

Two Polyene Amides Produced by Genetically Modified *Streptomyces diastaticus* var. 108

Elena M. Seco,¹ Trinidad Cuesta,¹ Serge Fotso,² Hartmut Laatsch,² and Francisco Malpartida^{1,*}

¹Centro Nacional de Biotecnología

Campus de la UAM

28049 Cantoblanco

Madrid, Spain

²Department of Organic and Biomolecular Chemistry

University of Göttingen

Tammannstrasse 2

Göttingen 37077, Germany

Summary

Streptomyces diastaticus var. 108, a newly isolated strain, was recently characterized as a producer of two polyene macrolide antibiotics (rimocidin and CE-108), and the biosynthetic gene cluster was partially characterized. When the producer strain was genetically modified by transformation with some engineered SCP2*-derived vectors carrying the *ermE* gene, two previously uncharacterized macrolides were detected in the fermentation broth of the recombinant strain and chemically characterized as the amides of the parental polyene carboxylic acids. The biological activity and some in vitro toxicity assays showed that this chemical modification resulted in pharmaceuticals with improved biological properties compared with the parental products.

Introduction

The polyenes are a group of macrolide polyketides that are interesting because of their antifungal activity. These compounds contain a macrolactone ring with several conjugated double bonds, forming chromophores with characteristic ultraviolet/visible light spectra; these features are responsible for the physical and chemical properties of the compounds (strong light absorption, photolability, and poor solubility in water) [1, 2]. Despite the importance of some polyenes, such as amphotericin B (1; Figure 1), as antifungal drugs, their precise mechanism of action is still not well understood; nevertheless, antifungal activity seems to be due to interactions between polyene molecules and sterol-containing membranes. This interaction results in an ion channel and the membranes become permeable, causing destruction of electrochemical gradients and consequent cell death [3]. These compounds show significantly higher affinity for ergosterol-containing membranes (the main sterol present in fungal membranes) than for cholesterol-containing membranes (mammalian cells) [4]. However, the interaction between polyenes and cholesterol-containing membranes is not negligible and causes some side effects, which, along with low solubility, makes the compounds not fully satisfactory for treating systemic fungal infections. De-

spite its undesirable properties, amphotericin B (1) has been used for more than 40 years and there is a consensus that there are no better alternatives available to fight emerging fungal diseases.

For this reason, the finding of new antifungal drugs or improving the pharmacological properties of the old ones has become an exciting challenge. With this aim, and using rational molecular modeling approaches, many semisynthetic derivatives of amphotericin B (1) have been generated and tested as efficient antifungal drugs. Two main targets were considered for structural modifications, among others: the side-chain carboxyl group and the amino group of the sugar moiety [5–11]. Whereas some of these semisynthetic derivatives still showed the same toxicity, others had improved pharmacological features compared with the parental amphotericin molecule: higher antifungal activity, water solubility, specificity for ergosterol-containing membranes, and less hemolytic activity, suggesting more specificity for ergosterol-containing membranes. Although the higher antifungal activity confers some advantage to those compounds, surprisingly these structural modifications are not widely represented within the natural polyenes isolated from microorganisms. One exception is the polyene AB-400 (2b; Figure 1), an amide of pimaricin (2a; Figure 1), recently reported as a natural product isolated from *Streptomyces costae* [12] along with other tetraene derivatives. AB-400 (2b) was also detected in a different strain isolated in our laboratory in a screening program for producers of antifungal compounds (*Streptomyces* sp. RGU5.3, unpublished data).

We have recently characterized a chromosomal region of *Streptomyces diastaticus* var. 108 involved in the biosynthesis of two related polyenes: rimocidin and CE-108 (3a and 4a, respectively; Figure 1) [13]. Both compounds are derived from the same biosynthetic pathway in which the *rimA* gene, coding for the loading module PKS, plays a pivotal role in the balance of the two polyenes through its choice of the starter unit (acetyl-CoA or butyryl-CoA). This versatility in the recognition of the building blocks for rimocidin or CE-108 biosynthesis makes this biosynthetic cluster a promising system in the effort to generate new bioactive molecules.

Results

Generation of a Recombinant *rimA* Gene

The *rimA* gene [13] was engineered with the aim of generating new recombinant molecules using the rimocidin and CE-108 gene cluster. Because *rimA* is coded within a polycistronic mRNA, the structural gene was cloned under the control of the *xysA* promoter (*xysAp*) of the xylanase gene of *Streptomyces halstedii* JM8 [14]. The *xysAp* was excised from pHis1 as a 547 bp BglII/SmaI fragment also carrying the terminator of the methylenomycin resistance gene (T1) [15] upstream of *xysAp*. After several cloning steps described in Table 1, a DNA

