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] Centro Nacional de Biotecnología, Campus de la UAM
28049 Cantoblanco,
Madrid, Spain.

[2]Department of Organic and Biomolecular Chemistry
University of Göttingen,
Tammannstrasse 2,
Göttingen D-37077, Germany.

Key words: polyene macrolides, selective toxicity, AB-400, recombinant Streptomycetes, polyene amides.

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 new 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 which 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 their physical and chemical properties (strong light absorption, photolability and poor solubility in water) (1, 2). Despite the importance of some members such as amphotericin B (Figure 1, **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 towards ergosterol-containing membranes (the main sterol present in fungal membranes) than to 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 the low solubility, makes the compounds not fully satisfactory for treating systemic fungal infections. Despite 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 anti-fungal 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 *semi*-synthetic 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). While some of these *semi*-

* Corresponding Author: fmalpart@cnb.uam.es; phone: +34 915854548; FX: +34 915854506

synthetic 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 haemolytic 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 (Figure 1, **2b**), an amide of pimaricin (Figure 1, **2a**), 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).

We have recently characterized a chromosomal region of *Streptomyces diastaticus* var. 108 involved in the biosynthesis of two related polyenes: rimocidin and CE-108 (Figure 1, **3a** and **4a**, respectively) (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 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 attempts to generate new bioactive molecules.

Results

Generation of a Recombinant *rimA* Gene

With the aim of generating new recombinant molecules using the rimocidin and CE-108 gene cluster, the *rimA* gene (13) was engineered. Because *rimA* is coded within a polycistronic mRNA, the structural gene was cloned under the control of the *xysA* promoter (*xysA*p) of the xylanase gene of *Streptomyces halstedii* JM8 (14). The *xysA*p was excised from pHis1 as a 547 bp *Bgl*II/*Sma*I fragment also carrying the terminator of the methylenomycin resistance gene (T1) (15) upstream of *xysA*p. After several cloning steps described in Table 1, a DNA fragment from the *rim* cluster containing *rimA* and the 3' end of the *rimI* gene (9336-15445 bp from the sequence deposited under accession number AY442225) (13) was fused with *xysA*p. 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 *Eco*RV site (inside the thiostrepton resistance marker) of a low copy number *Streptomyces* vector (pIJ922). The recombinant plasmid (pSM743B) (see 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 intra-specific 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*, so *rimA* expression is now 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 probably involves the efficiency of *xysA*p in driving expression of the *rimA* gene, suggesting that expression of *rimA* would be a limiting

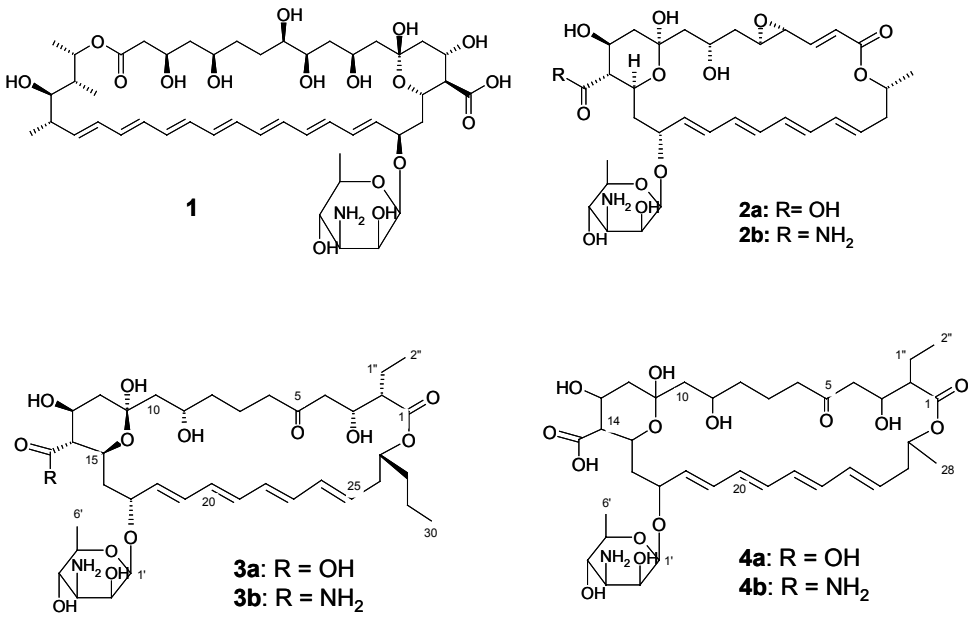


Figure 1
Chemical structures of the relevant tetraenes cited within the text. The structures were: 1: amphotericin B; 2a: pimaricin; 2b: AB-400; 3a: rimocidin; 3b: rimocidin B; 4a: CE-108; 4b: CE-108B

