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ORIGINAL PAPER

Improvement of sordarin production through process optimization: combining traditional approaches with DOE

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Abstract BMS-353645, also known as sordarin, was of interest based on its activity against pathogenic fungi. The objective of these studies was to provide high quality starting substrate for chemical modification aimed at further improving biological activity, with particular interest in the inhibition of *Aspergillus*. In the work presented here, Design of Experiments, or DOE, was successfully combined with traditional approaches to significantly improve sordarin yields in fermentation flasks. Overall, yields were increased 25-fold from <100 μg/g to as high as 2,609 μg/g in flasks through the use of various medium and conduction changes supplemented with DOE. The improved process was then successfully scaled to pilot plant tanks with the best batch producing 2,389 μg/g sordarin at the 250-l scale.

Keywords Sordarin · *Sordaria araneosa* · Factorial design · Central composite design · Fermentation optimization

Introduction

The natural product sordarin (1) is a diterpene glycoside produced by the fungus *Sordaria araneosa*. Although its antifungal activity was initially described in 1971 [13], more recent interest in this compound has been based on the identification of its fungal target as translation elongation factor EF-2 [6, 10]. Sordarin and its derivatives inhibit protein synthesis by selectively binding to the fungal EF-2-ribosome complex and blocking translocation [8, 16]. Despite the high amino acid sequence homology among eukaryotes, Shastry et al. [22] demonstrated that differences in sordarin activity were based on the amino acid composition at three specific positions in fungal EF-2. Specificity of the sordarin class of compounds for fungal protein synthesis, along with their multiple functional groups amenable to chemical modification, has presented an opportunity to develop much needed antifungal agents exhibiting novel mechanisms of action (Fig. 1).

Recent work has been directed toward the characterization of natural and semi-synthetic derivatives of sordarin [9, 12, 14, 15, 18], and Odds [19] has published an excellent review on the topic. Other examples of the many diverse structures reported recently include the azasordarin derivative shown in (2) which contains a morpholino group in place of the natural product's sugar moiety [14]. A primary goal of the Discovery Group at Bristol-Myers Squibb was to identify novel structures exhibiting improved activity, particularly against the genera *Cryptoccoccus* and *Aspergillus*. Synthesis and biological activities of several such analogues, including acetylenic ketones and oxazepine heterocycles, have been described [20, 21]. Since the preparation of these derivatives required chemical coupling to a secondary product, specifically the aglycone sordaricin (3), significant quantities of sordarin starting material were required. The process by which sordarin fermentation yields were improved is hence the primary focus of the studies presented here.

Principles of statistical analysis have been employed for the process optimization of many secondary metabolites

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and have been available for decades [2–4], with more recent applications including the enzymatic esterification of 2-chlorobutyric acid with 1,2-epoxy-5-hexene employing an immobilized lipase from *Mucor miehei* [11], optimization of phytase production by *Aspergillus ficuum* [1], and carotenoid production by *Rhodotorula glutinis* [5]. In their review, Kennedy and Krouse [17] have also discussed the pros and cons of component swapping and other medium design methods. This report describes one example of how such traditional approaches, when combined with the more modern DOE technology, were able to improve sordarin yields ca. 50-fold to 2,600 μg/g in flasks and 2,400 μg/g in tanks.

napolis, IN, USA), 0.15% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \cdot 7\ H_2O$, and 0.001% $FeSO_4 \cdot 7\ H_2O$.

SR5-M1 base medium 5.0% dextrose, 1.0% Tastone 154, 0.15% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4 \cdot 7\ H_2O$, and 0.001% $FeSO_4 \cdot 7\ H_2O$.
Additional modifications of SR5 had the following composition:

Medium	Cerelose (%)	Tastone-154 (%)	Pharmamedia (%)	NH_4NO_3 (%)	$(NH_4)_2SO_4$ (%)	$FeSO_4 \cdot 7\ H_2O$ (%)	$MgSO_4 \cdot 7\ H_2O$ (%)
SR5-M2	5.5	1.0	1.0	0.5	0.8	0.003	–
SR5-M3	6.5	1.0	0.5	0.3	0.5	0.001	–
SR5-M4	7.5	0.75	0.35	0.6	0.35	0.0003	–
SR5-M5	9.0	0.75	0.35	0.6	0.044	0.0003	–
SR5-M6	9.0	0.75	0.35	0.6	–	0.0003	0.0025

Materials and methods

Media

Media were prepared with the following ingredients.

SPV-M medium 1.0% peptone, 2.1% malt extract, 4.0% glycerol, 0.1% yeast extract.

SR2-M2 medium 4.0% sucrose, 1.25% corn steep solids, 0.5% Tastone 154 (Sensient Bionutrients, India-

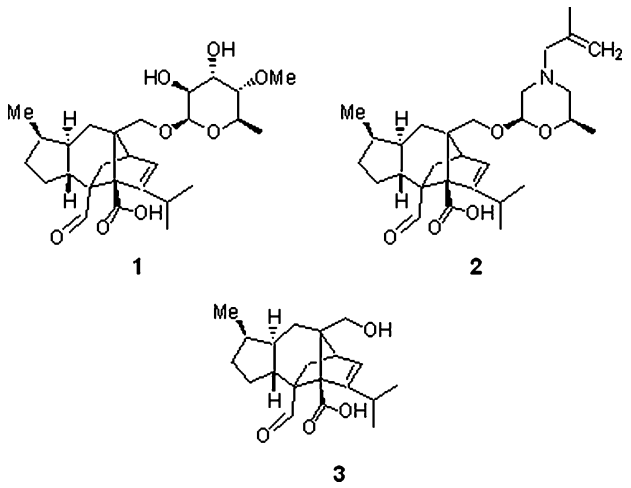

Fig. 1 Sordarin (1), azasordarin (2), and sordaricin (3)

All media were made up in deionized water and sterilized for 20–30 min at 121°C.

Microorganisms

Sordaria araneosa SC16361 was obtained from the Bristol-Myers Squibb (BMS) culture collection.