*Correspondence: fmalpart@cnb.uam.es

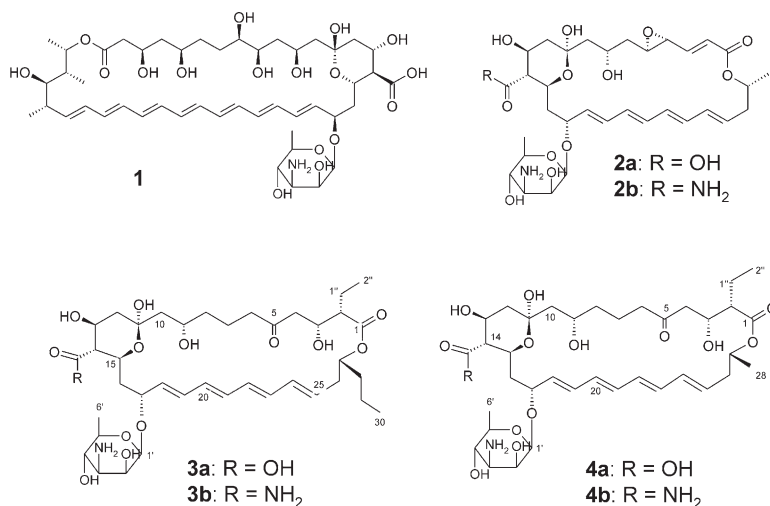


Figure 1. Chemical Structures of the Relevant Tetraenes Cited within the Text

The structures were: amphotericin B (1); pimaricin (2a); AB-400 (2b); rimocidin (3a); rimocidin B (3b); CE-108 (4a); and CE-108B (4b).

fragment from the *rim* cluster containing *rimA* and the 3' end of the *rimI* gene (9,336–15,445 bp from the sequence deposited under accession number AY442225) [13] was fused with *xysAp*. In order to allow selection

of recombinant strains, the *ermE* gene from pNAe-1 (see Table 1) was also cloned in the same fragment and the resultant construction was cloned into the EcoRV site (inside the thiostrepton resistance marker) of a low-

Table 1. Bacterial Strains and Vectors Used in this Study

Strain or Plasmid	Properties	Reference
<i>S. diastaticus</i> var. 108	WT, CE-108 and rimocidin producer	[17]
<i>S. diastaticus</i> var. 108/922	WT transformed with pIJ922 plasmid (WT, control)	This work
<i>S. diastaticus</i> var. 108/PM1-500	WT derivative with <i>rimA</i> -disrupted by integration of PM1-500; CE-108 and rimocidin non-producer	[13]
<i>S. diastaticus</i> var. 108/743B	WT derivative transformed with pSM73B; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
<i>S. diastaticus</i> var. 108::PM1-500/743B	WT derivative with <i>rimA</i> -disrupted by integration of PM1-500 and transformed with pSM743B; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
<i>S. diastaticus</i> var. 108/784	WT derivative transformed with pSM784; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
<i>Streptomyces</i> sp. RGU5.3	WT; pimaricin and AB-400 producer	This work
<i>E. coli</i> JM101	General cloning host	[35]
<i>S. lividans</i> TK21	General cloning host	[33]
<i>Penicillium chrysogenum</i> ATCC10003	Antifungal activity assays	ATCC
<i>Candida albicans</i> ATCC10231	Antifungal activity assays	ATCC
<i>C. krusei</i> ATCC14243	Antifungal activity assays	ATCC
<i>Aspergillus niger</i> ATCC1004	Antifungal activity assays	ATCC
<i>Cryptococcus neoformans</i> ATCC10226	Antifungal activity assays	ATCC
pIJ922	Vector based on the SCP2* replicon, <i>tsr</i> , 24 kb	[36]
pIJ941	Vector based on the SCP2* replicon, <i>tsr</i> , <i>hyg</i> , 25 kb	[36]
pNAe-1	Replicative vector in <i>E. coli</i> , <i>ermE</i> , Km ^R .	N. Allende, personal communication
pHis1	Replicative vector in <i>E. coli</i> carrying xylanase <i>xysA</i> promoter (<i>xysAp</i>), Ap ^R , 3.7 kb	[14]
pSM736	1.2 kb SacI-DraIII and 4.9 kb DraIII-BglII fragments containing <i>rimA</i> cloned simultaneously into the SacI/BamHI sites of pIJ2925	This work
pSM738	6.1 kb BglII-PstI fragment of pSM736 (<i>rimA</i> gene) and 1.7 kb PstI-SphI fragment of pNAe-1 (<i>ermE</i> gene resistance) cloned simultaneously into the SmaI/SphI sites of pHis1	This work
pSM743B	8.4 kb BglII fragment of pSM738 (containing <i>xysAp</i> , <i>rimA</i> , and <i>ermE</i>) cloned into the EcoRV site of pIJ922 (see Figure 2).	This work
pGAe-1	Replicative vector in <i>E. coli</i> , <i>ermE</i> , Ap ^R .	A. González, personal communication
pSM784	1.8 kb SspI-EcoRV fragment of pGAe-1 (<i>ermE</i> gene resistance) cloned into the EcoRV site of pIJ941 (see Figure 2)	This work

Ap, ampicillin; *ermE*, erythromycin resistance gene; *hyg*, hygromycin resistance gene; Km, kanamycin; *tsr*, thiostrepton resistance gene; WT, wild-type.

