMR and MS data with those published in refs. 11, 26, and 27. Compounds **7** and **21** were identified through their MS and NMR data (Tables 4 and 5, which are published as supporting information on the PNAS web site) in comparison with the known (11, 26) compounds **1** and 11,11′-dichlorochromopyrrolic acid **8**. Compound **23** was identified as 3-chloroarcyriaflavin by comparison of its NMR data (Table 6, which is published as supporting information on the PNAS web site) with those of arcyriaflavin **2** (10).

***In Vitro* Antiproliferative Activity.** The antitumor activity of selected compounds was tested against 14 tumor cell lines. Quantitative measurement of cell growth and viability was carried out by using a colorimetric assay with sulforhodamine reaction (28).

Results

Experimental Strategy for Combinatorial Biosynthesis. The genes of interest (Table 1) were isolated by PCR and organized in an operon-like fashion downstream of promoter *ermE**p (*ermE*p ΔTGG) from *Saccharopolyspora erythraea*, which allows constitutive expression in actinomycete hosts (29). These artificial operons were cloned in *E. coli–Streptomyces* shuttle vectors, consisting of either a high-copy number replicative plasmid or a site-specific integrative plasmid in *Streptomyces*. In some cases, to facilitate transcription of long operons, the required genes were organized in two sets (each of which was preceded by a copy of *ermE**p), and they were independently cloned into two compatible plasmids (one integrative and one replicative). A selection of the constructed gene combinations is shown in Fig. 2, and a full relation is given in Table 7, which is published as supporting information on the PNAS web site. Plasmids were introduced into a convenient actinomycete host, *S. albus*, and the transformed strains were analyzed for indolocarbazole production by HPLC–MS. The compounds generated (Table 2) were identified based on their HPLC elution time, UV-visible absorption characteristics, and mass spectra. Further structural elucidation of selected compounds was done by HRMS and NMR. In this article, the names for gene combinations are abbreviated when possible, for readability; for example, a gene combination such as *rebO* + *rebD* + *staC* is referred to as *rebOD*+*staC* hereafter.

Table 1. Genes used in this study

Gene	Protein function
*rebO**	Amino acid oxidase
*rebD**	Chromopyrrolic acid synthase
*rebC**	FAD-containing monooxygenase
*rebP**	P450 oxygenase
*rebG**	*N*-glycosyltransferase
*rebM**	Sugar *O*-methyltransferase
*rebH**	Tryptophan 7-halogenase
*rebF**	Flavin reductase
*rebT**	Integral membrane transporter
staC†	FAD-containing monooxygenase
staP†	P450 oxygenase
thaI‡	Tryptophan 6-halogenase
pyrH§	Tryptophan 5-halogenase

*From *L. aerocolonigenes*.
†From *S. longisporoflavus*.
‡From *S. albogriseolus*.
§From *S. rugosporus*.