step in the production of the polyenes. Surprisingly, in the *rimA*-disrupted mutant transformed with the engineered *rimA* gene, in addition to the expected complementation, new polyenes were also observed. A similar profile was also seen in the recombinant *S. diastaticus* var. 108 carrying the engineered *rimA*; the new compounds showed characteristic visible/UV tetraene spectra similar to rimocidin and CE-108 (see Figure 2C). Due to their proximity within the chromatogram in retention time to the native compounds, the new polyenes were named rimocidin B and CE-108B, respectively (Figure 1, **3b** and **4b**).

In order to exclude the possibility that production of the novel 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 profile on their production (data not shown), suggesting that the formation of the new 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.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Reference
S. diastaticus var. 108	Wild type (WT), CE-108 and rimocidin producer	(17)
S. diastaticus var. 108/922	Wild type (WT) transformed with pIJ922 plasmid (WT, control)	This work
S. diastaticus var. 108/PM1-500	WT derivative with *rimA* disrupted by integration of PM1-500; CE-108 and rimocidin non-producer	(13)
S. diastaticus var. 108/743B	WT derivative transformed with pSM73B; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
S. diataticus var. 108::PM1-500/743B	WT derivative with *rimA* disrupted by integration of PM1-500 and transformed with pSM743B; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
S. diastaticus var. 108/784	WT derivative transformed with pSM784; CE-108, rimocidin, CE-108B and rimocidin B producer	This work
S. sp. RGU5.3	Wild type (WT); pimaricin and AB-400 producer	This work
E. coli JM101	General cloning host	(35)
S. lividans TK21	General cloning host	(32)
Penicillium chrysogenum ATCC10003	Antifungal activity assays	ATCC
Candida albicans ATCC10231	Antifungal activity assays	ATCC
Candida krusei ATCC14243	Antifungal activity assays	ATCC
Aspergillus niger ATCC1004	Antifungal activity assays	ATCC
Cryptococcus neoformans ATCC10226	Antifungal activity assays	ATCC
pIJ922	Vector based on the SCP2* replicon, *tsr,* 24 kb	(36)
pIJ941	Vector based on the SCP2* replicon, *tsr, hyg*, 25 kb	(36)
pNAe-1	Replicative vector in *E. coli*, *ermE,* Km^R.	N. Allende, unpublished
pHis1	Replicative vector in *E. coli* carring xylanase *xysA* promoter (*xysA*p), Ap^R, 3.7 kb	(14)
pSM736	1.2 kb *Sac*I-*Dra*III and 4.9 kb *Dra*III-*Bgl*II fragments containing *rimA* cloned simultaneously into the *Sac*I/*Bam*HI sites of pIJ2925	This work
pSM738	6.1 kb *Bgl*II-*Pst*I fragment of pSM736 (*rimA* gene) and 1.7 kb *Pst*I-*Sph*I fragment of pNAe-1 (*ermE* gene resistance) cloned simultaneously into the *Sma*I/*Sph*I sites of pHis1	This work
pSM743B	8.4 kb *Bgl*II fragment of pSM738 (containing *xysA*p, *rimA* and *ermE*) cloned into the *Eco*RV site of pIJ922 (see Figure 2).	This work
pGAe-1	Replicative vector in *E. coli, ermE,* Ap^R.	A. González, unpublished
pSM784	1.8 kb *Ssp*I-*Eco*RV fragment of pGAe-1 (*ermE* gene resistance) cloned into the *Eco*RV site of pIJ941 (see Figure 2)	This work

Ap, ampicillin; Km, kanamycin; *ermE*, *tsr* and *hyg:* erythromycin, thiostrepton and hygromycin resistance genes.

