THE SORAPHENS: A FAMILY OF NOVEL ANTIFUNGAL COMPOUNDS FROM *Sorangium cellulosum* (MYXOBACTERIA)

I. SORAPHEN $A_{1\alpha}$: FERMENTATION, ISOLATION, BIOLOGICAL PROPERTIES†

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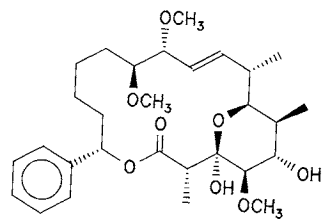
An antifungal activity was detected in the culture broth of *Sorangium cellulosum* (Myxococcales), strain So ce26. The activity was excreted into the supernatant during the log and early stationary phase. The active substance was quantitatively bound to XAD adsorber resin added to the medium at the beginning of the fermentation. The new secondary metabolite was called soraphen and is of special interest to plant disease control for its inhibitory activity against numerous phytopathogenic fungi.

In recent years, myxobacteria have become more and more of interest as a rich source of new secondary metabolites[1,2]. Strains of the species *Sorangium cellulosum*, however, were not yet intensively studied because of the difficulties in their isolation and cultivation.

Two groups of secondary metabolites have been described so far from *Sorangium* strains. The first one includes ambruticin S, an antifungal compound produced by a strain of *Sorangium cellulosum*[3], and the closely related compounds, ambruticins VS 1 to 5 which differ from ambruticin S by their nitrogen content. The latter have been isolated from *S. cellulosum* strain So ce10 and are intensively studied in our laboratory[4]. The second group comprises the sorangicins, which are powerful inhibitors of eubacterial RNA polymerase[5].

During the screening of new isolates of *S. cellulosum*, a strong antifungal activity was discovered in the supernatant of strain So ce26. The activity differed from that of ambruticins in its antimicrobial spectrum. The compounds responsible for the activity, the soraphens, turned out to be a novel class of at least 40 chemically related secondary metabolites. This paper deals with the fermentation process, some biological activities, and the mode of action of the main component, soraphen $A_{1\alpha}$ (Fig. 1). The structure elucidation of soraphen $A_{1\alpha}$ and its derivatives is reported elsewhere[6].

Fig. 1. The structure of soraphen $A_{1\alpha}$[6].



Materials and Methods

Culture Strain

Sorangium cellulosum So ce26 was deposited at

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the National Collection of Industrial and Marine Bacteria (NCIB), Torry Research Station, Aberdeen, Scotland, UK, under the number NCIB 12 411. Stock cultures were maintained on VY/2 agar (baker's yeast 0.5% (w/v) by fresh weight; $CaCl_2 \cdot 2H_2O$ 0.1%; agar 1.5%; pH 7.2). Plates were incubated at 30 °C.

Growth in Liquid Culture

Seed cultures were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of HESO medium with the following composition (in g/liter distilled water): potato starch (Maizena) 8; glucose (Maizena) 2, separately autoclaved; defatted soy bean meal (gift from Ciba-Geigy AG) 2; yeast extract (Marcor) 2; $MgSO_4 \cdot 7H_2O$ 1; $CaCl_2 \cdot 2H_2O$ 1; ethylenediaminetetraacetic acid iron(III)-sodium salt 0.008. The pH of the medium was adjusted to 7.2 with KOH before autoclaving. The inoculated flasks were incubated at 30°C on a rotary shaker at 160 rpm for 4 days.

Isolation of Single Cell Colonies

Cultures of So ce26 adapted to homogeneous growth were streaked on the following Plating agar: agar 1.4%; peptone from casein, tryptically digested (Marcor) 0.04%; glucose 0.35%; KNO_3 0.2%; $MgSO_4 \cdot 7H_2O$ 0.15%; $CaCl_2 \cdot 2H_2O$ 0.1%; K_2HPO_4 0.006%; ethylenediaminetetraacetic acid iron(III)-sodium salt 8 mg/liter; sodium dithionite 0.01%; HEPES 50 mM; pH 7.2. Colonies were isolated after 3 weeks of incubation at 30 °C.