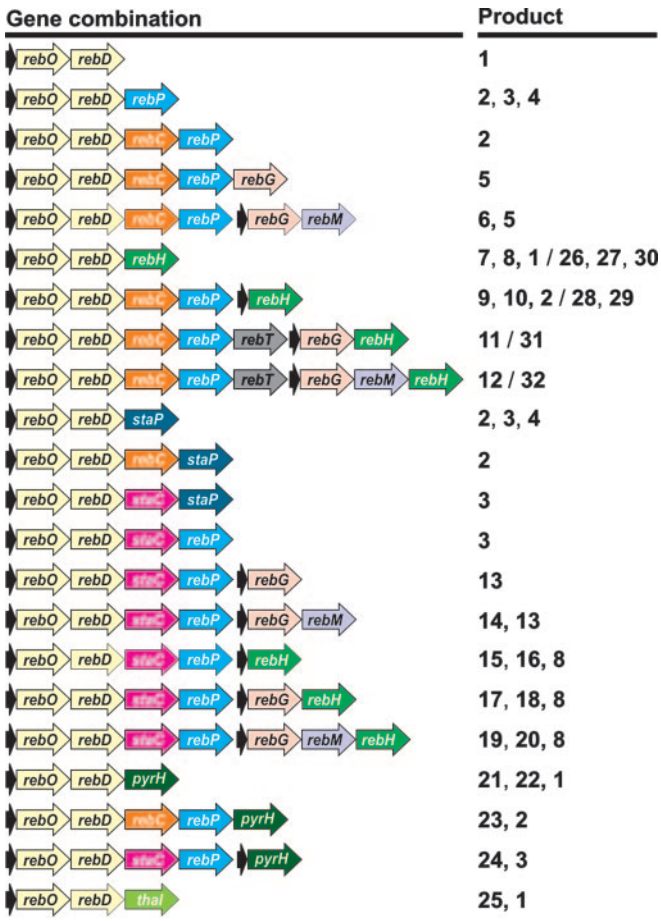

Fig. 2. A selection of gene combinations and the obtained products. Black arrows indicate the *ermE**p promoter.

Table 2. Products generated in this work

Product no.	Product	min (m/z)*
1	CPA†‡	19.0 (386)
2	AF‡	23.7 (326)
3	K252c†‡	21.0 (312)
4	7-hydroxy-K252c	19.3 (328)
5	Glucosyl-AF	19.1 (488)
6	Dideschlororebeccamycin‡	20.6 (502)
7	11-chloro-CPA†‡	20.5 (420)
8	11,11′-dichloro-CPA	21.9 (454)
9	1-chloro-AF‡	26.3 (360)
10	Rebeccamycin aglycon	29.5 (394)
11	4′-*O*-demethyl-rebeccamycin	20.8 (556)
12	Rebeccamycin‡	23.0 (570)
13	Glucosyl-K252c	16.6 (474)
14	Methylglucosyl-K252c	18.1 (488)
15	1-chloro-K252c	23.4 (346)
16	1,11-dichloro-K252c	26.7 (380)
17	Glucosyl-1-chloro-K252c	17.0 (508)
18	Glucosyl-1,11-dichloro-K252c	18.6 (542)
19	Methylglucosyl-1-chloro-K252c	18.7 (522)
20	Methylglucosyl-1,11-dichloro-K252c	20.4 (556)
21	9-chloro-CPA†	20.2 (420)
22	9,9′-dichloro-CPA	21.3 (454)
23	3-chloro-AF†	25.9 (360)
24	3-chloro-K252c	23.1 (346)
25	10-chloro-CPA	20.7 (420)
26	11-bromo-CPA	20.9 (464)
27	11,11′-dibromo-CPA	22.7 (542)
28	1-bromo-AF	26.8 (404)
29	1,11-dibromo-AF	30.5 (482)
30	11-bromo-11′-chloro-CPA	22.4 (498)
31	Glucosyl-1,11-dibromo-AF	21.1 (646)
32	Methylglucosyl-1,11-dibromo-AF	23.3 (658)

CPA, chromopyrrolic acid; AF, arcyriaflavin; K252c, staurosporine aglycone.
*Retention time and $[M+H]^+$ molecular ion in HPLC–MS.
†Structure also confirmed by HRMS and NMR.
‡Test for antitumor activity shown in Table 8, which is published as supporting information on the PNAS web site.

Dissection and Reconstitution of the Rebeccamycin Biosynthetic Pathway. We sought to dissect and reconstitute the complete rebeccamycin biosynthetic pathway in *S. albus* by expressing a collection of gene combinations that were constructed by successive assembling of rebeccamycin (*reb*) genes (Figs. 2 and 3*A*). Based on the functions proposed for the *reb* gene products (Table 1) (10–12), it was hypothesized that the simplest bisindole intermediate might be produced by the joint action of the two proteins RebO and RebD. The *rebO* and *rebD* genes were coexpressed in *S. albus*, and the resultant strain accumulated a compound that was purified and studied by MS and NMR. This product was identified as 3,4-bis(indol-3-yl)pyrrole-2,5-dicarboxylic acid **1** (Fig. 3*A*), identical to a natural product isolated from *Chromobacterium violaceum* (26) and from *Lycogala epidendrum* (30). This natural product has been called "chromopyrrolic acid" (used hereafter) or "lycogalic acid."