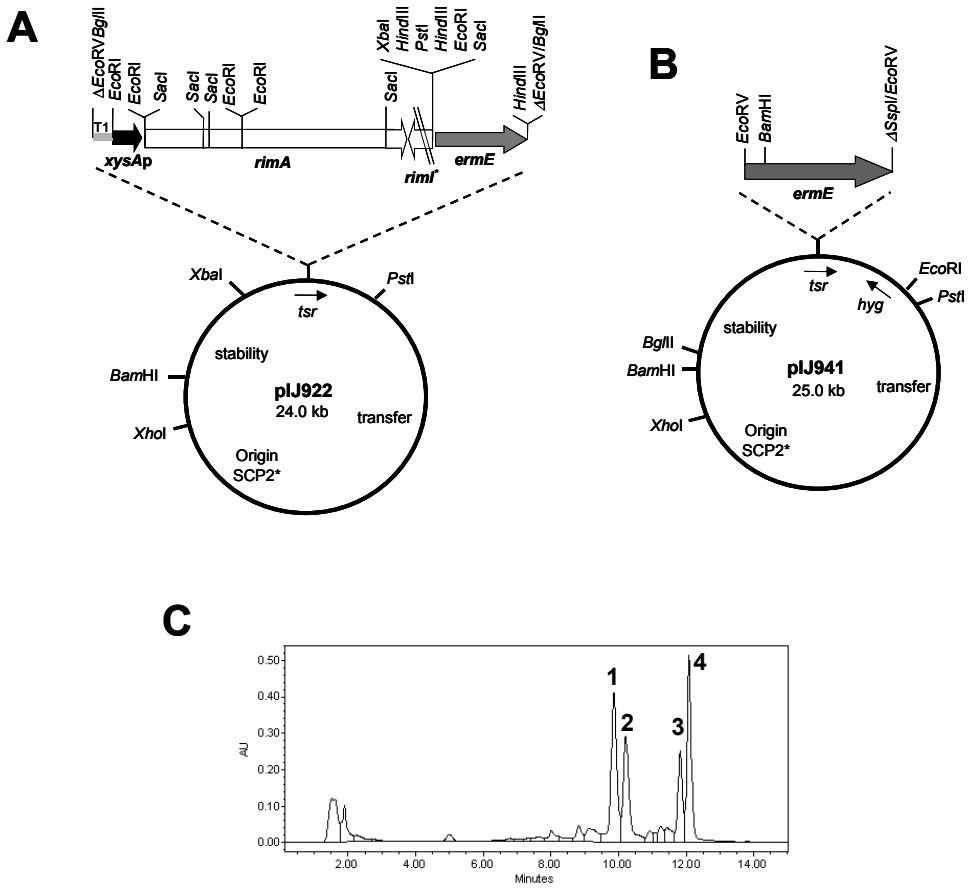


Figure 2

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 are: 1. CE-108B; 2. CE-108; 3. rimocidin B; and, 4. rimocidin.

(*xysA*p: *xysA* promoter; *rimA*: Type I PKS involved in CE-108 and rimocidin biosynthetic pathway; T1: terminator of methylenomycin resistance gene; *ermE*, *tsr* and *hyg:* erythromycin, thiostrepton and hygromycin resistance genes, respectively; *rimI**: *rimI* truncated at its N-terminus).

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 the new biosynthetic process.

A new construction was made 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 *Eco*RV 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 novel 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 for generating the new polyenes. It is noteworthy that neither erythromycin resistance (cloned for other purposes in different vectors such as pHJL401 (16), unpublished results) 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 new polyenes' formation.

Characterization of the new 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 novel tetraenes were 738 and 766 for the lower and higher retention time, respectively. In both cases the mass of the new polyenes is one unit lower than that of the polyenes with closest retention time, CE-108 (739) and rimocidin (767). Both the mass differences and the chromatographic mobility supported the idea that the two new polyenes were derived from the natural macrolides.
Chemical Structure Elucidation
With the aim of structure elucidation, the two new tetraenes were preliminarily characterized in order to develop a procedure for their purification. Both compounds interact not only with reverse phase silica gel such as C8 and C18 but also with an ion exchange resin like SP-Sepharose, 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 *Streptomyces diastaticus* var. 108/pSM743B (see Experimental Procedures).
Compound **4b** was obtained as a yellow powder with a typical tetraene UV spectrum (λ_{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. (+)-ESI MS afforded *pseudo*-molecular ions at *m/z* 739 ($[M+H]^+$) and 761 ($[M+Na]^+$), which delivered the molecular formula $C_{37}H_{58}N_2O_{13}$ by high resolution (found 739.40110, calcd. 739.40118 for $[M+H]^+$). 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 (see 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 mass spectrometry fixed the molecular weight of **3b** as 766, and high resolution delivered the molecular formula $C_{39}H_{62}N_2O_{13}$ (found 767.43254, calcd. 767.43301 DA for $[M+H]^+$). 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 Activities of the Compounds
Antifungal Activity Assays
The antifungal activity of the new tetraene amides was tested against several fungi: *Penicillium chrysogenum, Candida albicans, Aspergillus niger, Candida krusei* and *Cryptococcus neoformans*. Incressing concentrations of different tetraenes dissolved in methanol, were applied onto paper discs (9 mm diameter), dried and transferred to the bioassay plates. The