Influence of Temperature and Adsorber Resin on Growth and Production

Sorangium cellulosum So ce26 was grown at different temperatures in 250-ml Erlenmeyer flasks containing 50 ml HESO medium with or without XAD-1180 (Rohm and Haas) (2%). Generation times were determined by counting the cell numbers in a Thoma chamber after an appropriate dilution. After consumption of glucose, XAD-1180 was added to the cultures grown without the resin, and the flasks were incubated for 1 hour to adsorb the soraphen $A_{1\alpha}$ to the resin. The resin was then harvested on a sieve and eluted with methanol. The soraphen $A_{1\alpha}$ content was determined by HPLC.

Comparison of Soraphen $A_{1\alpha}$ Producer Strains

Soraphen $A_{1\alpha}$ production of strains So ce481, So ce521 and So ce539, was compared with that of strain So ce26. The cultures were inoculated into 250-ml Erlenmeyer flasks with 50 ml HESO medium and grown under the following conditions:

1) at 30°C in the presence of 2% XAD-1180
2) at 36°C in the presence of 2% XAD-1180
3) at 30°C in the presence of XAD-1180 and 0.2% peptone
4) at 30°C without XAD-1180

Fermentation

In 100-liter Bioreactor

The fermentor (Giovanola, Monthey, Switzerland) with 60 liters HESO medium was inoculated with 10 liters of a 4-day old preculture in the same medium grown in 1-liter Erlenmeyer flasks with 500 ml medium. To prevent foam formation, 10 ml silicone antifoam (Merck) was added. The fermentation was run for 3 days at 30°C, with an aeration rate of 300 liters air per hour and a stirrer speed of 500 rpm. The pH was maintained at 7.2 with 10% KOH or 5% H_2SO_4.

In 350-liter Bioreactor

The fermentor (Giovanola) containing 230 liters fermentation medium including 30 ml silicone antifoam was inoculated with 70-liter seed culture from the previous fermentation. The fermentation was carried out for 3 days at 30°C, pH 7.2, with an aeration rate of 1.5 m^3 air per hour and a stirrer speed of 400 rpm.

In 6,000-liter Bioreactor

The production fermentor with 4,400 liters medium was inoculated with 300 liters broth from the

350-liter fermentor. For continuous adsorption of the antibiotic produced, 1% of adsorber resin XAD-1180 was added before autoclaving. Foam formation was prevented by maintaining a 200 mbar back pressure and, if necessary, by adding silicone antifoam to the fermentor. The bioreactor was aerated with 20 m^3 air per hour, and agitated with a stirrer velocity of 230 rpm. The pH was maintained at 7.2, and the temperature at 30°C. On the 10th and 12th day of fermentation, 290 liters of feeding medium were added. The medium contained (in g/liter): starch 3.3; soy bean meal 0.7; yeast extract 0.7; $MgSO_4 \cdot 7H_2O$ 0.3; and $CaCl_2 \cdot 2H_2O$ 0.3. During the fermentation, glucose was assayed with glucotest strips (Boehringer, Mannheim), and starch with the iodine-starch reaction. After 15 days of fermentation, the adsorber resin was harvested from the culture broth by sieving in a process filter.

Isolation

The adsorber resin (about 50 liters) on the filter was washed with water to eliminate adherent cells. The adsorbed product was then eluted with methanol. The eluate was concentrated to the water phase (about 30 liters), and the soraphen reextracted twice with 60 liters ethyl acetate. After evaporation of the solvent, the highly viscous residue (approximately 1 kg) was dissolved in 2.5 liters *n*-butyl acetate, the solution was filtered, and soraphen $A_{1\alpha}$ crystallized over night at 0°C with a purity of approximately 85% soraphen $A_{1\alpha}$.