Coexpression of two additional genes *rebC* and *rebP* (together with *rebO* and *rebD*) was needed for efficient production of the indolopyrrolocarbazole core (i.e., dideschlororebeccamycin aglycone **2**). This compound is identical to "arcyriaflavin A," which is a natural product with antiviral properties isolated from different organisms (31). We also produced arcyriaflavin by feeding chromopyrrolic acid to a *S. albus* strain coexpressing *rebCP*. When *rebC* was omitted, lower yields of three versions of the indolopyrrolocarbazole core appeared: arcyriaflavin **2**, staurosporine aglycone **3**, and 7-hydroxystaurosporine aglycone **4** (Figs. 2 and 3*A*). However, when *rebP* was omitted, no structural modification of chromopyrrolic acid occurred.

The addition of *rebG*, encoding a glycosyltransferase, to a gene combination such as *rebOD* did not result in modification of chromopyrrolic acid. Similarly, when we fed chromopyrrolic acid to a *S. albus* strain expressing *rebG*, no glycosylated chromopyrrolic acid was observed. However, coexpression of *rebODCPG* successfully produced a glycosylated compound, glucosylarcyriaflavin **5**. Production of this compound was achieved also by feeding arcyriaflavin to a strain expressing *rebG*. When the *rebM* gene was added to the *rebODCPG* combination, the resulting strain produced dideschlororebeccamycin **6**.

Incorporation of chlorine atoms into rebeccamycin might take place at the first step in the pathway through tryptophan halogenation catalyzed by RebH. In agreement with this hypothesis, no chlorination of chromopyrrolic acid or arcyriaflavin was detected when we fed either of these compounds to a *S. albus* strain expressing *rebH*. In contrast, coexpression of *rebODH* or *rebODCPH* yielded chloro and dichloro versions of chromopyrrolic acid (compounds **7** and **8**) and arcyriaflavin (compounds **9** and **10**), respectively (Fig. 3*A*). To confirm the exact position of chlorination, compound **7** was purified and analyzed by HRMS and NMR studies that supported the structure of 11-chlorochromopyrrolic acid **7** (Table 4). Addition of *rebF*, encoding a putative reductase producing reduced flavin for halogenation, did not increase the yield of chlorinated compounds significantly. This result suggests that RebF, if needed, can be replaced by host enzymes, as has been shown for other $FADH_2$-dependent halogenases (32).

We have shown (10) that rebeccamycin inhibits the growth of *S. albus*, unless a protecting *rebT* gene (encoding a putative transmembrane transporter) is present. In fact, no transformants could be obtained when trying to coexpress either *rebODCPGH* or *rebODCPGMH*, suggesting that both 4′-*O*-demethylrebeccamycin and rebeccamycin were toxic for the host strain. We could obtain transformants that produced the otherwise toxic compounds **11** and **12** (Fig. 3*A*) only when *rebT* was also added to the gene combinations (Fig. 2). In summary, dissection and reconstitution of the rebeccamycin biosynthetic pathway yielded 12 compounds (Table 2).

Conversion of Rebeccamycin-Like into Staurosporine-Like Derivatives: Pivotal Role of RebC vs. StaC. One of the structural differences between rebeccamycin and staurosporine is found at the C-7 position of the aglycone; a carbonyl function is present in rebeccamycin but absent in staurosporine (Fig. 1). Comparison of the published gene clusters for biosynthesis of these indolocarbazoles revealed a common core of three genes encoding enzymes participating in early biosynthetic steps (i.e., *rebO-rebD-rebP* and *staO-staD-staP*), whereas a fourth gene, *rebC*, was described to be present only in the rebeccamycin cluster (10–13). However, after a careful examination of the available DNA sequences encoding staurosporine biosynthetic genes from *Streptomyces* sp. TP-A0274 (13) and *S. longisporoflavus* (14), we found, in both cases, a previously uncharacterized gene coding for a protein that is highly similar to RebC (65.7% and 64.7% identity, respectively). We named this gene *staC* (Table 1).