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*. (34); in our measurement, the signal was missing.

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 (see 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 haemolytic activity of the new molecules. Human erythrocytes were used as a cellular model for this study (7, 18). Haemolytic activity of the new 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 haemolytic activity of the tetraene amides [rimocidin B (**3b**) and CE-108B (**4b**)] was not significantly different from that of their corresponding parental tetraenes while its antifungal activity was clearly higher. Noteworthy are the strong differences in toxicity observed between CE-108 (**4a**) and CE-108B (**4b**) with that of rimocidin (**3a**) and rimocidin B (**3b**); while 50% of haemolysis is reached with 40 to 60 nanomols of these two last polyenes, six to seven folds more concentration of CE-108 and its amide are needed. Thus, the pharmacological properties of the amide polyene CE-108B (**4b**) are clearly enhanced: while CE-108 (**4a**) shows low antifungal activity, its structurally related amide **4b** has an increased antifungal activity nearly as high as that of rimocidin (**3a**), but not its haemolytic activity that is six to seven folds lower. These assays were also performed with horse blood showing similar results (data not shown).

Identical results were obtained using pimaricin (**2a**) and its amide AB-400 (**2b**). Both were purified from our 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 carbon source pimaricin (**2a**) was nearly 70% of the produced polyenes; in an acetate-containing medium, the production profile was reversed, with AB-400 (**2b**) as the mainly produced compound (see Figure 4). This effect was not observed in the engineered *S. diastaticus* var. 108 strain producing the amides. AB-400 (**2b**) was purified from this medium and tested for both antifungal and haemolytic activities. The results summarized in Figure 4C are in good agreement with the previous finding: while pimaricin (**2a**) gave undetectable antifungal activity at the concentration tested, the same amount of AB-400 (**2b**) was active. However, the haemolytic activity assays carried out with AB-400 (**2b**) and commercial pimaricin (**2a**) showed no significant differences between both polyenes. This is a new example 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 infections. Several factors contributed to the increased figures, such as patients with AIDS, those subjected to chemotherapy or those treated with immunosuppressant agents. Despite the need for new antifungal drugs, the numbers of these pharmaceuticals in the market for treating systemic infections is dangerously low. Most of them, such as azoles and amphotericin B (**1**), target the structural integrity of fungal membranes (3, 19), while in the last few years, new antifungal drugs (echinocandins) were developed by targeting specific components of 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 enhanced the clinical interest of this old drug; thus, **1** is undoubtedly a good model in attempts to generate new 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 in deciphering the physico-chemical interactions between the drug or its chemical derivatives and its target membranes, as well as the pharmacokinetic properties of its *semi*-synthetic derivatives (4-8, 11, 22-26). Although not conclusive, all the experimental data, as well as those based on molecular dynamic simulation (4), 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 *semi*-synthetic derivatives and generated by organic synthesis rather than by biotransformations. In this work, we describe the biosynthesis of two new polyenes with changes in the carboxylic group generated by genetic manipulation. *Streptomyces 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 *semi*-synthetic 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 the 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