Analysis of Soraphen $A_{1\alpha}$

The antibiotic was analyzed by thin-layer chromatography (Silica gel 60 F_{254}, Merck, Darmstadt) with the following solvent system: dichloromethane - acetone, 9 : 1. Soraphen $A_{1\alpha}$ was detected at an R_f value of 0.5, after spraying with anisaldehyde - sulfuric acid reagent and heating to 120 °C.

HPLC analysis was done on Nucleosil RP-18, 7 μm (Macherey & Nagel) using a 4 × 250 mm column with methanol - water, 75 : 25 as the solvent and a flow rate of 1.5 ml/minute. Soraphen $A_{1\alpha}$ was detected by its absorption at 210 nm at an R_t of 11.8 minutes.

Determination of the Antimicrobial Spectrum

The tests were carried out by the paper-disk method. Paper disks of 6 mm (Schleicher and Schuell) containing 1 μg of soraphen $A_{1\alpha}$ were placed on agar plates seeded with the respective test organism. Fungi and yeasts were assayed on Mycophil agar (Phytone peptone, BBL, 1% (w/v); glucose 1%; agar 1.6%). Bacteria were suspended in Peptone agar (peptone from casein, tryptically digested, Merck, 1%; $MgSO_4 \cdot 7H_2O$ 0.2%; agar 1.6%; pH 7.2). The diameters of inhibition zones were determined.

MIC Determination

Minimum inhibitory concentrations (MICs) were determined by serial dilution assay in Mycophil liquid medium (Phytone peptone, BBL, 1%; glucose 1%; pH 7.0) inoculated with 10^5 cells per ml of the respective test strain. Dilution series were prepared with dilution steps of 1 : 2 in 10 reagent tubes, each with 1 ml of seeded medium and the first one starting with 5 μg of soraphen $A_{1\alpha}$ per ml. The last tube was used as reference without antibiotic. After incubation at 30 °C over night, the optical densities were read at 623 nm in an Eppendorf spectrophotometer. The minimal inhibitory concentration (MIC) was defined as the lowest soraphen $A_{1\alpha}$ concentration, at which growth of the test organism stopped completely.

Test for Fungicidal Effects

Saccharomyces cerevisiae BT 27C-2A was grown over night in YPG-medium (yeast extract (Difco) 10 g; Bacto peptone (Difco) 10 g; glucose 20 g; in 1 liter aqua dest.) at 30°C. Fresh medium was inoculated with this preculture and incubated for two hours on a rotary shaker at 30°C. Experiments were performed with 20 ml cultures adjusted to a cell concentration of 10^6/ml in 100-ml Erlenmeyer flasks. The experiment was started by the addition of 2 μg/ml soraphen $A_{1\alpha}$ to two cultures, one of which was incubated on a water bath at 30°C, the other on an ice bath at 0°C. A control culture without soraphen $A_{1\alpha}$ was run at 30°C. During the following 4 hours, 1 ml samples were taken every hour, diluted 6 times in steps of 1 : 10, and 0.1 ml of each dilution step was plated on YPG agar. After incubation over night, the number of colonies was determined.

The experimental conditions for testing the influence of increasing soraphen $A_{1\alpha}$ concentrations on

viability were as described above. The cells were incubated for 3 hours in the presence of 1, 5, or 10 μg/ml soraphen $A_{1\alpha}$ before dilution and plating.

Incorporation of Radioactive Precursors

Saccharomyces cerevisiae BT 27C-2A was precultured as described above. The experiments were started by the addition of 2 μg/ml soraphen $A_{1\alpha}$ simultaneously with the radioactive precursors to 20 ml cultures with a cell concentration of 10^7 per ml. The control was without antibiotic.