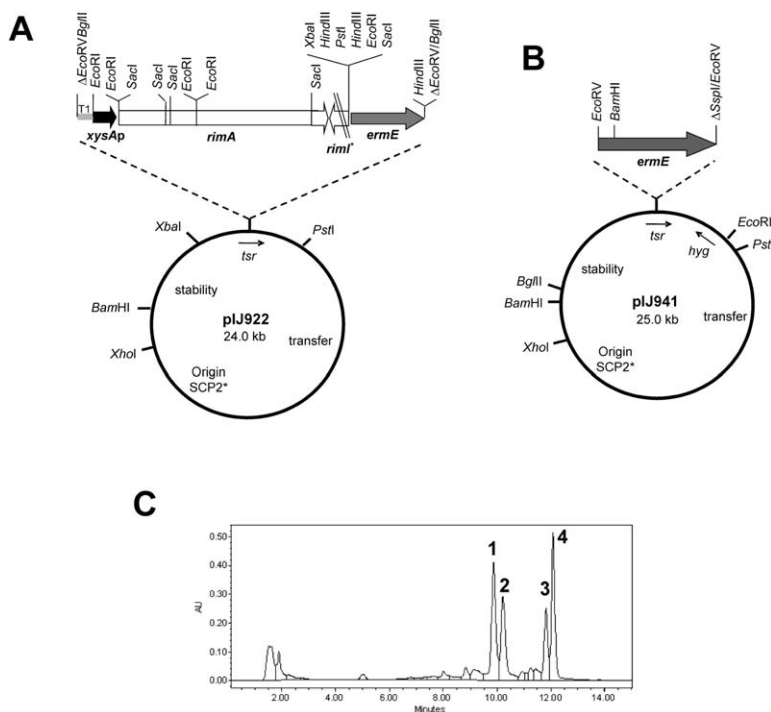


Figure 2. Physical Maps of Recombinant Plasmids and the Chromatographic Profile of the Fermentation Broth from Genetically Modified Strains

(A) Engineered *rimA* in pIJ922 (pSM743B).
(B) Engineered *ermE* in pIJ941 (pSM784).
(C) HPLC analysis of the fermentation broth of *S. diastaticus* var. 108/743B recombinant strain (see text).
The numbers represent: CE-108B (1); CE-108 (2); rimocidin B (3); and rimocidin (4). *ermE*, erythromycin resistance gene; *hyg*, hygromycin resistance gene; *rimA*, type I PKS involved in CE-108 and rimocidin biosynthetic pathway; T1, terminator of methylerythromycin resistance gene; *tsr*, thiostrepton resistance gene; *rimI**, *rimI* truncated at its N terminus; *xysAp*, *xysA* promoter.

copy number *Streptomyces* vector (pIJ922). The recombinant plasmid (pSM743B) (Figure 2A) conferred erythromycin but not thiostrepton resistance.

In order to test the functionality of the recombinant *rimA*, pSM743B was introduced by transformation into *S. diastaticus* var. 108 (wild-type), giving rise to *S. diastaticus* var. 108/743B (a wild-type strain carrying extra copies of the recombinant *rimA* gene). The recombinant plasmid was transferred from this strain to the *rimA*-disrupted mutant (*S. diastaticus* var. 108/PM1-500) [13] by intraspecific conjugation, producing *S. diastaticus* var. 108::PM1-500/743B. Plasmid extraction and Southern hybridization confirmed that the *rimA*-disrupted mutant carries, as expected, the engineered copy of the intact *rimA*; therefore *rimA* expression is under the control of the heterologous promoter.

The fermentation broths from the two recombinant strains (wild-type and disrupted mutant, both carrying the engineered *rimA*) were tested for rimocidin and CE-108 production by HPLC analysis; the chromatograms showed that the production of both tetraenes was restored in the disrupted mutant. Polyene production was significantly lower in the complemented *rimA*-disrupted mutant than that of the engineered wild-type strain and wild-type control (carrying the pIJ922 vector) (see Table 1); this lower production was not significantly altered when either glucose or xylane was added to the medium. The explanation for this finding probably involves the efficiency of *xysAp* in driving expression of the *rimA* gene, suggesting that expression of *rimA* would be a limiting step in the production of the polyenes. Surprisingly, in the *rimA*-disrupted mutant transformed with the engineered *rimA* gene, what we believe to be new polyenes were observed in addition to the expected complementation. A similar profile was also seen in the

recombinant *S. diastaticus* var. 108 carrying the engineered *rimA*; these apparently new compounds showed characteristic visible/UV tetraene spectra similar to rimocidin and CE-108 (see Figure 2C). Due to their proximity in retention time to the native compounds within the chromatogram, these polyenes were named rimocidin B and CE-108B, respectively (3b and 4b; Figure 1).

In order to exclude the possibility that production of these compounds was due to a possible degradation of CE-108 and/or rimocidin, the timing profile for production of both compounds was compared with that of the original compounds. The results clearly showed that all the compounds followed a similar production profile (data not shown), suggesting that the formation of the compounds was not due to degradation of the natural compounds. However, the tetraenes production in *S. diastaticus* var. 108/743B and *S. diastaticus* var. 108::PM1-500/743B strains was delayed compared with the wild-type control, *S. diastaticus* var. 108/922. Differences in temporal expression of the recombinant *rimA* compared with the wild-type could explain the delay in polyene production. This different temporal expression could alter the availability of some metabolites needed for this apparently new biosynthetic process.