As an initial hypothesis, we predicted that *staO* and *staD* would probably direct the formation of chromopyrrolic acid, as *rebO* and *rebD* did. Therefore, *staC* and/or *staP* might be responsible for the structural difference at the C-7 position between rebeccamycin and staurosporine. We generated a series of plasmids, including *rebOD* and different combinations of *rebC*, *rebP*, *staC*, and *staP* (Fig. 2), and these gene combinations were expressed in *S. albus*. The results showed that the replacement of *rebP* by *staP* in different constructs did not alter the compounds that were produced, indicating that both genes were functionally equivalent (Fig. 3*B*). However, when *rebC* was substituted by *staC* (as in *rebODP+staC* or *rebOD+staCP*), the staurosporine aglycone **3** was produced. This

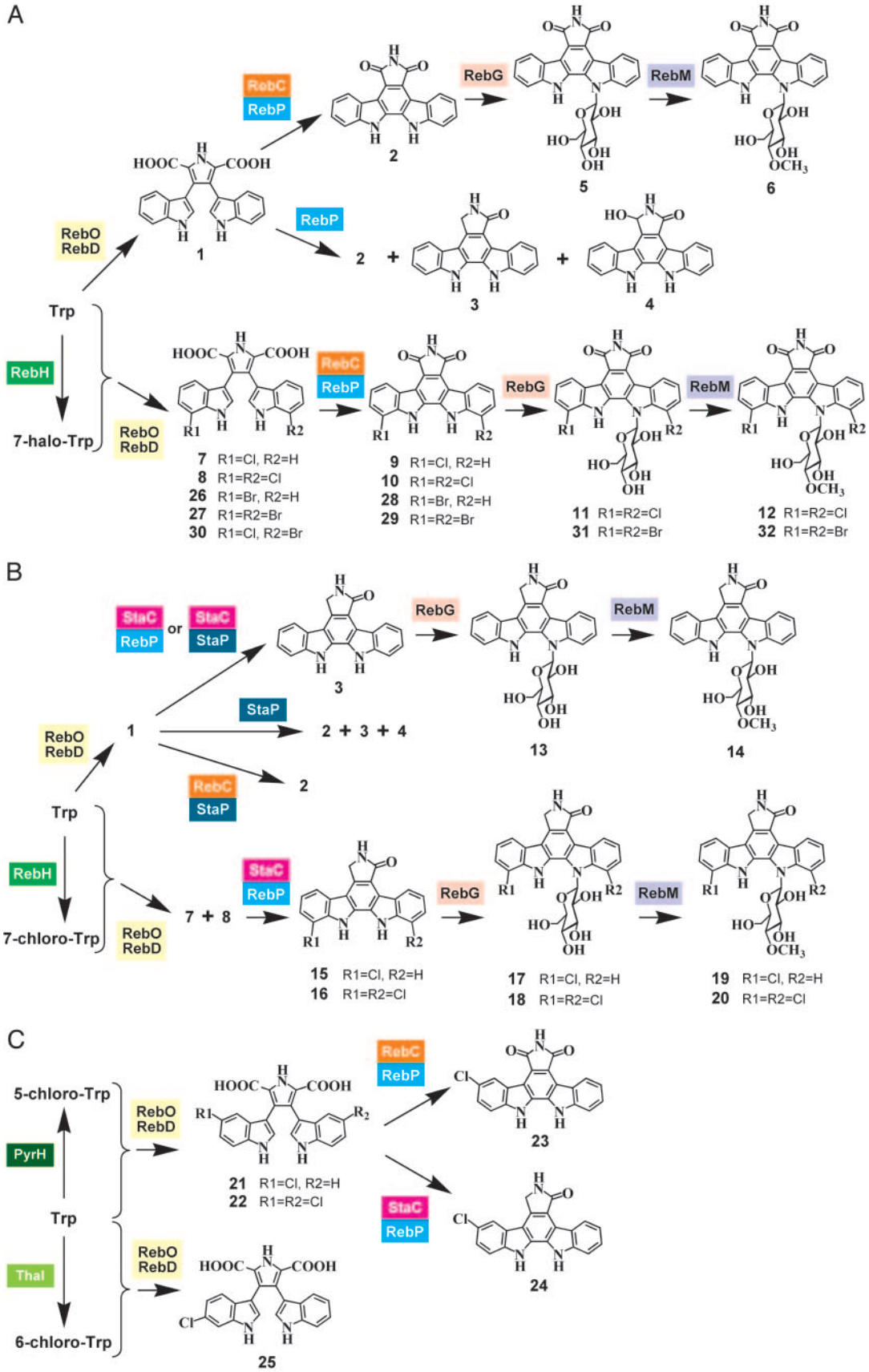

Fig. 3. Biosynthetic pathways for production of indolocarbazole compounds. (*A*) Dissection and reconstitution of rebeccamycin biosynthesis. (*B*) Pathways constructed by coexpression of rebeccamycin and staurosporine genes to generate derivatives of staurosporine aglycone. (*C*) Pathways constructed by coexpression of rebeccamycin, staurosporine, pyrroindomycin, and thienodolin genes to generate bisindole compounds with novel halogenations.

product was purified, and its structure was confirmed to be identical to the natural product K252c, or staurosporine aglycone (27). Therefore, *rebC* and *staC* seem to determine different oxidation states at position C-7 of the aglycone. Also, RebC (or StaC) seems to be needed for efficient completion of the reaction to yield a single product. Consequently, we were able to produce either rebeccamycin-type aglycones (by using *rebODCP*) or staurosporine-type asymmetrical aglycones (by using *rebODP+staC*).

Our next goal was to generate "hybrid" indolocarbazoles by modifying the staurosporine aglycone by using rebeccamycin chlorination, glycosylation, and sugar methylation activities. For this purpose, additional *reb* genes were incorporated to the previous combination *rebODP+staC*, which already produced K252c **3** (Fig. 2). Sequential addition of *rebG* and *rebM* yielded glucosyl-K252c **13** and methylglucosyl-K252c **14**, respectively (Fig. 