L-[*U*-^{14}C] proline, 9.25 GBq/mmol (Amersham Buchler), [2-^{14}C] uracil, 1.85 GBq/mmol and L-[*U*-^{14}C] glycerol 3-phosphate, ammonium salt, 3.7GBq/mmol (Amersham Buchler), were added at a concentration of 18.5 kBq/ml. The cultures were incubated on a reciprocal shaking water bath at 30°C. During the following 4 hours, duplicate 0.5 ml samples were pipetted every 30 minutes into 0.5 ml of ice cold 14% trichloro acetic acid and filtered over glass microfibre filters (GF/B, Whatman). After washing 3 times with 10 ml cold water, the filters were dried, and the radioactivity was determined in a scintillation counter (Beckmann LS 1801).

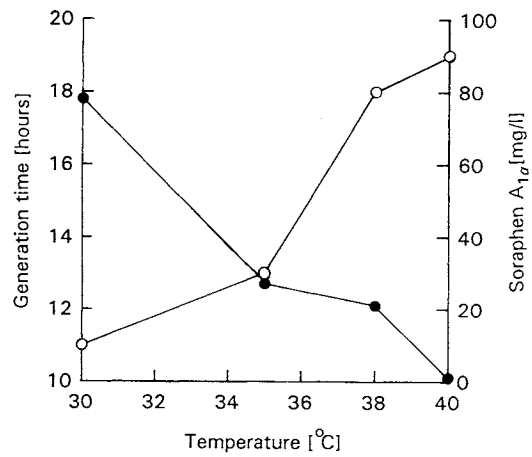
Results

Characterization of the Producing Organism

Strain So ce26 with yellow-orange to dark brown fruiting bodies is a typical *Sorangium* (*Polyangium*) *cellulosum* which belongs to the order Myxococcales, suborder Sorangineae, and family Polyangiaceae. It was isolated from a sample of goat dung collected on the island of Djerba, Tunesia. The strain grew in homogenous cell suspension only after many transfers in HESO liquid media. The soy bean meal used as nitrogen source was free from detectable iron(III) ions, therefore addition of iron(III) ions to this medium was essential, otherwise the growth declined after several transfers.

The yield of soraphen $A_{1\alpha}$ production by the wild strain was 2~4 mg/liter. Isolation of better producing clones and strain improvement by UV mutation as well as substrate and process development have increased the yield to 120~150 mg/liter. The cell growth and soraphen $A_{1\alpha}$ production were very much influenced by the incubation temperature (Fig. 2). When temperature was raised from 30 to 40 °C, the generation time increased from 11 to 18 hours, and soraphen $A_{1\alpha}$ production dropped to zero. XAD-1180 had no effect on the cell growth but was beneficial to the antibiotic production. In the absence of resin, the sum of all known soraphen components, as determined by HPLC (article in preparation), dropped to 30% of the control with XAD.

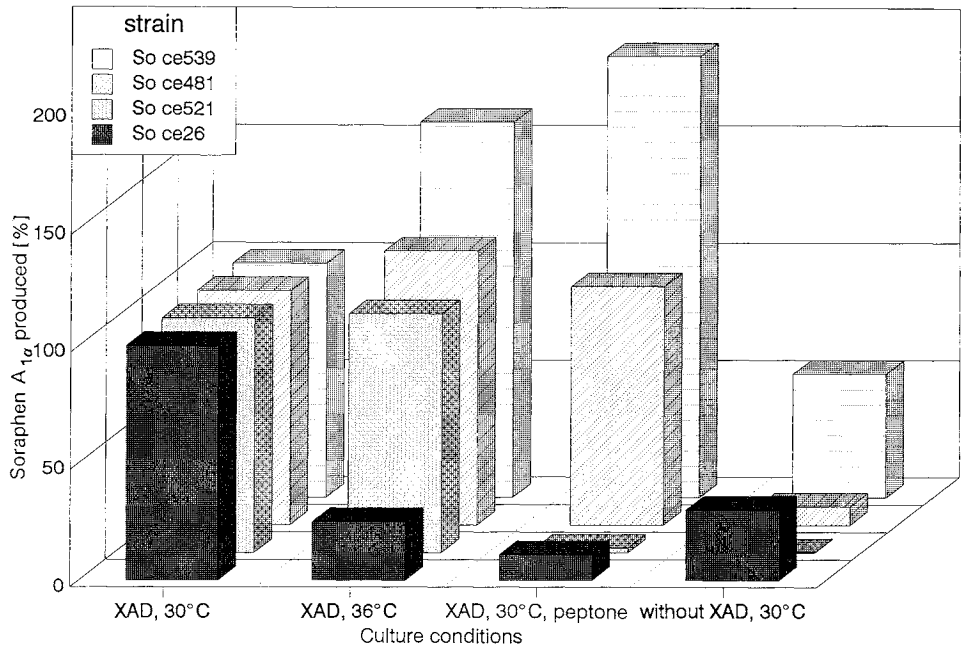
Fig. 2. The effect of incubation temperature on the cell growth and soraphen $A_{1\alpha}$ production in the presence of XAD (● soraphen $A_{1\alpha}$ content, ○ generation time).



