Preparation and Ultraviolet Light-Induced Transformation of an Antifungal Mixture of Heptaene Antibiotics of *Streptomyces surinam*  

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Conditions for the production and isolation of an antifungal antibiotic mixture (DJ400) were investigated. Different preparations of DJ400 may contain at least 12 different heptaenes, which were characterized by partition chromatography (peak number) and ultraviolet (UV) spectra (types A, B, C). Irradiation with UV light transformed the predominant UV spectrum of type B into A. Comparison of untreated and UV-irradiated products indicated that the main components may exist in two forms with identical structures except for an all *trans*-heptaene system in A compounds and one internal *cis* double bond in B compounds. The ratio of the major components 6B and 8B depended on the media composition. Component 4B is probably a precursor of 6B; 10B and 12 may be precursors of 8B.

Antifungal antibiotic DJ400 is a mixture of heptaene antibiotics and was first isolated by Johnson and Durrell (personal communication) from a strain of *Streptomyces surinam*, which was discovered in soil from a tropical river in Surinam, South America.

The strain was subsequently examined by Schering AG, Berlin, for the production of the antibiotic and its possible therapeutic use. We wish to report some of our results concerning the fermentative production and analysis of the polyene mixture.

The chemistry of the two major components (B₁ and B₂) of antibiotic DJ400 has been reported by Boehm and co-workers (1, 2).

**MATERIALS AND METHODS**

Fermentations. Medium 1 (500 ml) in a 2-liter Erlenmeyer flask was inoculated with a lyophilized stock culture of *S. surinam* and shaken for 3 days at 30°C. Fermentors with a capacity of 15 or 30 liters were inoculated with 5% seed culture either from shaken flasks or from a preceding fermentor and were stirred at 220 rev/min. Aeration was at 1 liter per min per liter of culture, air pressure was at 1.7 atm, and antifoam was Pluronic L 81. Medium 1 contained, per liter: 15 g of commercial glucose (hydrolyzed starch), 15 g of soybean meal, 5 g of casein, 1 g of CaCO₃, 5 g of NaCl (pH 6.3 to 6.5; the reaction was kept below pH 6.5 by HCl throughout fermentation), and 5 and 2.5 g of commercial glucose per liter were added at 60 and 108 hr after inoculation. Medium 1 is the same as used by Johnson and Durrell for the production of the antibiotic, except for the use of commercial glucose (hydrolyzed starch) and the two additions of sugar during the fermentation.

Medium 2 contained, per liter: 5 g of commercial glucose, 25 g of soybean meal, 3 g of NH₄NO₃, 1 g of CaCO₃ (pH adjustment as for medium 1). Five grams of commercial glucose per liter was added at 20, 36, 48, 60, 72, 82, 90, and 98 hr after inoculation. Medium 2 was developed by H. J. Koch in our laboratory. The total sugar was added in several small portions because high concentrations of sugars were growth-inhibiting.

Medium 3 contained, per liter: 20 g of olive or coconut oil, 15 g of soybean meal, 5 g of casein, 1 g of CaCO₃, and 5 g of NaCl (pH 6.5 with no pH adjustment during fermentation).

Medium 4 contained, per liter: 20 to 40 g of olive or coconut oil, 15 g of soybean meal, 3 g of NH₄NO₃, 1 g of CaCO₃, and 5 g of NaCl (no pH adjustment). Medium 4a had 20 g, 4b had 30 g, and 4c had 40 g of olive or coconut oil per liter.

**Determination of heptaene concentrations.** Culture (10 ml) was diluted with methanol to 50 ml and kept at room temperature for 1 hr with occasional shaking. After filtration and appropriate dilution with methanol, the solution was read at 378 to 383 nm. The specific extinction coefficient of the purest sample was ε₅ = 105 (g⁻¹ × 1 × cm⁻¹) and was used for calculations throughout this work.