3*B*). However, the single incorporation of *rebH* resulted in production of chloro-K252c **15** and dichloro-K252c **16**. However, this strain accumulated dichlorochromopyrrolic acid **8** also, indicating that carbazole ring closure catalyzed by StaC and RebP was not efficient on this substrate. Last, strains in which *rebGH* or *rebGMH* were incorporated into the starting combination *rebODP+staC* yielded the chloro and dichloro versions of glucosyl-K252c (compounds **17** and **18**) or methylglucosyl-K252c (compounds **19** and **20**), respectively (Fig. 3*B*).

Generation of Idolocarbazoles with Modified Halogenation Pattern. In an effort to increase the size of our combinatorial library, we used the following two additional genes: *pyrH*, encoding a tryptophan 5-halogenase involved in pyrroindomycin biosynthesis in *S. rugosporus* LL-42D005 (K. H. van Pée, personal communication), and *thal*, encoding a tryptophan 6-halogenase participating in thienodolin biosynthesis in *S. albogriseolus* (32) (Table 1). We anticipated that 5- and 6-chlorotryptophan intermediates, produced by PyrH and Thal, respectively, could be accepted as substrates by rebeccamycin enzymes to yield new compounds.

Coexpression of *rebOD+pyrH* yielded chlorochromopyrrolic acid **21** and dichlorochromopyrrolic acid **22**, whereas combination *rebODCP+pyrH* produced chloroarcyriaflavin **23** (Fig. 3*C*). We could also produce chloro-K252c **24** by using the gene combination *rebODP+staC+pyrH*. The exact position of chlorination was validated by solving the structures of compounds **21** and **23** (Tables 5 and 6), which were confirmed to be 9-chlorochromopyrrolic acid **21** and 3-chloroarcyriaflavin **23**. However, gene *thal* allowed production of chlorochromopyrrolic acid **25**. Because Thal is a tryptophan 6-halogenase, it was expected that the resulting analog would possess a chlorine atom at position C-10, although this hypothesis must be tested by means of additional characterization of the compound. The major bisindole products of all our strains expressing *pyrH* or *thal* were nonchlorinated compounds, probably reflecting a low production of 5- and 6-chlorotryptophan and/or an inefficient conversion of these substrates by rebeccamycin enzymes.