Comparison of So ce26 with Other Soraphen $A_{1\alpha}$ Producers

The effect of temperature, adsorber resin and peptone on the productivity of four soraphen $A_{1\alpha}$ producers was investigated (Fig. 3). In contrast to So ce26, an increase in incubation temperature had no effect on soraphen $A_{1\alpha}$ production by strains So ce481 and So ce521. On the other hand, soraphen $A_{1\alpha}$ production was stimulated by increasing the temperature with strain So ce539. Peptone strongly

Fig. 3. Production of soraphen $A_{1\alpha}$ by different soraphen producing strains under the following conditions: 1) With XAD at 30°C, 2) with XAD at 36°C, 3) with XAD and 0.2% peptone at 30°C, 4) without XAD at 30°C.



The productivity under conditions 1) was taken as 100%.

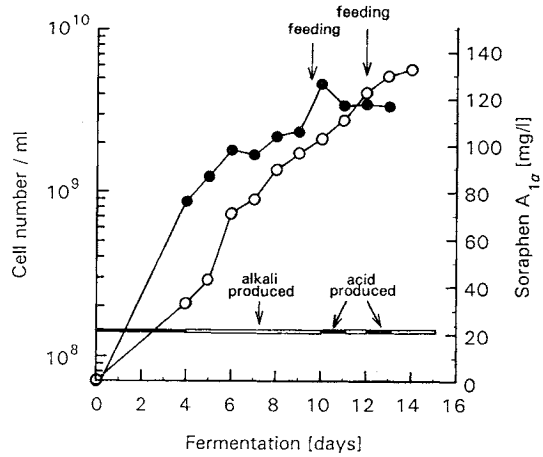
inhibited soraphen $A_{1\alpha}$ synthesis by strains So ce26 and So ce521 but stimulated the soraphen $A_{1\alpha}$ production of strain So ce539. No such effect could be seen with strain So ce481. Without XAD, the productivity of all tested strains was reduced.

Fermentation

S. cellulosum So ce26 was used for soraphen $A_{1\alpha}$ production. A fermentor with a final working volume of 5,300 liters was run at 30°C in the presence of 1% resin. During the first 4 days all free glucose in the fermentation broth was consumed with concomitant acid production (Fig. 4). Starch was slowly degraded under carbon limited growth during the following days. As a consequence, metabolism shifted to amino acid degradation accompanied by ammonia liberation. After 10 days of cultivation, starch was depleted. Feeding carbon and nitrogen sources at the 10th and 12th day (as specified in Materials and Methods) resumed acid production during the following 20 hours. The culture reached stationary phase after 12 days with a cell number of 4×10^9 per ml. Fifteen days after inoculation

Fig. 4. Production of soraphen $A_{1\alpha}$ in a $6\,m^3$ bioreactor.

● Cell number, ○ soraphen $A_{1\alpha}$ concentration.