Fermentation

Early studies

Initial studies typically employed the following protocol for sordarin production in flasks. A frozen vial was thawed and transferred into an F1 stage 500-ml flask containing 100 ml SR2-M2 medium, followed by incubation for 2–3 days at 26°C and 250 rpm. For the F2 stage, a 3% transfer was made into 500-ml flasks containing 100 ml of the same medium followed by an additional incubation period of 1–2 days at 26°C and 250 rpm. Incubation times were based on sufficient growth being present for transfer to the next stage. Production flasks were inoculated with a 2% transfer into 250-ml flasks containing 50 ml of SR5-M1 Base medium supplemented with 1% corn steep solids. In these early studies, flasks were incubated at 26°C and 250 rpm. Flasks were initially harvested after 3–4 days, although incubations were eventually extended to 10 days as described below. Data were analyzed and presented using the average of duplicate flasks, or triplicate flasks where possible.

DOE studies

The usual protocol for sordarin production at the start of the DOE studies which followed began with a frozen

vial which was thawed and transferred into an F1 stage 500-ml flask containing 100 ml SR2-M2 medium. This flask was incubated for 1 day at 26°C and 260 rpm. For the F2 stage, a 3% transfer was made into a 4-l flask containing 1 l of the same medium. After an additional incubation period of 1–2 days at 26°C and 250 rpm, a 4% transfer was made into 250-ml production flasks containing 50 ml of (initially) SR5-M2 medium. HPLC assays were usually performed following a 10–12 day production stage at 26°C and 280–300 rpm. Data points for each DOE study were based upon either single flasks or the average of duplicate flasks, as indicated in the corresponding figure or table. For 250-l tanks, F1 and F2 stages were prepared as above, followed by a 6% transfer into the production tank. The tank medium was SR5-M6 supplemented with 0.1% Dow Corning and SAG 5693 antifoams.

Analytical methods

Sordarin yields in whole broth were determined by extraction with ethyl acetate at acidic pH and quantitated by HPLC. Samples were prepared by adjusting 10 g of whole broth to pH 4.0 with 0.1 N HCl, followed by extraction with 20 ml ethyl acetate. The mixture was shaken in a glass tube sealed with a foil-lined cap for 15 min on a reciprocal shaker, then centrifuged for 10 min. From the separated phases, 10 ml of the ethyl acetate layer was dried under nitrogen, and the residue was taken up in one ml of acetonitrile for HPLC analysis. Sordarin and sordaricin elute at ca. 26 and 34 min, resepectively.

HPLC conditions:
Column: Whatman Partisil 5, ODS-3, 4.6 × 250 mm
Column temperature: Ambient
Injection volume: 20 µl
Flow rate: 1 ml/min, with a run time of 40 min
Detection: UV diode array monitored at 203 nm
Mobile phase: 0.1% TFA in H_2O**:**Acetonitrile (65**:**35)

DOE analysis

Eight designs were performed during this study: three 2^{5-1} fractional factorials with four center points, one 3^2 with three center points, one 2^4 full factorial with four center points, and three 2 factor central composite designs. Analyses of factorial and central composite designs are given in many standard texts [3, 4]. The four 2 level factorial designs were analyzed using a set of in-house custom designed Excel spreadsheets. The full 2^4 factorial analysis and three 2^{5-1} fractional factorials incorporated 16 factorial points plus four center points for a total of 20 runs or data points. The full factorial design was able to estimate all of the main effects as well as all 2, 3, and 4-way interactions. The three 2^{5-1} fractional factorials were capable of estimating main effects plus all two-way interactions. Macros were used to construct Main Effect and Two-Way Interaction Plots for all significant terms. The three level factorial and the central composite designs were analyzed using PROC GLM of SAS® (Version 8.2). The two factor central composite designs consisted of four factorial points, four axial points, and four center points. This allowed a full quadratic polynomial regression model to be fit to the data. For all analyses, P values less than 0.05 were considered significant whereas P values between 0.05 and 0.10 were considered marginally significant. Center-points for the factorial designs were used to test for overall curvature as well as the replication error for testing main and two-way interaction effects. Plots for the central composite designs were constructed using PROC G3D and PROC CONTOUR of SAS. These plots were based on the "best" model found by first fitting a full quadratic model (linear, cross product, and quadratic terms) and then removing non-significant terms (i.e., terms with P values greater than 0.10). Non-significant quadratic terms were removed first, followed by the cross product terms. If a higher order term was significant, then any sub-term was kept in the final model. For example, if $(\text{Cerelose})^2$ was significant, then Cerelose would be kept in the final model even if Cerelose was not significant.

Results and discussion

Traditional fermentation improvement methods

Early studies employing more traditional methods, while designed to increase product yields, were also critical to achieving process robustness and reproducibility of results. The experiments summarized here culminated in a protocol that was thus more amenable to DOE statistical analysis. The medium utilized at the start of the project was SR5-M1 Base supplemented with 1% corn steep solids (CSS). Product yields were low in this medium, below 100 µg/g, and supplemental nitrogen sources were investigated. It was found that yields could be improved by replacing corn steep solids with Pharmamedia at 1.0%, and this modification improved sordarin production after 4 days from our starting level of 23 µg/g to 53 µg/g. Aeration and inoculum level were found to be key factors, and it was observed that higher aeration (330 rpm) in combination with a higher inoculum level (4%) and an extension of

incubation time to 6 days increased the sordarin yield to 156 μg/g. The inorganic nitrogen component of the modified Pharmamedia-based medium was also investigated, leading to a further increase to 202 μg/g by replacement of NH_4NO_3 with 0.83% $(NH_4)_2SO_4$.

Additional studies helped to confirm and establish optimal aeration conditions (minimum of 280 rpm) and a standard inoculum generation protocol (2 day incubation for both the F1 and F2 stages); these changes gave a reproducible yield of 260 μg/g. At this time it was also found that the effects of varying the level of Tastone 154 (our preferred source of yeast extract) were dependent upon whether NH_4NO_3 or $(NH_4)_2SO_4$ was also present in the medium. Interestingly, it was determined that in the presence of 0.15% NH_4NO_3, increasing Tastone-154 from 1.0 to 2.0% reduced the yield by 80%. In contrast, in the presence of 0.83% $(NH_4)_2SO_4$, the same change in Tastone 154 levels increased sordarin production by 62% to 294 μg/g. The effects of nitrate and ammonium ion were isolated by the testing of $NaNO_3$ and NH_4Cl during the course of these studies, and it was ultimately determined that $(NH_4)_2SO_4$ and NH_4NO_3, at 0.8 and 0.5%, respectively, provided the best results. Incorporation of both inorganic components simultaneously reduced the Tastone 154 requirement back to 1.0%. This modified formulation, along with an extension of the fermentation time from 7 to 10 days, facilitated a new sordarin high of 498 μg/g.