Previously, a bromo analog of rebeccamycin was obtained from *L. aerocolonigenes* when grown in a medium supplemented with potassium bromide (33). In a similar way, we intended to produce brominated versions of our compounds by replacing chloride with bromide in the fermentation medium of the *S. albus* strains. This approach led to the production of bromo derivatives of chromopyrrolic acid (compounds **26** and **27**), arcyriaflavin (compounds **28** and **29**), glucosylarcyriaflavin (compound **31**), and methylglucosylarcyriaflavin (compound **32**) (Fig. 3*A*). Some products containing both a bromine and a chlorine atom could also be identified, such as bromo-chloro-chromopyrrolic acid **30**.

***In Vitro* Antiproliferative Activity.** Eight representative compounds were subjected to cytotoxicity assays against 14 tumor cell lines corresponding to nine cancer types. A summary of the results is given in Table 8. The analyzed compounds can be divided into the following four groups of decreasing activity: (*i*) staurosporine (included as a control); (*ii*) rebeccamycin and dideschlororebeccamycin; (*iii*) arcyriaflavin, K252c, and 1-chloroarcyriaflavin; and (*iv*) chromopyrrolic acid and 1-chlorochromopyrrolic acid.

Discussion

Natural products, especially from plants and microorganisms, are a rich source for drug leads, with biomedical and industrial applications (34). Although organic chemistry methods are routinely used to synthesize and modify natural products, harvesting the product (or a modifiable precursor) from the natural source is often the only cost-effective way of production. Furthermore, chemical-synthetic methods can cause substantial environmental problems. Therefore, biological processes are of growing importance as an addition to chemical synthesis. In particular, metabolic engineering is becoming a helpful tool for the discovery, development, and scale-up of useful compounds (19, 35).

In this article, we have provided proof-of-principle for the rational generation of indolocarbazole alkaloids in genetically engineered microorganisms. By using a combinatorial approach, we have dissected and reconstituted the entire rebeccamycin pathway in a heterologous host, with concomitant identification of 12 bisindole intermediates. Biosynthesis of indolocarbazole natural products can be divided into five stages catalyzed by different sets of enzymes (Fig. 3), which are (*i*) tryptophan modification (halogenation) by RebH; (*ii*) dimerization by RebO/RebD or StaO/StaD; (*iii*) decarboxylative ring closure by RebC/RebP or StaC/StaP; (*iv*) glycosylation by RebG or StaG; and (*v*) sugar modification by RebM or several enzymes for staurosporine before and/or after glycosylation (refs. 10–13 and this article). Stages *ii* and *iii* constitute the central reactions in the pathway, whereas the other stages can be considered as accessories for "tailoring" the alkaloid skeleton. As a result of dimerization (stage *ii*), chromopyrrolic acid **1** (for staurosporine) or dichlorochromopyrrolic acid **8** (for rebeccamycin) seem to be the first bisindole intermediates in indolocarbazole biosynthesis. Therefore, carbazole ring closure occurs after pyrrole formation, in contrast to the hypothesis given in ref. 12. In both rebeccamycin and staurosporine biosyntheses, decarboxylative ring closure appears to be catalyzed by a P450 protein (RebP or StaP) acting on a chromopyrrolic intermediate to yield the indolopyrrolocarbazole core. However, in the absence of a crucial monooxygenase (RebC or StaC), the P450 enzyme seems to be unable to determine the oxidation state at position C-7 of the molecule, because three versions of the indolopyrrolocarbazole appeared: arcyriaflavin **2**, staurosporine aglycone **3**, and 7-hydroxystaurosporine aglycone **4** (Fig. 3 *A* and *B*). However, when RebC was present, efficient production of arcyriaflavin as a single product occurred. Also, we have identified a gene (*staC*), which codes for a monooxygenase highly similar to RebC, in staurosporine-producing microorganisms. When *rebC* was replaced by *staC*, a single indolopyrrolocarbazole was also obtained, but this time consisting of staurosporine aglycone. In conclusion, a monooxygenase (RebC or StaC) was needed for efficient production of a single product, and more interestingly, the choice of *rebC* vs. *staC* diverted the pathway into either rebeccamycin- or staurosporine-type compounds at stage *iii*. This finding allowed us to produce eight staurosporine aglycone derivatives by using *staC* in combination with several rebeccamycin genes.