Acid and alkali production was monitored by the consumption of KOH and H_2SO_4 required to maintain a constant pH.

Table 1. Antifungal spectrum of soraphen $A_{1\alpha}$.

Test organism	Inhibition zone diameter (mm)*	MIC** (μg/ml)
Debaryomyces hansenii DSM[a] 70238	29	0.4
Candida albicans CBS[b] 1893	25	0.06
Nadsonia fulvescens CBS 2596	—	3.0
Nematospora coryli CBS 2608	35	0.05
Saccharomyces cerevisiae BT 27C-2A YGSC[c]	20	1.0
S. cerevisiae BT 27C-2A M1	N.D.	40.0
S. cerevisiae BT 27C-2A M2	N.D.	>100
Byssochlamys fulva DSM 1808	39	0.2
Kloeckera corticis DSM 2249	35	0.4
Schizosaccharomyces pombe Tü[d] 501	—	>100
Torulopsis glabrata DSM 70398	—	3.0
Rhodotorula glutinis DSM 70398	21	1.0
Mucor hiemalis DSM 2655	30	0.03
Rhizopus arrhizus DSM 905	10	4.0
Ustilago zeae DSM 3121	10	4.0
Botrytis cinerea SM 877	30	0.15
Ceratocystis ulmi CBS H6	23	0.5
Alternaria solani DSM 62028	27	N.D.
Pythium debaryanum DSM 62946	45	0.1

* Agar diffusion assay: The volume of agar was 10 ml per Petri dish of 9 cm diameter. Soraphen concentration was 1 μg per filter disk of 6 mm.
** The MICs were determined in liquid culture as described in the text.
[a] Deutsche Sammlung von Mikroorganismen Braunschweig.
[b] Centraalbureau voor Schimmelcultures Baarn.
[c] Yeast Genetic Stock Center Berkeley.
[d] Strain collection University Tübingen.

the cells began to lyse and the fermentation was terminated. The culture produced soraphen $A_{1\alpha}$ during the growth as well as the early stationary phase, and reached a yield of 133 mg/liter. The overall yield of soraphen $A_{1\alpha}$ adsorbed to XAD was 700 g.

Antimicrobial Spectrum

A broad spectrum of yeasts and hyphomycetes was inhibited by low concentrations of soraphen $A_{1\alpha}$ (Table 1). Strains which appeared resistant to the antibiotic in the agar diffusion test were sensitive to soraphen $A_{1\alpha}$ at higher concentrations in liquid medium. Only *Schizosaccharomyces pombe* was completely resistant. In contrast, prokaryotes were not inhibited at all even at 100 μg per filter disk. The producer strain was insensitive to the antibiotic.

Biological Effects of Soraphen $A_{1\alpha}$

The growth of *Saccharomyces cerevisiae* was comparable to that of the control culture during the first two hours after the addition of soraphen $A_{1\alpha}$ to a log phase culture. With longer incubation times, however, the number of living cells decreased significantly (Fig. 5). No fungicidal effect was observed when the cells were incubated at 0°C: the cell number remained constant. The killing rate depended on the soraphen $A_{1\alpha}$ concentration used (Fig. 6). An increase in soraphen $A_{1\alpha}$ concentration from 1 to 10 μg/ml resulted in a doubling of the number of cells killed within 3 hours.

The effects of soraphen $A_{1\alpha}$ on the basic metabolism of *S. cerevisiae* were investigated. The incorporation of radioactive proline (Fig. 7) and uracil (data not shown) into high molecular weight material began

Fig. 5. Effect of soraphen $A_{1\alpha}$ on the growth of *Saccharomyces cerevisiae*. 2 μg/ml soraphen $A_{1\alpha}$ was added at zero time.

○ Untreated control culture, ● culture with soraphen $A_{1\alpha}$ added and incubated at 30°C, ▽ culture with soraphen $A_{1\alpha}$ and incubated at 0°C.