**Isolation of crude antibiotic mixture.** The mycelium was separated by filtration or centrifugation, washed four times with 3 volumes of water, and extracted four times at room temperature, once with 3 volumes...
each of methanol and three times with methanol-water (4:1). The combined extracts were concentrated
under reduced pressure at 35 C to 20 to 25% of the volume. The crude mixture of heptaines, which
precipitated at 0 C, was washed with water and stirred with 200 volumes of ethyl acetate for 3 hr at room
temperature. After filtration and drying, the crude product contained 60 to 90% heptaines (40 to 70%
yields of the initial culture).
Partition chromatography. The following procedure was developed. Sodium borate buffer at 0.1 m, pH
8.4 (1.0 liter), dimethylformamide (1.0 liter), 1-butanol (0.7 liter), and toluene (1.3 liter) were equilibrated
at room temperature, and the phases were separated.
A column (110 by 2.1 cm) was filled with a suspension of Sephadex G25 medium (100 g) in the lower phase,
washed with the lower phase (400 ml), and then washed with the upper phase (approximately 1,400
ml) until no more of the lower phase was eluted. Sephadex (3 g) was suspended in the upper phase
and layered on top of the column. Five milliliters of a solution of the antibiotic mixture in the lower phase
with an absorbancy at 386 nm of 200 to 250 was adsorbed on the column, which was eluted with the
upper phase (1.7 to 1.9 liter; 350 to 380 fractions of 5 ml each) at a rate of 0.8 to 1.0 ml/min. The absorbancy
of the eluate at 386 nm was recorded with a Unicam SP600 spectrophotometer and a Unicam SP22
logarithmic recorder. A rest of 5 to 20% of the original heptaine mixture was only eluted with the lower
phase (200 to 250 ml). For quantitation the areas of the different peaks were measured and recorded as
percentage of the total area of the elution curve (including the material eluted with the lower phase).
UV spectra. Spectra of the crude antibiotic mixture were recorded in methanol with a Beckman DB
spectrophotometer and a 10-inch (25.4 cm) logarithmic recorder over the range of 300 to 450 nm. Spectra
of fractions after partition chromatography were measured directly in the upper phase of the elution
mixture and were then shifted by 4 to 6 nm to longer wavelengths.
UV irradiation. Solutions of the antibiotic mixture (1 g/liter) in methanol-water (4:1) or the crude methanolic
extracts (3 to 8 liters) obtained from the mycelium (see above) were irradiated at room temperature
with UV burner immersed into the solution [burner type S81, supplied by Quarzlampen GmbH, Hanau
(Main), West Germany] until the transformation of spectra was complete (1 to 4 hr; cf. Fig. 2). Samples
were taken at intervals of 10 to 30 min and diluted 100-fold with methanol for UV spectroscopy. The
isolation procedure for the crude transformed antibiotic mixture was the same as described above for
untreated antibiotic.

RESULTS AND DISCUSSION
Maximal concentrations of antibiotic. The maximal concentrations of the DJ400 antibiotic which were obtained by growing S. surinam in the different media (see above) are given in Table 1. No significant difference was observed with casein or NH4NO3 as N source (compare media 1 and 2 in Table 1).

| Table 1. Maximal concentrations of the DJ400 antibiotic produced in different media* |
|---------------------------------|----------------------------------|
| Medium no. | Maximal concn (mg/liter)* |
|--------|-----------------|
| 1 | 650-1,050 |
| 2 | 730-920 |
| 3 | 1,100-1,300 |
| 4a | 1,750-1,850 |
| 4b | 2,100-2,400 |
| 4c | 2,400-3,100 |

* Length of fermentation for maximal production of antibiotic varied between 4 and 6 days.
* For simplicity, the concentrations of antibiotic were expressed as milligrams per liter, though it has not yet been proven that the specific extinction coefficient used for calculation (see Materials and Methods) is the same for all the heptaines of the mixture.

In contrast, by replacing glucose as carbon source in media 1 and 2 with natural oils (olive and coconut oil in media 3 and 4), the concentrations of total antibiotic mixture could be increased up to three times. Similarly, high yields of fungichromin (4) and filipin (3) had been obtained by the use of fatty acids and natural fats as carbon source.