Sordarin yields were further improved by increasing the $FeSO_4{\cdot}7H_20$ to 0.003% (622 μg/g). For scale up considerations, dextrose was also replaced at this time with Cerelose (glucose monohydrate), KCl was eliminated, and the resulting medium was designated SR5-M2. Complexity of the interactions seen in the above studies served as an impetus for the DOE experiments described below. Even higher levels were achieved by changing the medium in which the frozen vial lot was prepared. SPV-M, the medium used to this point, was replaced by the same medium used for generating the F1 and F2 inoculum stages, SR2-M2. Not surprisingly, it was observed that the F1 stage could be reduced from 2 days to 1 day, and this modified inoculum protocol also provided the highest pre-DOE yield at 945 μg/g. A summary of these improvements is shown in Fig. 2, and the starting medium for the DOE studies described below was SR5-M2.

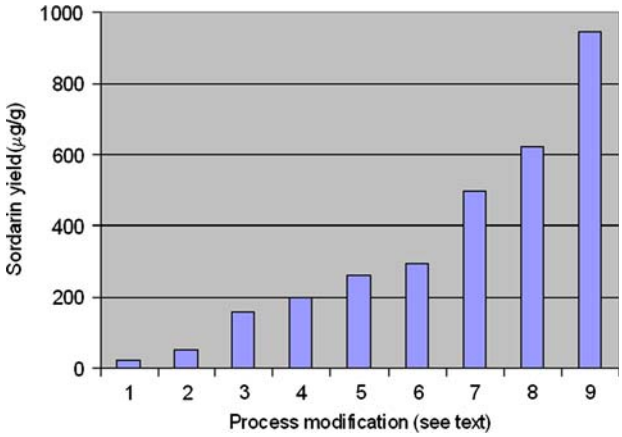

Fig. 2 Summary of traditional (non-DOE) process improvements. *1* Starting medium: SR5-M1 base w/corn steep solids; *2* CSS replaced with Pharmamedia in medium; *3* Initial aeration, inoculum, incubation conditions established; *4* Ammonium nitrate is replaced with ammonium sulfate; *5* Further establish aeration conditions and inoculum age for F1 and F2 stages; *6* Increase Tastone 154 to 2.0% [at 0.83% $(NH_4)_2SO_4$]; *7* Preliminary optimization of $(NH_4)_2SO_4$ [0.8%], NH_4NO_3 [0.5%], and Tastone 154 [1.0%], extension of fermentation time to 10 days; *8* Increase $FeSO_4{\cdot}7\ H_2O$ to 0.003%; *9* Modify vial preparation procedure, decrease F1 inoculum stage

DOE improvements

In order to further increase yields, a set of statistical experiments was performed to evaluate the interactions between the medium components of SR5-M2. To initially reduce the number of experiments (i.e. flasks) and still have the ability to estimate all two-way interactions for five of the components, $FeSO_4{\cdot}7\ H_2O$ was kept constant at 0.003%. Based on earlier studies, this was believed to be the component least likely to interact with the others and also have a minimal effect upon product yield. The remaining five medium components were then evaluated using a 2^{5-1} fractional factorial design. After the first study was completed, the results suggested a re-evaluation of $FeSO_4{\cdot}7\ H_2O$, and a second 2^{5-1} fractional factorial experiment was conducted holding Tastone 154 constant at 1.0%. (It is noted that a single 2^{6-1} fractional factorial design could also have been performed using all six medium components. However, this would have resulted in a requirement for 32 flasks which *at the time of the first study* exceeded available resources.) The factors and yields for both studies are displayed in Tables 1 and 2, with the results showing that the medium could indeed be improved. One plot of a marginal interaction based on the data in Table 1 is illustrated in Fig. 3 for Cerelose and Pharmamedia, where the *P* value (0.0746) demonstrated that the effect of Pharmamedia concentration was essentially independent of Cerelose concentration. In contrast, the plot of Pharmamedia versus NH_4NO_3 in Fig. 4 showed a much more significant effect (*P* 0.0002). In each study, two levels of each component were examined, with four possible combinations for each two-way interaction. Analysis of the data for both studies

Table 1 Initial four variable two level factorial experiment: $FeSO_4 \cdot 7\ H_2O$ was kept constant at 0.003%

Run #	Cerelose	Pharmamedia	Tastone	NH_4NO_3	$(NH_4)_2SO_4$	Sordarin
1	6.5	0.5	1.5	0.7	0.5	910
2	4.5	1.5	0.5	0.7	1.1	184
3	4.5	0.5	0.5	0.7	0.5	865
4	4.5	0.5	0.5	0.3	1.1	174
5	*5.5*	*1.0*	*1.0*	*0.5*	*0.8*	*581*
6	6.5	1.5	1.5	0.3	0.5	644
7	6.5	0.5	0.5	0.3	0.5	216
8	6.5	1.5	0.5	0.7	0.5	233
9	*5.5*	*1.0*	*1.0*	*0.5*	*0.8*	*580*
10	6.5	1.5	1.5	0.7	1.1	323
11	6.5	0.5	0.5	0.7	1.1	161
12	6.5	1.5	0.5	0.3	1.1	170
13	6.5	0.5	1.5	0.3	1.1	320
14	*5.5*	*1.0*	*1.0*	*0.5*	*0.8*	*587*
15	4.5	0.5	1.5	0.7	1.1	437
16	4.5	1.5	0.5	0.3	0.5	341
17	*5.5*	*1.0*	*1.0*	*0.5*	*0.8*	*542*
18	4.5	1.5	1.5	0.3	1.1	374
19	4.5	1.5	1.5	0.7	0.5	361
20	4.5	0.5	1.5	0.3	0.5	242

Center point values are italicized. Medium component concentrations are presented in units of percent, sordarin levels in μg/g. Each data point was based on a single flask

Table 2 Follow-up four variable two level factorial experiment: Tastone-154 level was kept constant at 1.0%