A construction was developed in order to investigate the possibility that this phenotype could be due to the erythromycin used for selection in the culture medium. A blunt-ended fragment carrying the *ermE* gene (see Table 1) was inserted into the *EcoRV* site of pIJ941 (a vector similar to pIJ922, which was used for generating the recombinant *rimA* gene); pSM784, carrying *ermE* in the same orientation as in pSM743B, was selected (Figure 2B). This plasmid was introduced by transformation into the wild-type strain. Unexpectedly, when HPLC analysis was performed with this strain, the tetraenes

(CE-108B and rimocidin B) were also detected. These results clearly indicate that the *ermE* gene in the SCP2*-derived vector plays a pivotal role in generating the polyenes. It is noteworthy that neither erythromycin resistance (cloned for other purposes [unpublished data] in different vectors, such as pHJL401 [16]) nor SCP2*-derived vectors, independently, are enough for production of the new structures. Further experiments are in progress in order to clarify the mechanism of the polyenes' formation.

Characterization of the Polyenes

HPLC-MS Analysis

HPLC-MS analysis of the fermentation broth from *S. diastaticus* var. 108/743B and *S. diastaticus* var. 108::PM1-500/743B were carried out; the deduced masses for the two tetraenes were 738 and 766 Da for the lower and higher retention times, respectively. In both cases the mass of the polyenes is one unit lower than that of the polyenes with the closest retention time, CE-108 (739 Da) and rimocidin (767 Da). Both the mass differences and the chromatographic mobility supported the idea that the two polyenes were derived from the natural macrolides.

Chemical Structure Elucidation

The two tetraenes were preliminarily characterized in order to develop a procedure for their purification with the aim of structure elucidation. Both compounds interact not only with reverse-phase silica gel, such as C8 and C18, but also with an ion exchange resin, like SP-Sephacrose, suggesting that these compounds have an accessible positive charge. This preliminary characterization allowed us to design a straightforward purification procedure from the fermentation broth of *S. diastaticus* var. 108/pSM743B (see [Experimental Procedures](#)).

Compound **4b** was obtained as a yellow powder with a typical tetraene UV spectrum ($\lambda_{\text{max}} = 317,302,289$ nm), similar to that of **4a** [17]. The proton NMR spectrum with three signals in the sp^2 range at δ 6.25 (dd, 14.9, 10.9 Hz), a multiplet at δ 6.00–6.15 and a doublet of doublet at δ 5.87 (15.2, 8.4 Hz) was similar to that of **4a** as well. Two exchangeable protons appeared at δ 7.30 and 6.83 as broad singlets. In the aliphatic range of δ 1.40–2.50, the spectrum displayed a complex multiplet pattern, and signals of three methyl triplet and doublets, respectively, appeared at δ 1.17, 1.15, and 0.83. Electrospray ionization mass spectrometry (ESI MS) afforded pseudomolecular ions at m/z 739 ($[\text{M}+\text{H}]^+$) and 761 ($[\text{M}+\text{Na}]^+$), which delivered the molecular formula $\text{C}_{37}\text{H}_{58}\text{N}_2\text{O}_{13}$ by high resolution (found 739.40110, calculated 739.40118 for $[\text{M}+\text{H}]^+$). The ^{13}C -NMR spectrum indicated 37 carbon signals as in **4a** and as demanded by the molecular formula. The ^{13}C data of **4a** and **4b** were closely related ([Table 2](#)) and permitted the conclusion that **4a** and **4b** possess the same carbon skeleton, including the amino sugar. According to these data, the second nitrogen must be attributed to an amide function, which identifies CE-108B (**4b**) as the amide of **4a**.

The yellow powdery **3b** was readily soluble in DMSO. ESI MS fixed the molecular weight of **3b** as 766 Da, and NMR delivered the molecular formula $\text{C}_{39}\text{H}_{62}\text{N}_2\text{O}_{13}$ (found 767.43254 Da, calculated 767.43301 Da for

$[\text{M}+\text{H}]^+$). The proton NMR spectrum was similar to that of **4b** and displayed two H/D exchangeable protons at δ 7.30 and 6.83, two doublet of doublets and a multiplet in the range of δ 6.40–5.80. The aliphatic region was very complex due to less resolution, but a triplet and a doublet at δ 1.83 and 1.16, attributed to methyl signals, were easily identified. The ^{13}C NMR spectrum indicated the presence of 39 carbon signals. Comparison with that of CE-108B (**4b**) revealed the presence of three carbonyl signals at 208.8, 174.1, and 172.1, in addition to signals of eight sp^2 carbon atoms in the range of 136.7–128.3 and of two acetal groups. The close similarity with **4b** identified this compound finally as rimocidin amide (**3b**).

Biological Activity of the Compounds

Antifungal Activity Assays

The antifungal activity of the tetraene amides was tested against several fungi: *Penicillium chrysogenum*, *Candida albicans*, *Aspergillus niger*, *Candida krusei*, and *Cryptococcus neoformans*. Increasing concentrations of different tetraenes dissolved in methanol were applied to paper discs (9 mm diameter), dried, and transferred to the bioassay plates. The activity of these tetraene amides was compared with that of the parental molecules (CE-108 [**4a**] and rimocidin [**3a**]), showing that the biological activity of the amides on all fungi tested was substantially higher compared with their corresponding parental tetraenes ([Figure 3](#)). In both cases, substitution of the free carboxylic group for the amide group increased antifungal activity approximately four times.