To increase the number of generated compounds, we worked also on the first biosynthetic stage, i.e., tryptophan modification. Two additional genes were used, *pyrH* and *thal*, that encoded tryptophan halogenases with regioselectivities different to that displayed by RebH. This approach allowed the production of five derivatives containing chlorine atoms located at novel positions (Fig. 3*C*). Moreover, modifying the first biosynthetic stage was also achieved by fermentation of recombinant strains in a medium containing bromide instead of chloride, resulting in five bromo analogs (Fig. 3*A*).

Note that some compounds identified in this work might actually consist of a mixture of isomers that could not be separated by HPLC. For example, asymmetrical molecules such as staurosporine aglycone **3** could be glycosylated either at the 12-*N* or at the 13-*N* position by RebG, but only one of the possible structures **13** is shown in Fig. 3. In relation to this possibility, it has been reported that a *rebC* mutant of *L. aerocolonigenes* produced 7-deoxo-7-hydroxyrebeccamycin consisting of a mixture of 12-*N*- and 13-*N*-glycosides that are not separable by HPLC (11).

Studies have shown that some indolocarbazole compounds, such as rebeccamycin, inhibited the growth of streptomycete strains, whereas other indolocarbazole compounds, such as staurosporine, allowed growth but affected cell differentiation in the tested strains (36, 37). Therefore, a possibility existed at the beginning of this study that some of the generated derivatives could affect the growth of the host, *S. albus*. Actually, we observed toxic effects associated to the production of two of them: rebeccamycin **12** (noted in refs. 10 and 37) and 4′-*O*-demethylrebeccamycin **11**. However, these toxicity problems were overcome by coexpressing *rebT*, which is a gene conferring rebeccamycin resistance to the microbial host (10).

Also, a selected group of compounds was tested for antitumor activity (Table 8). The most active compounds were the glycosides, followed by the aglycones, and last, the chromopyrrolic derivatives. The importance of the sugar moiety for the biological activity of indolocarbazoles has been reported (3, 38).

In summary, this approach provides microbial hosts capable of supplying precursors for production of potentially useful indolocarbazole alkaloids, as exemplified by production and identification of >30 different compounds. Extending combinatorial biosynthesis of indolocarbazoles through further modifications at the sugar moieties by following procedures reported for other glycosylated natural products (17, 39) may prove to be useful.