Run #	Cerelose	Pharmamedia	$FeSO_4 \cdot 7H_2O$	NH_4NO_3	$(NH_4)_2SO_4$	Sordarin
1	6.5	0.5	0.01	0.7	0.5	307
2	4.5	1.5	0.001	0.7	1.1	309
3	4.5	0.5	0.001	0.7	0.5	552
4	4.5	0.5	0.001	0.3	1.1	684
5	*5.5*	*1.0*	*0.003*	*0.5*	*0.8*	*844*
6	6.5	1.5	0.01	0.3	0.5	807
7	6.5	0.5	0.001	0.3	0.5	1,158
8	6.5	1.5	0.001	0.7	0.5	362
9	*5.5*	*1.0*	*0.003*	*0.5*	*0.8*	*855*
10	6.5	1.5	0.01	0.7	1.1	403
11	6.5	0.5	0.001	0.7	1.1	238
12	6.5	1.5	0.001	0.3	1.1	269
13	6.5	0.5	0.01	0.3	1.1	362
14	*5.5*	*1.0*	*0.003*	*0.5*	*0.8*	*852*
15	4.5	0.5	0.01	0.7	1.1	368
16	4.5	1.5	0.001	0.3	0.5	560
17	*5.5*	*1.0*	*0.003*	*0.5*	*0.8*	*789*
18	4.5	1.5	0.01	0.3	1.1	441
19	4.5	1.5	0.01	0.7	0.5	192
20	4.5	0.5	0.01	0.3	0.5	158

Center point values are italicized. Medium component concentrations are presented in units of percent, sordarin levels in μg/g. Each data point was based on a single flask

showed that best results were usually achieved at one concentration for any particular component, e.g. 0.001% $FeSO_4 \cdot 7\ H_2O$ or 0.5% $(NH_4)_2SO_4$. The primary exceptions to this were Tastone 154 and NH_4NO_3, in which both high and low levels supported high product yields. These components would therefore be the subject of the next study. Based on the new peak value of 1,158 μg/g shown in Table 2, medium SR5-M2 was modified to SR5-M3. This new medium composition then became the basis for the next factorial analysis, although 1.5% (rather than 1.0%) Tastone 154 was selected as a center point in order to better evaluate a higher concentration range. A level of 0.3% NH_4NO_3 was selected as the other center point.

The resulting 3^2 factorial is shown in Fig. 5a, b, where Tastone 154 and NH_4NO_3 levels were each varied in basal SR5-M3. The data suggested that at ca. 0.6% NH_4NO_3 one should be able to further increase sordarin yields in conjunction with lowering Tastone 154 below 1.0%. It was noteworthy however, that a new sordarin high of 1,286 μg/g was in fact achieved at levels of 0.3% NH_4NO_3 (rather than 0.6%) and 1.0% Tastone 154. Despite this actual flask result, the suggested 0.6% level was selected as a constant for the third 2^{5-1} fractional factorial shown in Table 3. At this point, it was clear that higher product yields were obtained by increasing carbon (Cerelose) and decreasing all three of the experimental nitrogen sources

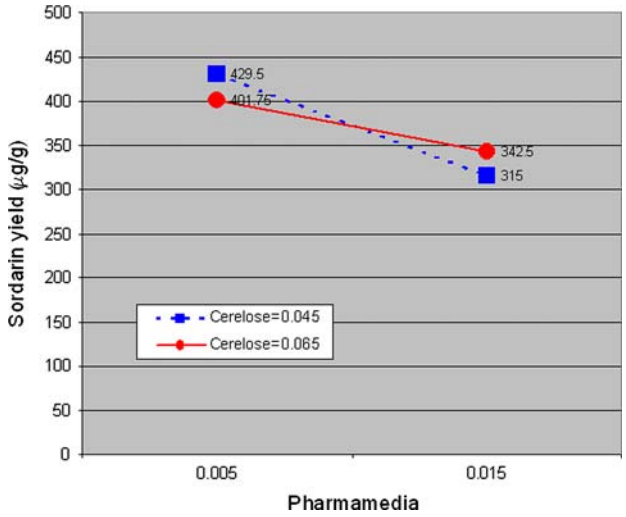

Fig. 3 Sordarin yield interaction means ($N = 4$) for Cerelose and Pharmamedia (P 0.0746). Nutrient levels in this and other interaction plots, unless stated otherwise, are expressed in decimal form for percent (i.e. 0.005 = 0.5%)

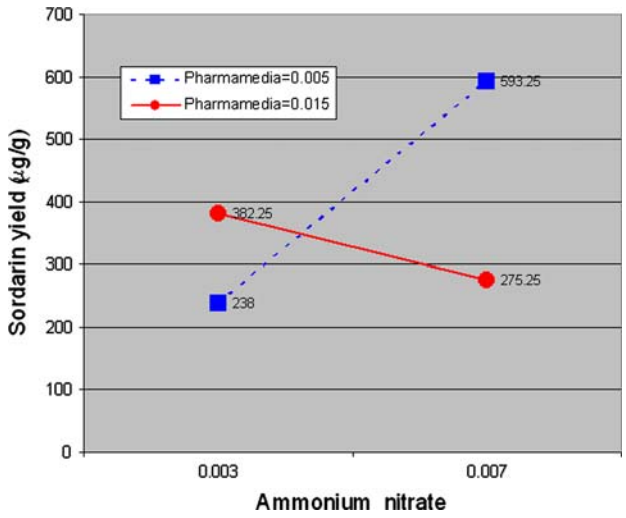

Fig. 4 Sordarin yield interaction means ($N = 4$) for Pharmamedia and ammonium nitrate (P 0.0002)

(Pharmamedia, Tastone 154, and $(NH_4)_2SO_4$) in SR5-M3. In this study, a high of 1,476 µg/g was achieved, with the greatest significance observed for the interaction between Cerelose and $(NH_4)_2SO_4$ (P 0.0287) as shown in Fig. 6. Accordingly, SR5-M3 was modified to SR5-M4 for the next study, with Cerelose and $(NH_4)_2SO_4$ undergoing further analysis.

At this stage, a two factor central composite for these two components was performed. Employing SR5-M4 center points of 7.5 and 0.35% for Cerelose and $(NH_4)_2SO_4$ respectively, the best sordarin yield achieved was 1,631 µg/g at 8.0% Cerelose and 0.25% $(NH_4)_2SO_4$. According to the response surface plot illustrated in Fig. 7, the maximum *theoretical* value was even higher (1,854 µg/g) at 8.35% Cerelose and 0.18% $(NH_4)_2SO_4$, and these component levels were selected as SR5-M4 center points for the follow-up central composite shown in Fig. 8. Here, the best flask value of 2,454 µg/g surpassed expectations and was achieved at 8.35% Cerelose and 0.044% $(NH_4)_2SO_4$. It was noteworthy that the center point average of 2,285 µg/g actually obtained in this study exceeded the previous theoretical value of 1,854 µg/g by 23%. The latter study also predicted a peak value (3,269 µg/g) at 9.03% Cerelose and 0.044% $(NH_4)_2SO_4$, and these concentrations (implemented into medium SR5-M5) were successfully used in later experiments as well as scale-up into tanks. Time constraints prevented additional efforts to determine which factor(s) were responsible for the eventual production limit of ca. 2,600 µg/ml (see below) with the described medium composition.