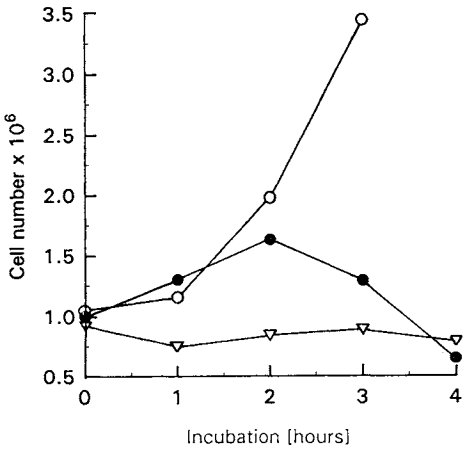


Fig. 6. The effect of soraphen $A_{1\alpha}$ concentration on the killing of *Saccharomyces cerevisiae* within 3 hours.

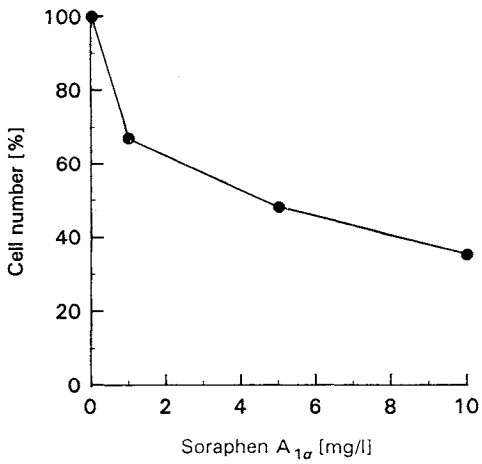


Fig. 7. The effect of soraphen $A_{1\alpha}$ on the incorporation of [^{14}C] proline by *Saccharomyces cerevisiae* into high molecular weight material.

○ Untreated control, ● culture with 2 μg/ml soraphen $A_{1\alpha}$.

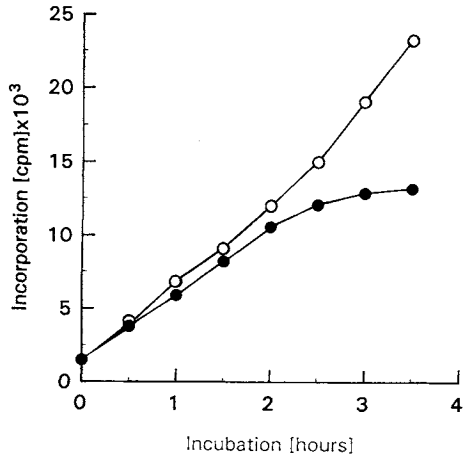
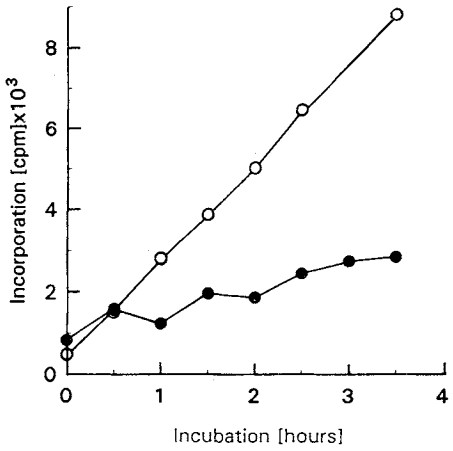


Fig. 8. The effect of soraphen $A_{1\alpha}$ on the incorporation of [^{14}C] glycerol phosphate by *Saccharomyces cerevisiae*.

○ Untreated control, ● culture with 2 μg/ml soraphen $A_{1\alpha}$.



to level off about two hours after the addition of soraphen $A_{1\alpha}$. In contrast, the incorporation of [^{14}C]glycerol phosphate (Fig. 8) was much reduced by the antibiotic from the very beginning.