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1. Gribble, G. W. & Berthel, S. J. (1993) in *Studies in Natural Products Chemistry* (Elsevier, Amsterdam), Vol. 12, pp. 365–409.
2. Akinaga, S., Sugiyama, K. & Akiyama, T. (2000) *Anti-Cancer Drug Design* **15,** 43–52.
3. Prudhomme, M. (2003) *Eur. J. Med. Chem.* **38,** 123–140.
4. Mucke, H. A. (2003) *IDrugs* **6,** 377–383.
5. Denny, W. A. (2004) *IDrugs* **7,** 173–177.
6. Smith, B. D., Levis, M., Beran, M., Giles, F., Kantarjian, H., Berg, K., Murphy, K. M., Dauses, T., Allebach, J. & Small D. (2004) *Blood* **103,** 3669–3676.
7. Campochiaro, P.A. & C99-PKC412–003 Study Group. (2004) *Invest. Ophthalmol. Vis. Sci.* **45,** 922–931.
8. Pearce, C. J., Doyle, T. W., Forenza, S., Lam, K. S. & Schroeder, D. R. (1988) *J. Nat. Prod.* **51,** 937–940.
9. Meksuriyen, D. & Cordell, G. A. (1988) *J. Nat. Prod.* **51,** 893–899.
10. Sánchez, C., Butovich, I. A., Braña, A. F., Rohr, J., Méndez, C. & Salas, J. A. (2002) *Chem. Biol.* **9,** 519–531.
11. Onaka, H., Taniguchi, S., Igarashi, Y. & Furumai, T. (2003) *Biosci. Biotechnol. Biochem.* **67,** 127–138.
12. Hyun, C. G., Bililign, T., Liao, J. & Thorson, J.S. (2003) *Chembiochem.* **4,** 114–117.
13. Onaka, H., Taniguchi, S., Igarashi, Y. & Furumai, T. (2002) *J. Antibiot.* **55,** 1063–1071.
14. Schupp, T., Engel, N., Bietenhader, J., Toupet, C. & Pospiech, A. (1997) World Intellectual Property Organization Patent WO9708323.
15. Khosla, C. & Zawada, R. J. (1996) *Trends Biotechnol.* **14,** 335–341.
16. Cane, D. E., Walsh, C. T. & Khosla, C. (1998) *Science* **282,** 63–68.
17. Méndez, C. & Salas, J. A. (2001) *Trends Biotechnol.* **19,** 449–456.
18. Rix, U., Fischer, C., Remsing, L. L. & Rohr, J. (2002) *Nat. Prod. Rep.* **19,** 542–580.
19. Khosla, C. & Keasling, J. D. (2003) *Nat. Rev. Drug Discov.* **2,** 1019–1025.
20. Shen, B. (2004) *Sci. STKE* **2004,** pe14.
21. Chater, K. F. & Wilde, L. C. (1980) *J. Gen. Microbiol.* **116,** 323–334.
22. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, New York).
23. Quirós, L. M., Aguirrezabalaga, I., Olano, C., Méndez, C. & Salas, J. A. (1998) *Mol. Microbiol.* **28,** 1177–1185.
24. Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood D. A. (2000) *Practical Streptomyces Genetics* (The John Innes Foundation, Norwich, U.K.).
25. Fernández, E., Weissbach, U., Sánchez Reillo, C., Braña, A. F., Méndez, C., Rohr, J. & Salas, J. A. (1998) *J. Bacteriol.* **180,** 4929–4937.
26. Hoshino, T., Kojima, Y., Hayashi, T., Uchiyama, T. & Kaneko, K. (1993) *Biosci. Biotechnol. Biochem.* **57,** 775–781.
27. Yasuzawa, T., Iida, T., Yoshida, M., Hirayama, N., Takahashi, M., Shirahata, K. & Sano, H. (1986) *J. Antibiot.* **39,** 1072–1078.
28. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bokesch, H., Kenney, S. & Boyd, M. R. (1990) *J. Natl. Cancer Inst.* **82,** 1107–1112.
29. Bibb, M. J., White, J., Ward, J. M. & Janssen, G. R. (1994) *Mol. Microbiol.* **14,** 533–545.
30. Fröde, R., Hinze, C., Josten, I., Schmidt, B., Steffan, B. & Steglich, W. (1994) *Tetrahedron Lett.* **35,** 1689–1690.
31. Slater, M. J., Cockerill, S., Baxter, R., Bonser, R. W., Gohil, K., Gowrie, C., Robinson, J. E., Littler, E., Parry, N., Randall, R. & Snowden, W. (1999) *Bioorg. Med. Chem.* **7,** 1067–1074.
32. van Pée, K. H. (2001) *Arch. Microbiol.* **175,** 250–258.
33. Lam, K. S., Schroeder, D. R., Veitch, J. M., Matson, J. A. & Forenza, S. (1991) *J. Antibiot.* **44,** 934–939.
34. Newman, D. J., Cragg, G. M. & Snader, K. M. (2003) *J. Nat. Prod.* **66,** 1022–1037.
35. Burkart, M. D. (2003) *Org. Biomol. Chem.* **1,** 1–4.
36. Hong, S. K., Matsumoto, A., Horinouchi, S. & Beppu, T. (1993) *Mol. Gen. Genet.* **236,** 347–354.
37. Sancelme, M., Fabre, S. & Prudhomme, M. (1994) *J. Antibiot.* **47,** 792–798.
38. Bailly, C., Qu, X., Graves, D. E., Prudhomme, M. & Chaires, J. B. (1999) *Chem. Biol.* **6,** 277–286.
39. Rodríguez, L., Aguirrezabalaga, I., Allende, N., Braña, A. F., Méndez, C. & Salas, J. A. (2002) *Chem. Biol.* **9,** 721–729.