Additional factors affecting the sordarin fermentation

As the above medium formulations resulting in improved yields evolved, reproducibility also improved, especially in flasks. However, scale-up into tanks remained problematic as fermentations frequently died out prematurely with very low productivity. One of the first factors to be examined was that of sterilization time and its effect upon SR5-M4 medium. It was found that versus a sterilization time of 20 min in flasks, there was a reduction in sordarin yields of 83% for 40 min and 88% for 1 h. However, it was discovered during a parallel experiment examining the effects of $(NH_4)_2SO_4$ and $FeSO_4{\cdot}7H_2O$ within the improved SR5-M5 medium that sterilization time became less of a factor. As shown in Table 4, $FeSO_4{\cdot}7H_2O$ was clearly required for significant sordarin production. However, it is also shown that $(NH_4)_2SO_4$ could be completely eliminated from this medium and that it was in fact inhibitory, but only in the absence of $FeSO_4{\cdot}7H_2O$. This $FeSO_4{\cdot}7H_2O$ requirement was much more significant for SR5-M4 than SR5-M5 (data not shown), presumably due to the eightfold higher levels of $(NH_4)_2SO_4$ in the former , which might also explain the minimal effect of sterilization time upon SR5-M5. In fact, it was found that as long as ferrous sulfate was a component of the medium, production yields in SR5-M5 were not only more stable, but actually higher at longer (60 vs. 30 min) sterilization times. On this basis, the use of ferrous sulfate was continued upon scale-up into larger tanks. The elimination of $(NH_4)_2SO_4$ at this point was ironic in that its addition to the medium during early studies led to the single most significant breakthrough, having provided us with the first indication that high yields (>50 µg/g) were even possible.

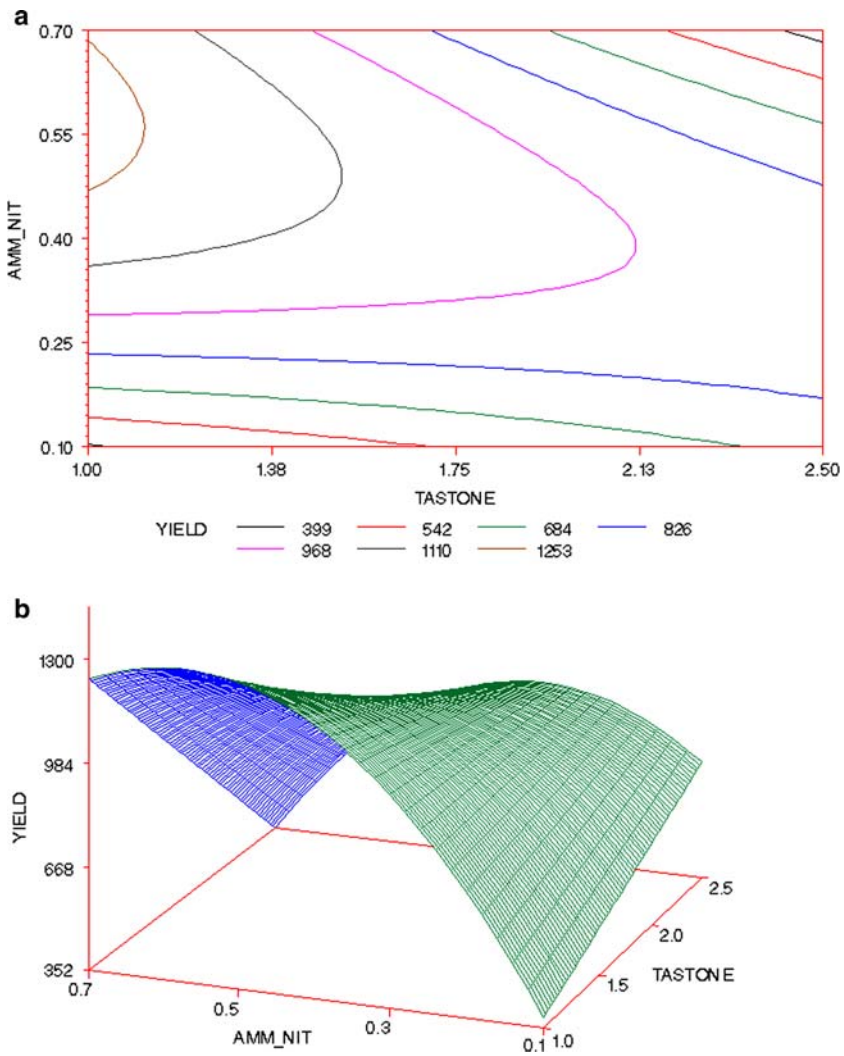

Fig. 5 **a**, **b** Results from a 3^2 factorial study, shown in both 2-D and 3-D format, where Tastone 154 and NH_4NO_3 levels were varied. All axes are presented in units of percent for each medium component, and sordarin yields are in units of μg/g. Data were based on the results from single flasks

The above factors turned out to be not only critical to the sordarin fermentation scale-up but also served to demonstrate the importance of interactions between such components and the value of DOE. Preliminary studies had suggested that not only Fe^{2+}, but also Mg^{2+}, Ca^{2+}, and Mn^{2+} might affect product formation. A 2^4 full factorial was subsequently performed for these four trace metals in medium SR5-M5, and as shown in Table 5, the best results (2,411 μg/g) were coincidentally obtained as an average of the four center point concentrations. Since Fe^{2+} and Mg^{2+} exhibited both the strongest main effects (*P* 2.883E−06 and 5.487E−07, respectively) as well as the most significant two-way interaction (*P* 4.118E−06), they were selected for further analysis in the form of a two factor central composite. One important observation made during this study from a visual perspective is shown by a further analysis of the data according to Table 6. Of those flasks having the lowest Fe^{2+} level (eight data points at 0.003 mM), there was a strong trend whereby only that subset of flasks *also* having the highest Mg^{2+} level (four data points at 0.3 mM) "blackened" early (usually associated with product formation) and gave the best final results at 1,779 μg/g. In the presence of lower Mg^{2+} (four data points at only 0.03 mM) yields were reduced to 895 μg/g. The impact of Mg^{2+} was not as significant at the higher 0.03 mM Fe^{2+} level. The additional Mg^{2+} appeared to reduce cell lysis and facilitate product formation, similar to the "stabilizing" effect of Fe^{2+} in the presence of ammonium ion previously observed.