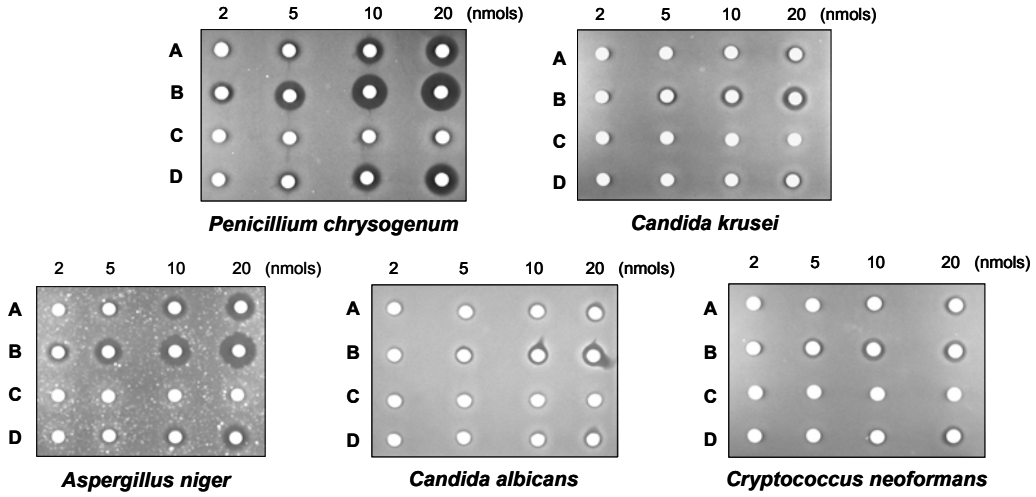


Figure 3

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).

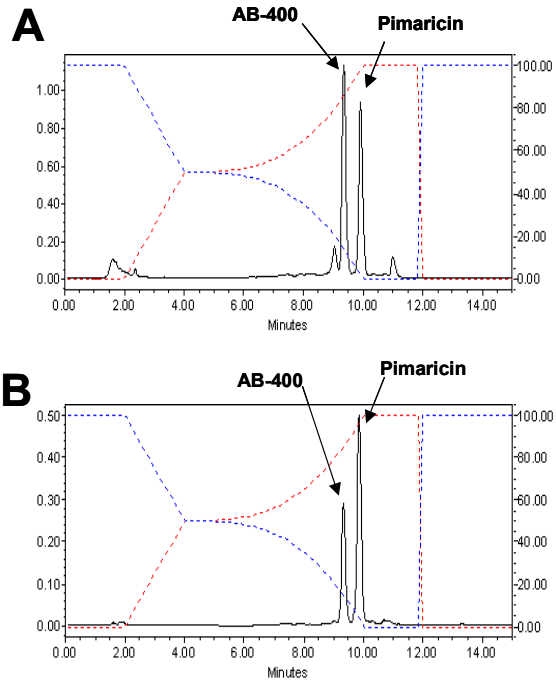
Table 3. Comparative Haemolytic Activity of Several Polyenes (see Experimental Procedures)**.**

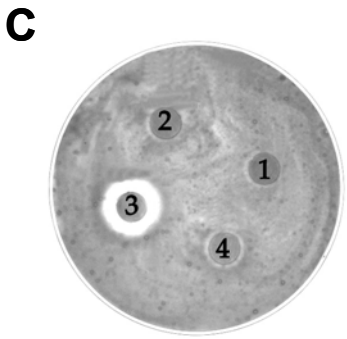
	Amphotericin B (**1**)	Nystatin A	Rimocidin (**3a**)	Rimocidin B (**3b**)	CE-108 (**4a**)	CE-108B (**4b**)
1	1,97					
2	4,47					
3	82,26					
4	100					
20		2,20	10,00	10,00		
40		49,53	21,18	33,51		
60		76,80	65,02	84,80		
80		92,79	95,59	94,80		
100		100,00	100,00	100,00		
120					11,72	12,01
160					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

was obtained with pimaricin (**2a**) and its derivative AB-400 (**2b**), which reinforces the idea that the substitution of the carboxylic group for an amide group will

improve the selective toxicity also of other polyenes.

What are the origins of the new amidated tetraenes? Analysis of polyene production in *Streptomyces 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 newly identified polyene amides are likely being produced by the wild type strain, although at an extremely low level. Genetic manipulation 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 interesting pharmaceuticals.