An additional advantage of media 3 and 4 was that all the oil could be added at the beginning of fermentation and that no pH adjustment was necessary during fermentation. The pH remained constant or it decreased, sometimes as low as pH 4.5 to 5.0, which seemed to be favorable for the antibiotic production, but it did not rise as in the sugar-containing media.

Analysis by partition chromatography and UV spectroscopy. Figure 1 represents a typical elution curve obtained by partition chromatography of the crude antibiotic mixture. The peaks were numbered as shown. The material eluted with the lower phase, which may consist of several compounds, was marked as peak 12.

For further characterization of the heptaine components, one fraction of each side of the main peaks was usually selected for UV spectroscopy. We recognized three types of spectra, which differed by the ratio and the position of the absorption maxima as shown in Table 2 (cf. also Fig. 2).

The different heptaines of the mixture were designated by the peak number and the type of spectrum. Altogether the following compounds were detected in different preparations, 2A, 2B, 4A, 4B, 4C, 6A, 6B, 7A, 8A, 8B, and 10B, the material of peak 12 was not further characterized. The components, called B1 and B2 by Bohlmann et al. (1), are identical with our compounds 6B and 8B. (Samples of the purified components B1
and B₂ were from Dehmlow, Technische Universität, Berlin.) On partition chromatography these compounds gave one, single peak each in the positions of components 6B and 8B, respectively, and had UV spectra of type B.

Compound 4C was found only twice in products obtained after relatively short fermentations, and it disappeared on prolonged incubation. The antibiotic preparations from fermentors consisted mainly of B-heptanes but often contained small and variable amounts of A-heptanes. In contrast, preparations isolated from shaken flasks, which required much longer fermentation times (up to 17 days) for maximal production of the antibiotic, usually contained A-heptanes as the main components.

Compounds 4A and 6A were eluted a little faster than 4B and 6B, respectively, and sometimes gave rise to a shoulder in peaks 4 and 6 (e.g., peak 4 in Fig. 1).

Isomerization by UV light. The differences between spectra of type A and B may be due to the presence of an all trans heptaene system in type A and one internal cis double bond in type B (5). It was therefore attempted to isomerize crude antibiotic preparations of type B by irradiation with UV light. Figure 2 shows the spectra of a methanolic solution before (B) and after (A) irradiation. A transformation of type B into type A has taken place in such a way that the extinction of the second maximum has remained nearly constant (the small decrease is probably due to destruction), whereas the extinctions of maxima 1, 3, and 4 are either raised (1) or lowered (3, 4). On prolonged irradiation, the peak ratio of type A remained constant, but the heptaene system was gradually destroyed until the absorption spectrum had completely disappeared.

Some analyses of the antibiotic preparations obtained from crude methanolic extracts with and without UV irradiation are compared in Table 3. These results may be best explained by assuming that the first transformations induced by UV light are the following: 4B → 4A, 6B → 6A, 8B → 7A, and perhaps 10B → 8A. It therefore seems likely that the main heptanes of antibiotic DJ400 can exist in two forms which differ from each other only by the configuration of one internal double bond. Additional reactions and the subsequent destruction of the heptanes probably take place on irradiation.

Effect of media and fermentation time on the composition of the DJ400 antibiotic. From a fairly large number of analyses of the antibiotic preparations, the following conclusions concerning the effect of the media composition on the composition of the antibiotic mixture could be drawn. Replacement of casein by NH₄NO₃ (medium 1 → 2, 3 → 4) causes a small decrease, and replacement of glucose by oil (medium 1 → 3,
TABLE 3. Composition of the crude antibiotic preparations before and after ultraviolet (UV) irradiation

| Medium and prep no. | Percentage and spectral type in peak no. a |
|--------------------|-------------------------------------------|
|                    | 2  | 4  | 6  | 7  | 8  | 10 | 12 |
| Medium 1           |    |    |    |    |    |    |    |
| 1a Control         | 2.7| 10.7| 14.2| 8.2| 42.0| 11.5| 10.7|
| 1b UV irradiation  | 3.5| A  | 9.1 | A  | 13.7| 24.0| 20.5| 28.2|
| Medium 3           |    |    |    |    |    |    |    |
| 2a Control         | 3.2| 26.9| 21.2| 3.2| 27.0| 11.3| 7.2 |
| 2b UV irradiation  | 2.9| 22.0| 15.3| 14.9| 22.1| 22.8|   |
| Medium 4           |    |    |    |    |    |    |    |
| 3a Control         | 3.4| 25.8| 28.6| 4.8 | 22.8| 6.7 | 7.8 |
| 3b UV irradiation  | 5.9| 22.6| 37.0| 25.4| 9.1 |   |    |

a Where no letter is given, the spectral type was not determined.