The best flask data for the SR5-M5 derived central composite shown in Fig. 9 was 2,609 μg/g at 0.0042 mM Fe^{2+} and 0.158 mM Mg^{2+} for the average of two factorials. Clearly but surprisingly, Mg^{2+} no longer appeared to play a critical role in sordarin production. Although time constraints precluded further investigation, this was attributed to the fact that SR5-M5 had been modified to completely eliminate $(NH_4)_2SO_4$ by the time this study was initiated, i.e. SR5-M6 was used. Despite these results, $MgSO_4 \cdot 7\ H_2O$ had already been incorporated into the SR5-M5 based tank production

Table 3 A five variable, two level factorial study was performed with four center points and NH_4NO_3 was kept constant at 0.6%

Run #	Cerelose	Pharmamedia	$(NH_4)_2SO_4$	FeSO4·7 H_2O	Tastone	Sordarin
1	7.5	0.35	0.65	0.003	0.75	570
2	5.5	0.65	0.35	0.003	1.25	142
3	5.5	0.35	0.35	0.003	0.75	205
4	5.5	0.35	0.35	0.0003	1.25	515
5	*6.5*	*0.5*	*0.5*	*0.001*	*1*	*406*
6	7.5	0.65	0.65	0.0003	0.75	251
7	7.5	0.35	0.35	0.0003	0.75	1,476
8	7.5	0.65	0.35	0.003	0.75	670
9	*6.5*	*0.5*	*0.5*	*0.001*	*1*	*629*
10	7.5	0.65	0.65	0.003	1.25	172
11	7.5	0.35	0.35	0.003	1.25	195
12	7.5	0.65	0.35	0.0003	1.25	1,143
13	7.5	0.35	0.65	0.0003	1.25	1,116
14	*6.5*	*0.5*	*0.5*	*0.001*	*1*	*593*
15	5.5	0.35	0.65	0.003	1.25	139
16	5.5	0.65	0.35	0.0003	0.75	967
17	*6.5*	*0.5*	*0.5*	*0.001*	*1*	*660*
18	5.5	0.65	0.65	0.0003	1.25	716
19	5.5	0.65	0.65	0.003	0.75	400
20	5.5	0.35	0.65	0.0003	0.75	1,006

Center point values are italicized. Using the new center point for NH_4NO_3, a new record yield of 1476 μg/g was achieved. Cerelose and $FeSO_4$ exhibited significant main effects ($P < 0.05$, data not shown). Medium component concentrations are presented in units of percent, sordarin levels in μg/g. Each data point was based on the average of triplicate flasks

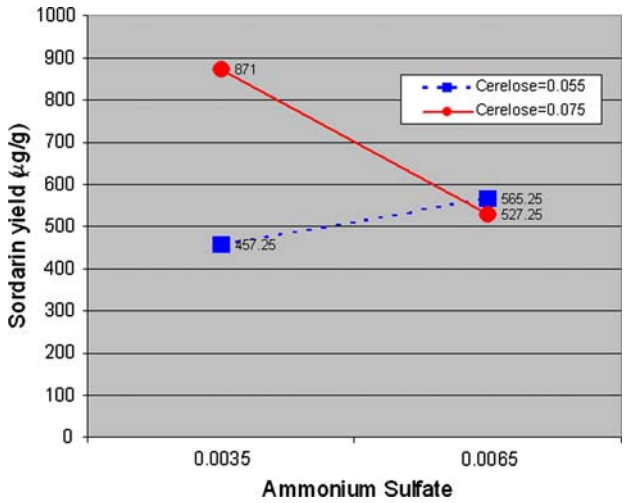

Fig. 6 Sordarin yield interaction means ($N = 4$) for Cerelose and ammonium sulfate (P 0.0287)

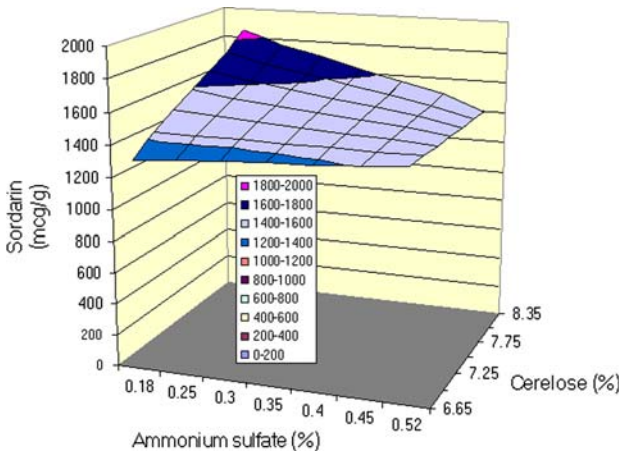

Fig. 7 Response surface plot of a two factor central composite analysis for sordarin versus ammonium sulfate and Cerelose. Center points were 7.5% Cerelose and 0.35% $(NH_4)_2SO_4$. Each data point was based on the average of duplicate flasks

medium at 0.1 mM (0.0025%) along with Fe^{2+} at 0.01 mM (0.0003%). This formulation, which yielded 2,389 μg/g at the 250-l tank scale, was designated SR5-M6 and no longer contained ammonium sulfate. The impact of trace metal as well as ammonium levels upon secondary metabolite production is not unusual, e.g., in the case of rapamycin biosynthesis ammonium (as well as phosphate and Mg^{2+}) was inhibitory while Fe^{2+} was stimulatory [7]. The continued addition of both trace metals appeared to yield consistent tank results while possibly neutralizing the potential effects of prolonged sterilization times and/or ammonium ion accumulation. This was suggested by flask data and became the basis for our standard production medium. Representative improvements in sordarin production

Table 4 Effect of $FeSO_4$·7 H_2O and $(NH_4)_2SO_4$ upon sordarin production in SR5-M5 medium. Results are based on the average of triplicate flasks