Toxicity Assays

From the previous experiments it was clear that modification of the polyenes produced by *S. diastaticus* var. 108 gave rise to compounds with higher antifungal activity. In order to test if toxicity was also enhanced, we carried out some preliminary determinations of the hemolytic activity of the molecules. Human erythrocytes were used as a cellular model for this study [7, 18]. Hemolytic activity of the compounds was evaluated (see [Experimental Procedures](#)) versus rimocidin (**3a**) and CE-108 (**4a**); amphotericin B (**1**) and nystatin A were also included. As shown in [Table 3](#), the hemolytic activity of the tetraene amides (rimocidin B [**3b**] and CE-108B [**4b**]) was not significantly different from that of their corresponding parental tetraenes, whereas its antifungal activity was clearly higher. Noteworthy are the strong differences in toxicity observed between CE-108 (**4a**)/CE-108B (**4b**) and rimocidin (**3a**)/rimocidin B (**3b**); whereas 50% of hemolysis is reached with 40–60 nmol of these two last polyenes, a 6- to 7-fold greater concentration of CE-108 and its amide are needed. Thus, the pharmacological properties of the amide polyene CE-108B (**4b**) are clearly enhanced: although CE-108 (**4a**) shows low antifungal activity, its structurally related amide **4b** has an increased antifungal activity level nearly as high as that of rimocidin (**3a**), but its hemolytic activity is 6- to 7-fold lower. These assays were also performed with horse blood, resulting in similar findings (data not shown).

Identical results were obtained using pimarin (**2a**) and its amide AB-400 (**2b**). Both were purified from our

Table 2. ^{13}C -NMR Chemical Shifts of Rimocidin B (3b), CE-108 (4a), and CE-108B (4b) in DMSO-d_6

C No.	4a	4b	3b	C No.	4a	4b	3b
1	173.4	173.3	172.1	17	78.2	78.2	74.2
2	56.3	57.4	56.0	18	136.0	137.4	136.7
1''	22.4	23.4	22.6	19	133.6	134.6	133.1
2''	10.9	12.0	11.7	20	129.3	130.0	128.3
3	68.5	69.7	68.3 ^a	21	133.4	133.6	132.8
4	48.8	49.6	48.3	22	132.3	134.4	131.3
5	210.6	211.5	208.8	23	132.3	133.2	131.5
6	44.3	45.2	43.5	24	132.6	134.4	131.8
7	19.6	20.6	21.8	25	131.1	132.2	130.8
8	37.6	38.6	37.6	26	40.0	41.0	37.4
9	68.4	69.5	67.5	27	70.8	71.8	72.4
10	46.5	47.1	44.7	28	20.0	21.0	37.1 ^a
11	97.8	98.8	96.9	29	—	—	17.7
12	44.1	45.1	45.3 ^a	30	—	—	13.7
13	69.9	69.9	69.6	1'	98.2	99.0	97.1
14	60.5	58.6	56.7	2'	68.0	69.4	64.7
14-COOH	179.3	—	—	3'	56.1	57.4	56.5
14-CONH ₂	—	177.7	174.1	4'	69.5	71.2	72.9
15	66.6	66.8	65.3	5'	73.5	74.6	73.0
16	38.7	39.0	36.4	6'	16.7	17.8	17.9

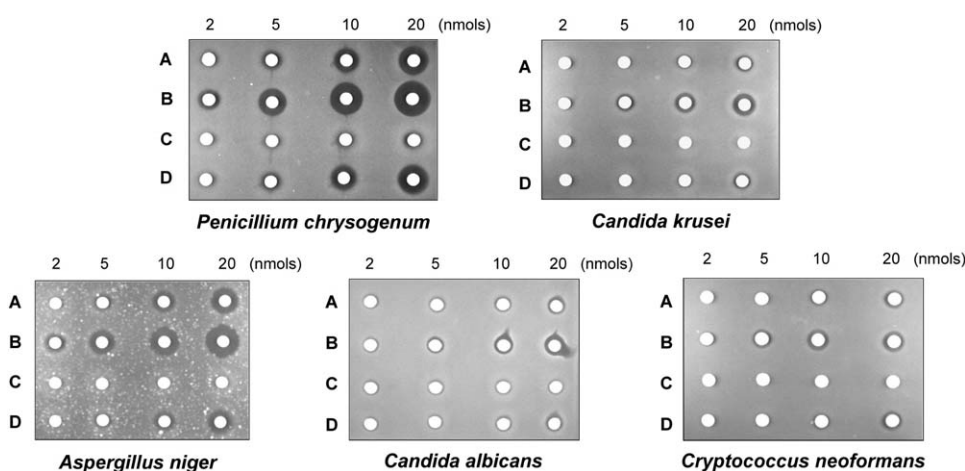
^a Expected value according to Sowinski et al. [37]; in our measurement, the signal was missing.

isolated strain RGU5.3 as indicated in the [Experimental Procedures](#). The strain was cultivated in a medium supplemented with either glucose or sodium acetate. Using a fermentation broth containing glucose as a carbon source, pimarin (2a) comprised nearly 70% of the produced polyenes; in an acetate-containing medium, the production profile was reversed, with AB-400 (2b) being the compound that was mainly produced ([Figure 4](#)). This effect was not observed in the engineered *S. diastaticus* var. 108 strain producing the polyene amides. AB-400 (2b) was purified from this medium and tested for both antifungal and hemolytic activities. The results, summarized in [Figure 4C](#), are in good agree-

ment with the previous finding: whereas pimarin (2a) displayed no detectable antifungal activity at the concentration tested, the same amount of AB-400 (2b) was active. However, the hemolytic activity assays carried out with AB-400 (2b) and commercial pimarin (2a) showed no significant differences between the two polyenes. This is an instance in which the polyene carboxamide has a clear advantage over the free acid.