Discussion

Strain So ce26 was discovered to produce a novel antifungal compound during the screening of a large number of *Sorangium* strains. About 4% of the 600 isolates tested turned out to produce this inhibitor which we called soraphen $A_{1\alpha}$. Although the various soraphen $A_{1\alpha}$ producers all belonged to the species *S. cellulosum*, differences in production behavior reflect substantial physicological versatility of the

isolates. So ce26, our first soraphen $A_{1\alpha}$ producer, was used exclusively for all further experiments. Under the given conditions of fermentation, soraphen $A_{1\alpha}$ is the main component. It is a macrocyclic polyketide with a molecular weight of 520, and is characterized by an unsubstituted phenyl ring[6]. It has been shown, that inexpensive technical grade substrates can be used for the growth and antibiotic production of most species of myxobacteria[7]. Therefore the media costs are no longer restrictive for large scale fermentations of myxobacteria. A problem is, however, the dependence of certain species, like those of *Myxococcus*, on amino acids as sole carbon and nitrogen source. Ammonium consequently accumulates in the culture broth and may become inhibitory for growth and antibiotic production[8]. *S. cellulosum* differs in its physiology metabolizing polysaccharides and sugars. Therefore it was possible to develop a cheap fermentation medium that supported growth and stimulated soraphen $A_{1\alpha}$ production.

Like most other myxobacteria, the optimum temperature for the growth and antibiotic production of So ce26 was 30°C. The culture grew even at 40°C. In fact, some of the strains of *S. cellulosum* isolated, grew even better at this temperature (unpublished results).

Continuous recovery of antibiotics from myxobacteria fermentation with XAD resin in a bypass system has been described earlier[9]. However, addition of the polystyrene resin directly into the production fermentor simplified the fermentation process, increased the yield and reduced the risk of contamination. Growth of So ce26 was not affected at all by the presence of XAD-1180, but the yield of soraphen $A_{1\alpha}$, increased substantially.

This procedure also greatly simplified the down stream processing of soraphen $A_{1\alpha}$ recovery. The resin could be harvested by sieving the culture broth, and eluted with methanol. Soraphen $A_{1\alpha}$ was isolated and purified in two steps by crystallization and recrystallization from concentrated crude XAD-1180 eluates without chromatographic steps. The process is cost-effective, avoids utilization of large volumes of organic solvents, and is simple to scale up.

Soraphen $A_{1\alpha}$ inhibited yeasts and molds at low MIC concentrations, $0.003 \sim 4\,\mu g/ml$ (Table 1). Different classes of phytopathogenic fungi, like Fungi imperfecti (*e.g.*, *Botrytis* or *Alternaria*); Basidiomycetes (*e.g.*, *Rhizoctonia*); Ascomycetes (*e.g.*, *Venturia* or *Erysiphe*); and Oomycetes (*e.g.*, *Phytophthora*) were inhibited by the antibiotic[10]. The effects seems to be specific to yeasts and fungi. The antibiotic toxicity to mouse fibroblasts was about $10\,\mu g/ml$.

Preliminary study on the mode of action of soraphen $A_{1\alpha}$ was carried out with *Saccharomyces cerevisiae*. The viability of yeast cells was affected by the antibiotic after a delay of 2 hours (Fig. 5). Soraphen $A_{1\alpha}$ seemed to interfere with a metabolic pathway necessary only for growing cells, while resting cells were not affected. In accordance with the delayed killing, both RNA and protein syntheses were not inhibited within 2 hours after the addition of soraphen $A_{1\alpha}$. On the other hand, the immediate inhibition of glycerol phosphate incorporation by the antibiotic suggested that the primary site of action of soraphen $A_{1\alpha}$ was on the membrane biosynthesis. Further study in our laboratory has shown that soraphen $A_{1\alpha}$ is a potent inhibitor of fungal acetyl-CoA carboxylase, while the respective enzymes of plants and bacteria are insensitive to the antibiotic[11].

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