0.044% $(NH_4)_2SO_4$	0.0003% $FeSO_4$·7 H_2O	BMS-353645 (μg/g)
+	+	2,157
–	+	2,177
+	–	311
–	–	893

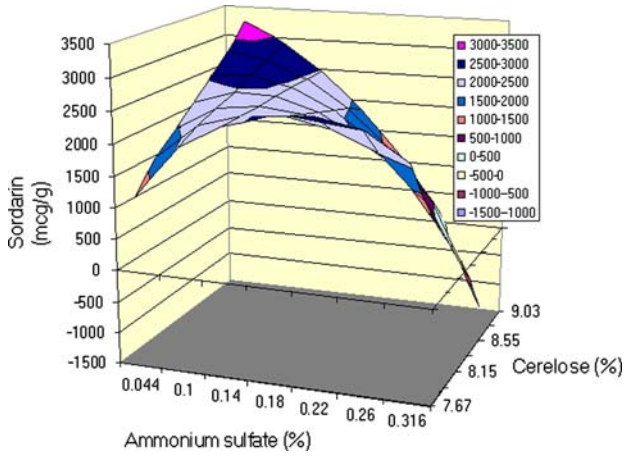

Fig. 8 Follow-up response surface plot of a two factor central composite analysis for sordarin versus ammonium sulfate and Cerelose. Center points were 8.35% Cerelose and 0.18% $(NH_4)_2SO_4$. Each data point was based on the average of duplicate flasks

Table 5 Four variable two level factorial experiment for four trace metals in SR5-M5 medium

RUN #	$FeSO_4 \cdot 7\ H_2O$	$MgSO_4 \cdot 7\ H_2O$	$CaCl_2 \cdot 2\ H_2O$	$MnCl_2$	Sordarin
1	0.003	0.03	0.03	0.1	810
2	*0.01*	*0.1*	*0.1*	*0.03*	*2,419*
3	0.003	0.03	0.03	0.01	419
4	0.003	0.3	0.3	0.1	1,420
5	0.03	0.3	0.3	0.1	1,712
6	0.003	0.3	0.03	0.01	1,271
7	0.003	0.03	0.3	0.1	1,989
8	*0.01*	*0.1*	*0.1*	*0.03*	*2,416*
9	0.03	0.03	0.03	0.1	1,525
10	0.03	0.3	0.3	0.01	1,857
11	0.003	0.3	0.03	0.1	2,296
12	0.03	0.3	0.03	0.1	1,850
13	0.003	0.3	0.3	0.01	2,129
14	*0.01*	*0.1*	*0.1*	*0.03*	*2,404*
15	0.03	0.3	0.03	0.01	1,848
16	0.003	0.03	0.3	0.01	360
17	0.03	0.03	0.3	0.1	1,079
18	0.03	0.03	0.3	0.01	1,897
19	*0.01*	*0.1*	*0.1*	*0.03*	*2,406*
20	0.03	0.03	0.03	0.01	1,620

Center point values are italicized. Trace metal concentrations were based on and presented in units of mM for more effective comparison; sordarin levels in μg/g. 0.01 mM corresponds to the 0.0003% $FeSO_4 \cdot 7\ H_2O$ described earlier. Each data point was based on the average of duplicate flasks

obtained by these DOE studies are highlighted in Fig. 10.

Conclusions

Although significant improvements in sordarin production were achieved through incorporation of traditional optimization methods, it was found that supplementation with statistical analysis yielded product levels beyond our initial expectations. In particular, the central composite studies revealed the critical nature of both Cerelose and ammonium sulfate levels. The high final carbon source level (greater than 9.0%) would probably not have been approached during routine experimentation had the multiple surface plots not suggested potentially higher yields. It was also interesting to observe the evolution of the medium composition from lower to higher C/N ratio since the first medium to demonstrate any significant production early on was one in which inorganic nitrogen (ammonium and nitrate) levels were much higher. However, early successes were difficult to reproduce, both in flasks and tanks, and it was not until optimal C/N ratios were more clearly established that yields became reproducible. In our experience, it was also relatively unusual to observe such significant influences upon production yields by factors such as sterilization time and Mg^{2+} levels. However, once the interactions involving ammonium ion or Fe^{2+} were observed and

Table 6 Analysis of two-way interactions for $FeSO_4 \cdot 7\ H_2O$ and $MgSO_4 \cdot 7\ H_2O$ from the four variable two level factorial experiment

$FeSO_4$	$MgSO_4$	Mean (N = 4)	Broth	*P* value	Significance
0.003	0.03	895	Light		
0.003	0.3	1779	Dark		
0.03	0.03	1530	Dark		
0.03	0.3	1817	Dark		
				4.1184E−06	***

Broths were usually light to medium brown prior to initiation of sordarin production, at which time they became nearly black

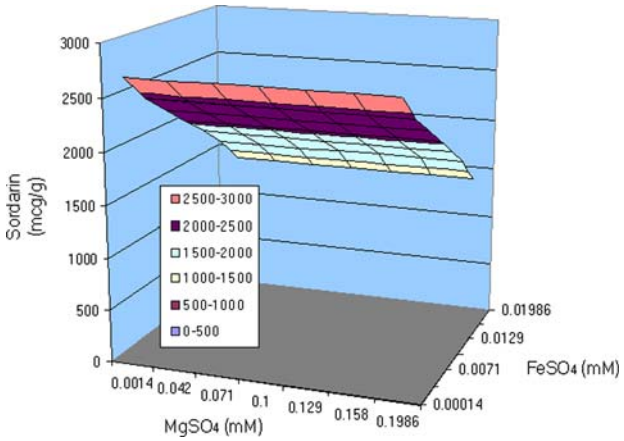

Fig. 9 Response surface plot of a two factor central composite analysis for sordarin versus magnesium sulfate and ferrous sulfate in SR5-M6 medium. Center points were 0.1 and 0.01 mM for magnesium and ferrous ion, respectively. Each data point was based on the average of duplicate flasks