Discussion

In recent decades we have witnessed dangerous increases in the incidence of nosocomial fungal infec-


Figure 3. Antifungal Activity of the Four Tetraenes Produced by *S. diastaticus* var. 108/743B

- (A) Rimocidin (3a).
- (B) Rimocidin B (3b).
- (C) CE-108 (4a).
- (D) CE-108B (4b).

The applied quantities for each polyene are expressed in nanomoles. The target organisms were *P. chrysogenum*, *C. krusei*, *A. niger*, *C. albicans*, and *C. neoformans* (see [Table 1](#)).

Table 3. Comparative Hemolytic Activity of Several Polyenes (See [Experimental Procedures](#))

Hemolytic Activity (% of Total Hemolysis)						
Test Value (nmol)	Amphotericin B (1)	Nystatin A	Rimocidin (3a)	Rimocidin B (3b)	CE-108 (4a)	CE-108B (4b)
1	1.97					
2	4.47					
3	82.26					
4	100.00					
20		2.20	10.00	10.00		
40		49.53	21.18	33.51		
60		76.80	65.02	84.80		
80		92.79	95.59	94.80		
100		100.00	100.00	100.00		
120					11.72	12.01
160					14.48	15.86
200					16.60	23.29
240					26.89	30.22
280					32.47	34.86
320					44.25	40.39
360					63.56	61.34
400					82.12	88.26
440					100.00	100.00

tions. Several factors have contributed to these increases, such as with the effects of AIDS, chemotherapy, and treatment with immunosuppressant agents. Despite the need for new antifungal drugs, the number of pharmaceuticals currently available for treating systemic infec-

tions is dangerously low. Most of them, such as azoles and amphotericin B (1), target the structural integrity of fungal membranes [3, 19]; although, in the last few years, new antifungal drugs (e.g., echinocandins) have been developed by targeting specific components of

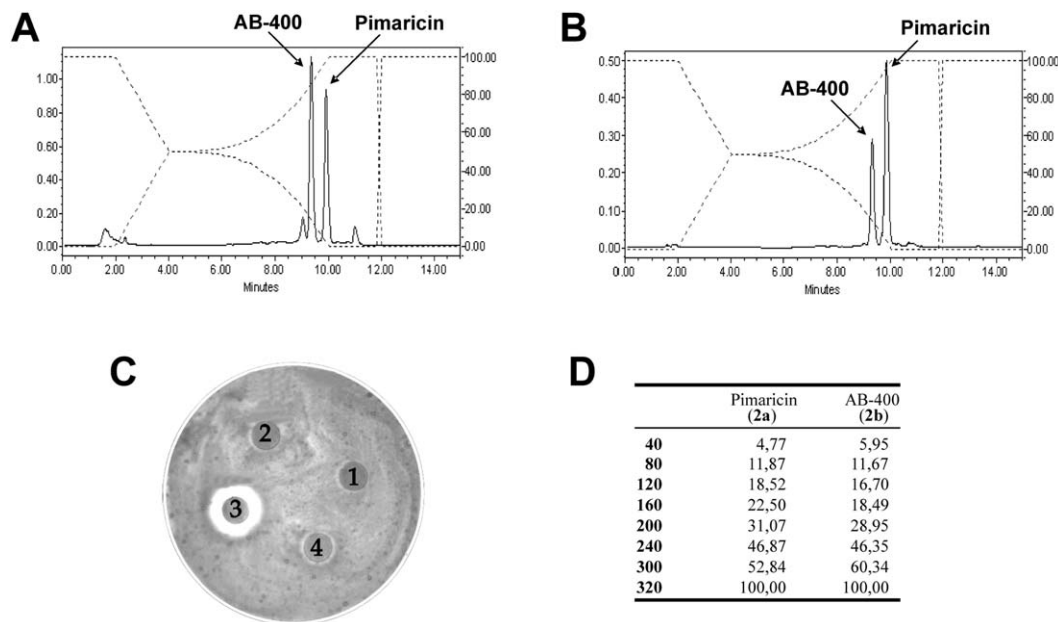


Figure 4. Biological Activities of Pimaricin and AB-400 Polyenes Extracted from *Streptomyces* sp. RGU5.3

(A) HPLC analysis of the fermentation broths from *Streptomyces* sp. RGU5.3 in media with acetate.
 (B) HPLC analysis of the fermentation broths from *Streptomyces* sp. RGU5.3 in media with glucose as carbon source.
 (C) Antifungal activity of pimaricin (2a) and AB-400 (2b) against *P. chrysogenum*. The polyene samples applied were: commercial pimaricin (1) (Calbiochem 527962), total extract from *Streptomyces* sp. RGU5.3 fermentation broth grown in glucose medium (2), purified AB-400 from *Streptomyces* sp. RGU5.3 (3) and purified pimaricin from *Streptomyces* sp. RGU5.3 (4); a total of 200 ng were added at each test.
 (D) Hemolytic activity for pimaricin (Calbiochem 527962, 98.8% purity) and AB-400 (HPLC-purified as indicated in [Experimental Procedures](#)). The values of each polyene are expressed in nanomols (left column) and the corresponding hemolytic activities are given as percentage of total hemolysis (see [Experimental Procedures](#)).