D

	10 µg	20 µg	40 µg	60 µg	80 µg
Pimaricin	0	0.090	0.858	0.963	1.038
AB-400	0	0	0.016	0.305	1.034

Figure 4

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

HPLC analysis of fermentation broths from *Streptomyces* sp. RGU5.3 in medium with acetate (**A**) and glucose (**B**) as carbon source respectively. **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 *S.* sp. RGU5.3 fermentation broth grown in glucose medium (2), purified AB-400 from *S.* sp. RGU5.3 (3) and purified pimaricin from *S.* sp. RGU5.3 (4); a total of 200 ng were added on each test. **D:** Haemolytic activity for pimaricin (Calbiochem 527962) and AB-400. The values of each polyene are expressed in nanomols (left column) and the corresponding haemolytic activities are given as percentage of total haemolysis (see text for experimental procedures).

Although the precise mechanism of the biosynthesis of these new 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. A second possibility would imply that malonamyl-CoA would be incorporated by module 7 of the corresponding PKS instead of methylmalonyl-CoA as proposed in the biosynthetic model for CE-108 (**4a**) and rimocidin (**3a**) production (13). In this last case, a non-decarboxylating condensation would be required for the incorporation of malonamide which would be a rare event not described so far for elongation steps of polyketide biosynthesis. 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), whose postulated starter unit is malonamide (29), so this metabolite would be easily available for secondary metabolite production in this strain.

Undoubtedly we are far from 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 sub-inhibitory concentrations of erythromycin can modulate bacterial transcription (30). 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 opens the possibility that the *ermE* product (a methylase) might be targeting another intermediate gene, coded within the plasmid DNA, whose final target 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 process if successfully applied to the biosynthetic pathway of commercial polyenes would undoubtedly be a straightforward process for production of improved pharmaceuticals.

Significance

Through genetic manipulation of a polyene producer (*Streptomyces diastaticus* var. 108) we could generate a strain that produces new bioactive compounds. The novel polyenes are amides of the parental tetraenes with higher selectivity toward 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 which may well compete with *semi*-synthetic methods described in the literature.

Experimental Procedures

Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are described in Table 1. *Streptomyces diastaticus* var. 108 and its engineered derivatives were routinely grown in liquid and solid SYM2 medium (31) for tetraene production analysis, and liquid TSB medium (Oxoid) for plasmid and total DNA extraction. *Streptomyces lividans* TK21 was used as general cloning host and grown in solid R5 medium and liquid YEME medium (32). *E. coli* strains were grown in Luria-Bertani (LB) agar or in LB broth (33). *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 (33). *Streptomyces* strains were manipulated as previously described (32). Intra-specific 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*. (33).

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 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 source and a positive ionization mode. The chromatographic conditions were the same as described above.

Purification of the Compounds

Streptomyces diastaticus var. 108/743B was cultivated in either solid or liquid SYM2 medium (17). After six 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 hour and clarified at 5,000 g for 20 minutes to remove solid particles. The clear supernatant was concentrated by rota-evaporation to 10-20 x 10^6 units per microliter 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 x 24 cm) yielded up to 40 mg of tetraene-containing samples. 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 Cat 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, while 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 pH5 in 20% methanol. The fractions containing the mixtures of the 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 *semi*-preparative column (Supelcosil PLC-8, 250 X 21.2 mm). The chromatographic parameters and the mobile phases, controlled with a Waters Automated Gradient Controller, were: 12 minutes with 100% of B (ammonium acetate 20 mM pH 5, ethanol 20%), 43 minutes of a binary gradient up to 50% of A (methanol) and 50% B (curve 6); 35 minutes 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.

Haemolytic 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 bloods. After incubation at 37 ^{o}C for 30 min without agitation, cells were pelleted by centrifugation, and haemolysis evaluated by measuring the absorbance at 545 nm. The values corresponding to total haemolysis 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 numbers, A-4888 and N-3503 respectively);

pimaricin, from Calbiochem (527962). All of them were tested directly from the manufacturer sources without further purification.

Acknowledgements

This work was supported by grants from the Spanish MCyT BIO2002-01445 and European Union QLK3-CT2000-00131. We thank Dr. N. Allende and Dr. A. González for making available pNAe-1 and pGAe-1 respectively; Dr. R. Santamaría for providing pHis1; Sir Prof. D.A. Hopwood for critical reading the manuscript and to Waters Cromatografia Servicio de Aplicaciones, Madrid Spain for the helpful assistance with mass spectra.

Received: December 9, 2004
Revised: February 11, 2005
Accepted: February 15, 2005
Published: May 20, 2005

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