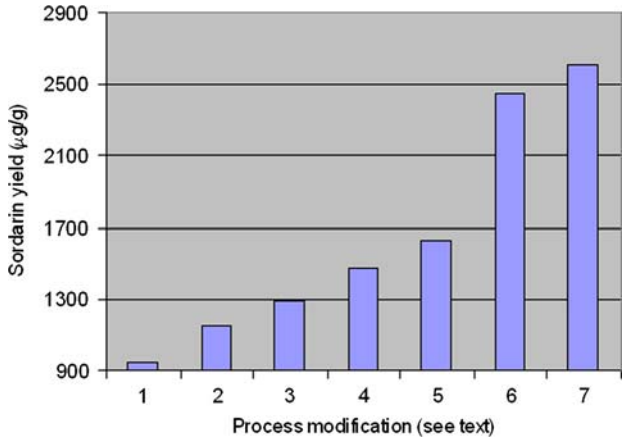

Fig. 10 Summary of improved yields obtained during DOE studies. *1* Sordarin level at start of DOE studies; *2* SR5-M3 medium developed on basis of five variable two level factorial; *3* Result obtained during 3^2 factorial for Tastone-154 versus ammonium nitrate; *4* Follow-up five variable two level factorial; *5* Initial two factor central composite for Cerelose and ammonium sulfate: peak obtained at 8.0% Cerelose, 0.25% ammonium sulfate; *6* Second two factor central composite for Cerelose and ammonium sulfate: peak obtained at 8.35% Cerelose, 0.044% ammonium sulfate; *7* Third central composite, result obtained at 0.0042 mM ferrous sulfate, 0.158 mM magnesium sulfate

investigated, reproducibility at both the flask and (even more so) tank scale was no longer problematic. The most noteworthy aspect of the results presented here was the time investment for the DOE (weeks) versus non-DOE (months) improvements. Although the latter still has its place in fermentation optimization—as evidenced by the improvements shown here—it was clear that the DOE-mediated strategy was far more time and resource efficient, especially when dealing with multiple critical variables.

References

1. Bogar B, Szakacs G, Linden JC, Pandey A, Tengerdy RP (2003) Optimization of phytase production by solid substrate fermentation. J Ind Microbiol Biotechnol 30:183–189
2. Box GEP, Behnken DW (1960) Some new three-level designs for the study of quantitative variables. Technometrics 2:455–475
3. Box GEP, Draper NR (1987) Empirical model-building and response surfaces. Wiley, New York
4. Box GEP, Hunter WG, Hunter JS (1978) Statistics for experimenters: an introduction to design, data analysis, and model building, 2nd ed: Statistics for experimenters: design, innovation and discovery, 2005. Wiley, New York
5. Buzzini P (2000) An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source. J Ind Microbiol Biotechnol 24:41–45
6. Capa L, Mendoza A, Lavandera JL, Gomez de las Heras F, Garcia-Bustos JF (1998) Translation elongation factor 2 is part of the target for a new family of antifungals. Antimicrob Agents Chemother 42:2694–2699
7. Cheng YR, Hauck L, Demain AL (1995) Phosphate, ammonium, magnesium and iron nutrition of *Streptomyces hygroscopicus* with respect to rapamycin biosynthesis. J Ind Microbiol 14:424–427
8. Dominguez JM, Gomez-Lorenzo MG, Martin JJ (1999) Sordarin inhibits fungal protein synthesis by blocking translocation differently to fusidic acid. J Biol Chem 274:22423–22427
9. Dominguez JM, Kelly VA, Kinsman OS, Marriott MS, Gomez de las Heras F, Martin JJ (1998) Sordarins: a new class of antifungals with selective inhibition of the protein synthesis elongation cycle in yeasts. Antimicrob Agents Chemother 42:2274–2278
10. Dominguez JM, Martin JJ (1998) Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. Antimicrob Agents Chemother 42:2279–2283
11. Garcia R, Martinez M, Aracil J (2002) Enzymatic esterification of an acid with an epoxide using an immobilized lipase from *Mucor miehei* as catalyst: Optimization of the yield and isomeric excess of ester by statistical analysis. J Ind Microbiol Biotechnol 28:173–179
12. Gomez-Lorenzo MG, Garcia-Bustos JF (1998) Ribosomal p-protein stalk function is targeted by sordarin antifungals. J Biol Chem 273:25041–25044
13. Hauser D, Sigg HP (1971) Isolierung und Abbau von Sordarin. Helvetica Chim Acta 54:1178–1190
14. Herreros E, Almela MJ, Lozano S, Gomez de las Heras F, Gargallo-Viola D (2001) Antifungal activities and cytotoxicity studies of six new azasordarins. Antimicrob Agents Chemother 45:3132–3139
15. Herreros E, Martinez CM, Almela MJ, Marriott MS, Gomez de las Heras F, Gargallo-Viola D (1998) Sordarins: in vitro activities of new antifungal derivatives against pathogenic yeasts, *Pneumocystis carinii*, and filamentous fungi. Antimicrob Agents Chemother 42:2863–2869
16. Justice MC, Hsu M-J, Tse B, Ku T, Balkovec J, Schmatz D, Nielsen J (1998) Elongation Factor 2 as a novel target for selective inhibition of fungal protein synthesis. J Biol Chem 273:3148–3151
17. Kennedy M, Krouse D (1999) Strategies for improving fermentation medium performance: a review. J Ind Microbiol Biotechnol 23:456–475
18. Martinez A, Aviles P, Jimenez E, Caballero J, Gargallo-Viola D (2000) Activities of sordarins in experimental models of candidiasis, aspergillosis, and pneumocystosis. Antimicrob Agents Chemother 44:3389–3394
19. Odds FC (2001) Sordarin antifungal agents. Exp Opin Ther Patents 11:283–294
20. Quesnelle CA, Gill P, Dodier M, St Laurent D, Serrano-Wu M, Marinier A, Martel A, Mazzucco CE, Stickle TM, Barrett JF, Vyas DM, Balasubramanian BN (2003) Sordaricin antifungal agents. Bioorg Med Chem Lett 13:519–524
21. Serrano-Wu MH, St. Laurent DR, Chen Y, Huang S, Lam K-R, Matson JA, Mazzucco CE, Stickle TM, Tully TP, Wong HS, Vyas DM, Balasubramanian BN (2002) Sordarin oxazepine derivatives as potent antifungal agents. Bioorg Med Chem Lett 12:2757–2760
22. Shastry M, Nielsen J, Ku T, Hsu M-J, Liberator P, Anderson J, Schmatz D, Justice M (2001) Species-specific inhibition of fungal protein synthesis by sordarin: identification of a sordarin-specificity region in eukaryotic elongation factor 2. Microbiology 147:383–390