the cell wall [20]. Despite the toxic side effect of amphotericin B (1), this old drug remains the preferred antifungal agent for treating most systemic infections; some of the undesirable effects can be minimized by delivering the drug in a liposomal formulation [21, 22]. This successful reduction in the toxicity level of amphotericin B (1) with the new formulations has increased the clinical interest in this old drug; thus, amphotericin B (1) is undoubtedly a good model to use in attempts to generate new and improved pharmaceuticals.

These attempts have attracted large efforts in academic laboratories as well as within the pharmaceutical industry, resulting in the generation of several new derivatives of amphotericin B (1). Most attention has focused on deciphering the physicochemical interactions between the drug or its chemical derivatives and its target membranes, as well as the pharmacokinetic properties of its semisynthetic derivatives [4–8, 11, 22–26]. Although not conclusive, all the experimental data, as well as those based on molecular dynamic simulation [4], have allowed some preliminary conclusions about the relationships between chemical structure and biological activity of these derivatives. All these techniques elicited novel approaches for designing a new generation of drugs following a more rational design of their chemical structure [27]. As a result of these efforts, there is a clear conclusion: two structural changes within the amphotericin B molecule seem to be important for improving its pharmacological properties. These changes are based on modifications of the carboxyl group and the sugar moiety [7, 11, 26, 28]. All the polyene compounds referred to in the literature as improved pharmaceuticals are semisynthetic derivatives and are generated by organic synthesis rather than by biotransformations. In this work, we describe the biosynthesis of two polyenes with changes in the carboxylic group generated by genetic manipulation.

S. diastaticus var. 108, a producer of two natural tetraenes [17], has the ability to naturally produce, as major compounds, the corresponding amides if it is appropriately engineered by genetic manipulation. As with other semisynthetic polyene derivatives, conversion of the free carboxylic group into the amide group results in a clear improvement of some pharmacological properties (substantial increase of antifungal but not hemolytic activities), thus giving an apparent advantage over native tetraenes. This chemical modification in both derivatives caused an increase in the selective toxicity toward ergosterol-containing membranes. A similar result was obtained with pimarin (2a) and its derivative, AB-400 (2b), which reinforces the idea that the substitution of the carboxylic group for an amide group will also improve the selective toxicity of other polyenes.

What are the origins of the new amidated tetraenes? Analysis of polyene production in *S. diastaticus* var. 108 wild-type by HPLC revealed that some minority tetraene compounds are also produced along with the major compounds. Using HPLC analysis coupled with a mass detector, we could detect tetraenes with a mass and retention time identical to the amidated derivatives. This allowed us to conclude that the identified polyene amides are likely being produced by the wild-type strain, although at an extremely low level. Genetic ma-

nipulation of the producer strain elicits overproduction by a mechanism that is not yet fully understood. One important lesson is noteworthy: paying attention to minor active metabolites in the fermentation broth of producer strains might be a good alternative for isolating new and interesting pharmaceuticals.

Although the precise mechanism of the biosynthesis of these metabolites is still unknown, at least two plausible mechanisms can be postulated. In one, the amides would be the result of an amidotransferase activity, as a tailoring function (post-PKS modification), which, under natural conditions, is poorly expressed. In the other, malonamyl-CoA would be incorporated by module 7 of the corresponding PKS instead of methylmalonamyl-CoA, as proposed in the biosynthetic model for CE-108 (4a) and rimocidin (3a) production [13]. In this latter case, a type of nondecarboxylating Claisen condensation would be required for the incorporation of malonamide, as it happens in the biosynthetic thiolases [29]. The availability of malonamide as a condensing unit would be crucial for good incorporation into the growing polyketide chain. It is noteworthy that the producer strain also biosynthesizes oxytetracycline [17], the postulated starter unit of which is malonamide [30], so this metabolite would be easily available for secondary metabolite production in this strain.

Undoubtedly we are far from having complete elucidation of the genetic mechanism leading to CE-108B (4b) and rimocidin B (3b) production. So far it is clear that both the *ermE* gene and either pIJ922 or pIJ941 plasmids are simultaneously required to trigger biosynthesis of the new amides. It has been recently described that subinhibitory concentrations of erythromycin can modulate bacterial transcription [31]. Nevertheless, the possibility that erythromycin could modulate the expression of a possible transcriptional regulator, making possible the amidation step in our polyene producer, can be ruled out because the amides were also detected in cultures with no erythromycin added. This allows the possibility that the *ermE* product (a methylase) might be targeting another intermediate gene, coded within the plasmid DNA, the final target of which would be activation of a chromosomal gene responsible for the amidation. Insight into the mechanism of this modification will provide an interesting tool for approaching the exciting challenge of generating new polyene amides by biotransformation. This would undoubtedly be a straightforward process for production of improved pharmaceuticals if successfully applied to the biosynthetic pathway of commercial polyenes.