TABLE 4. Time course of production of compounds 4B and 6B in medium 3 a, b

| Length of fermentation (days) | Total heptaenes in culture (mg/liter) a | Percentage in isolated heptaene mixture of | Sum (%) |
|------------------------------|----------------------------------------|-------------------------------------------|---------|
|                              |                                        | 4B (%)  | 6B (%)  |        |
| 2                            | 700                                    | 25.1    | 25.9    | 51.0   |
| 4                            | 1,140                                  | 14.4    | 32.9    | 47.3   |
| 6                            | 1,220                                  | 12.1    | 38.9    | 51.0   |

a From samples of 2 to 3 liters taken from a fermentor at the times indicated, crude DJ400 was isolated and analyzed.

b See Table 1.

TABLE 5. Time course of production of compounds 8B(+7A), 10B, and 12 in medium 1 a, b

| Length of fermentation (days) | Total heptaenes in culture (mg/liter) a | Percentage in isolated heptaene mixture of | Sum (%) |
|------------------------------|----------------------------------------|-------------------------------------------|---------|
|                              |                                        | 8B (+7A) (%) | 10B (%) | 12 (%) |      |
| 3                            | 360                                    | 33.7      | 17.2    | 22.6   | 73.5  |
| 8                            | 880                                    | 44.1      | 15.8    | 13.0   | 72.9  |
| 11                           | 950                                    | 48.6      | 13.1    | 10.9   | 72.6  |

a Analysis as in Table 4.

b See Table 1.

2 → 4) causes a larger decrease, not only of the content of 8B but also of 10B and 12 and a corresponding increase of 4B and 6B (cf. preparations 1a, 2a, and 3a in Table 3).

The time course of production of 4B and 6B in medium 3, where these two compounds are the major products, is shown in Table 4. An increase in 6B is accompanied by a similar decrease in 4B which is compatible with the assumption that 4B is a precursor of 6B. In this experiment, the changes of the remaining components were comparatively small.

Similarly, Table 5 shows the changes of the contents of 8B (+7A), 10B, and 12 in antibiotic preparations obtained from medium 1, where component 8B is the major product. The values indicate that 10B and 12 may be precursors of 8B. In this case, the values for 4B and 6B changed very little.

Therefore, on prolonged fermentation, 6B is the main product in media 3 and 4, whereas 8B predominates in media 1 and 2. Structural formulas for these two compounds have been proposed (2).

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LITERATURE CITED

1. Bohmenn, F., E. V. Dehmlow, H.-J. Neuhahn, R. Brandt, and B. Reinicke. 1970. Neue Heptaen-Makrolide I, Charakterisierung und Abbau. Tetrahedron 26:2191-2198.
2. Bohmenn, F., E. V. Dehmlow, H.-J. Neuhahn, R. Brandt, and H. Bethke. 1970. Neue Heptaen-Makrolide II, Grundsklekt, Stellung der funktionellen Gruppen und Struktur der Aglykone. Tetrahedron 26:2199-2207.
3. Brock, T. D. 1956. The effect of oils and fatty acids on the production of filipin. Appl. Microbiol. 4:131-133.
4. McCarthy, F. J., W. P. Fisher, J. Charnay, and A. A. Tytell. 1954-1955. Effects of oils and fatty acids on the production of fungichromins. Antibiot. Ann., p. 719-723.
5. Oroshnik, W., and A. D. Mebane. 1963. The polyene antifungal antibiotics. Fortschr. Chem. Org. Naturst. 21:17-79.