Significance

Through genetic manipulation of a polyene producer (*Streptomyces diastaticus* var. 108), we could generate a strain that produces, to our knowledge, new bioactive compounds. The polyenes are amides of the parental tetraenes with higher selectivity for fungal membranes. This finding opens the possibility of generating recombinant strains, producing polyene amides by biotransformation, and thus new antifungal drugs with improved pharmacological properties. The

biosynthetic process would undoubtedly be an interesting tool that may well compete with semisynthetic methods described in the literature.

Experimental Procedures

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are described in Table 1. *S. diastaticus* var. 108 and its engineered derivatives were routinely grown in liquid and solid SYM2 medium [32] for tetraene production analysis, and liquid TSB medium (Oxoid) for plasmid and total DNA extraction. *Streptomyces lividans* TK21 was used as a general cloning host and grown in solid R5 medium and liquid YEME medium [33]. *Escherichia coli* strains were grown in Luria-Bertani (LB) agar or in LB broth [34]. *P. chrysogenum*, *C. krusei*, *A. niger*, *C. albicans*, and *C. neoformans*, used for testing antifungal activity, were grown in MPDA medium (2% malt extract, 2% glucose, 0.1% Bacto peptone).

Genetic Procedures

E. coli strains were grown and transformed as described elsewhere [34]. *Streptomyces* strains were manipulated as previously described [33]. Intraspecific conjugation was carried out by growing together the donor and recipient strains in solid R5 medium without selection and then selecting for the corresponding antibiotic resistances of the plasmids and genetic markers of the recipient strains. DNA manipulations were performed as described by Maniatis et al. [34].

Assay for Tetraenes Production

Tetraenes production was analyzed by extracting whole culture with methanol as previously described [13]. The extracts were filtered and applied to an HPLC with a Waters 600S Controller, equipped with a Waters 996 PDA; quantitative determination and chromatographic conditions were as previously described [17].

HPLC-MS Assays

The mass spectra were determined in an 1100MSD HPLC connected to a quadrupole Agilent Technology Detector, using electrospray as the source and a positive ionization mode. The chromatographic conditions were the same as described above.

Purification of the Compounds

S. diastaticus var. 108/743B was cultivated in either solid or liquid SYM2 medium [17]. After 6 days, the whole solid medium was fragmented through a 50 ml syringe and extracted with four volumes of methanol and 25 mM formic acid; cultures from liquid medium were freeze-dried before similar extraction with methanol. The aqueous suspension was stirred for 1 hr and clarified at 5000 × g for 20 min to remove solid particles. The clear supernatant was concentrated by rotaevaporation to 10–20 × 10⁶ U/μl measured at a wavelength of 304 nm; the sample was then stored in 80% methanol/water until use. Two hundred milliliters liquid culture or one plate (24 × 24 cm) yielded up to 40 mg of tetraene-containing sample. The methanol-extracted samples were brought to 20% methanol with water and filtered to remove precipitated material. The clear filtrate was slowly applied to an Omnifit column (250 × 25 mm, Supelco Catalog No. 56010) packed with SP-Sepharose, Phast Flow (Pharmacia) equilibrated in the same solution. Under these conditions, rimocidin (3a) and CE-108 (4a) eluted with the unbound material, such as some pigment, whereas the amides CE-108B (4b) and rimocidin B (3b) were completely retained. The column was exhaustively washed with the same solution. The polyene amides were eluted from the column with 300 mM ammonium acetate pH 5 in 20% methanol. The fractions containing the mixtures of amides were desalted using Sep-Pak C18 (Waters) cartridges and reconstituted in 20% methanol. The tetraene mixtures (15 mg) were finally separated by HPLC using a semipreparative column (Supelcosil PLC-8, 250.0 × 21.2 mm). The chromatographic parameters and the mobile phases, controlled with a Waters Automated Gradient Controller, were: 12 min with 100% of B (ammonium acetate 20 mM pH 5, ethanol 20%), 43 min of a binary gradient up to 50% of A (methanol) and 50% of B (curve 6); 35 min of a binary gradient up to 100% of A (curve 8), and a constant flow of 5 ml/

min. Fractions were collected at regular intervals (5 ml per fraction) and those carrying the purified isolated compounds were pooled and subjected to an additional desalting step, as above, and finally freeze-dried twice. AB-400 was also purified from *Streptomyces* sp. RGU5.3 liquid cultures as above.

Hemolytic Activity Assays

The assays were carried out according to the method described by Gómez-Gómez et al. [18]. The polyene samples were weighed and dissolved in DMSO at 30 mg/ml. Increasing quantities of the different polyenes were brought to a final volume of 100 μl of DMSO and mixed by gently shaking with 500 μl of PBS buffer [18] containing either 2.5% human or horse blood. After incubation at 37°C for 30 min without agitation, cells were pelleted by centrifugation, and hemolysis evaluated by measuring the absorbance at 545 nm. The values corresponding to total hemolysis were estimated with a suspension of 2.5% horse blood in distilled water. Human blood (mostly erythrocytes) was provided by the Blood Bank of Ramón y Cajal Hospital (Madrid); horse blood was from Oxoid (defibrinated blood). Amphotericin B and nystatin A used were from Sigma (catalog Nos. A-4888 and N-3503, respectively); pimarinic acid was from Calbiochem (527962). All were tested directly from the manufacturer sources without further purification.

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