PNEUMOCANDINS FROM *Zalerion arboricola*

II. MODIFICATION OF PRODUCT SPECTRUM BY MUTATION AND MEDIUM MANIPULATION

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Zalerion arboricola ATCC 20868 produces pneumocandin A_0 (L-671,329), a cyclic hexapeptide with a dimethylmyristic acid side chain. This compound has anti-candida and anti-pneumocystis activities. We were interested in looking for other related compounds produced by this organism. To facilitate this search, a simple medium (S2) composed of D-mannitol, peptonized milk, lactic acid, glycine, KH_2PO_4 and trace elements, which supported the production of a number of such compounds, was designed. For the isolation of mutants, either spores or growing mycelia were treated with *N*-nitroso-*N*-methylurethane or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and survivors were screened for changes in the product spectrum. From approximately 1,500 survivors tested, 5 mutants were isolated. Mutants ATCC 20957, 74030, 20958 and 20988 exclusively produce various pneumocandins other than A_0. These compounds were active against *Candida* and *Pneumocystis carinii*. The yield of A_0 was found to be increased 2.5-fold over that of the parent in the fifth mutant, MF5415. Further medium studies indicated that the addition of soybean oil to S2 medium improved the yields. Subsequent development of another series of media containing Pharmamedia as a nitrogen source resulted in increase in production by 10-~20-fold. Overall, these studies resulted in substantial improvement in the production of A_0 as well as discovery and/or facile production of 7 other related compounds.

Zalerion arboricola (ATCC 20868) was found to produce L-671,329 which has anti-candida activity.[1] Later it was shown in the rat model to be active against *Pneumocystis carinii*, the causative organism of pneumonia in immune-compromised patients.[2] The compound was determined to be a lipopeptide and a structural analogue of echinocandin B.[3] In the early studies, it was observed that this organism when grown on solid medium produced other related compounds ("minors") with antifungal activity.[4] This group of compounds was designated as "pneumocandins" (Fig. 1) and L-671,329 was named pneumocandin A_0.[4] The yields of these compounds were low in the solid medium fermentations, which furthermore, are difficult to scale-up. Therefore, it was necessary to develop fermentation processes based on liquid medium to produce any one of these compounds selectively and in high yields. The approaches used to accomplish this included mutation and medium developments. This paper describes the results of these studies.

Materials and Methods

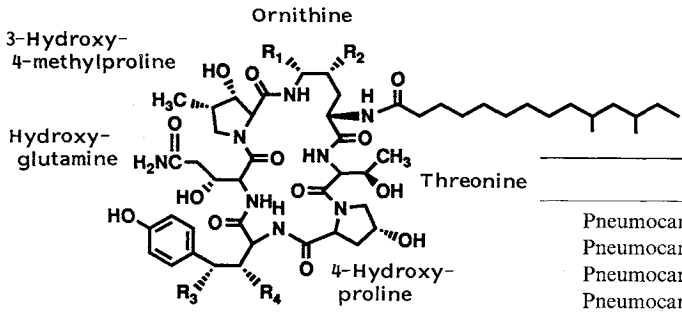
Cultures

Z. arboricola ATCC 20868 and *Candida albicans* Merck Culture Collection No. MY1028 were used.

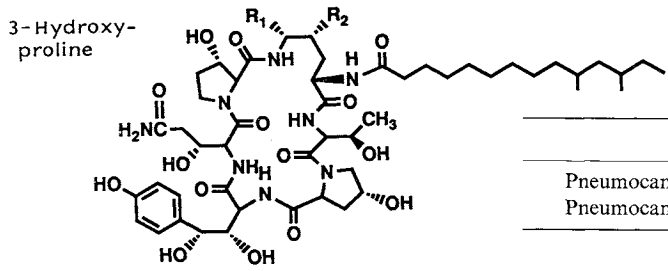
Media

Seed medium was the same as that described by SCHWARTZ *et al.* (1989).[1] Production media are

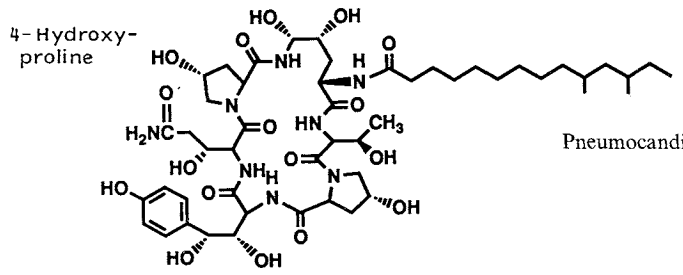
Fig. 1. Structure and nomenclature of pneumocandins.



	R_1	R_2	R_3	R_4
Pneumocandin A_0	OH	OH	OH	OH
Pneumocandin A_1	OH	OH	OH	H
Pneumocandin A_2	H	H	OH	OH
Pneumocandin A_3	OH	H	H	H
Pneumocandin A_4	H	H	H	H



	R_1	R_2
Pneumocandin B_0	OH	OH
Pneumocandin B_2	H	H



Pneumocandin C_0

described in Table 1.

Chemicals

Potato-Dextrose broth and agar were obtained from Difco Laboratories (Detroit, MI). Peptonized milk nutrient and NZ Amine type E are products of Sheffield Products (Norwich, NY). Traders Protein (Memphis, TN) was the supplier of Pharmamedia. Corn steep liquor was obtained from Grain Processing Corp. (Muscatine, IO). Lard water is a product of Inland Molasses Co. (Ames, IO). Pfalz and Bauer (Stamford, CT) was the supplier of *N*-nitroso-*N*-methylurethane (NMU) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG). Other chemicals and solvents were of analytical grade and along with sugars and medium ingredients were obtained from commercial sources.

Fermentation

To prepare the seed, 50 ml of the seed medium in 250-ml unbaffled Erlenmeyer flasks was inoculated with 1 ml of vegetative mycelium, which was stored frozen (FVM) or with a plug from the culture grown on Potato-Dextrose agar slants. The flasks were shaken at 25°C and 220 rpm (5 cm throw) for 72 hours, if inoculated with FVM or for 120 hours in case of plug inoculum. Two ml were used to inoculate a second

Table 1. Composition of the production media.

Components	Media (g/liter)			
	S2	S6	SP-5	SP-7
D-Mannitol	44	44	80	100
KH_2PO_4	2	2	9	9
Glycine	2	2	—	—
Peptonized milk	15	15	—	—
Pharmamedia	—	—	20	35
Cerelose	—	—	10	10
Lactic acid	2	2	—	—
Trace elements[a]	10 ml	10 ml	—	—
Soybean oil	—	10	—	—

Pre-sterilization pH of S2 and S6 was adjusted to 7.0 and that of SP-5 and SP-7 to 7.3.

[a] Trace elements.

	Per liter of 0.6 N HCl
$FeSO_4 \cdot 7H_2O$	1.0 g
$MnSO_4 \cdot 4H_2O$	1.0 g
$CuCl_2 \cdot 2H_2O$	0.025 g
$CaCl_2$	0.1 g
H_3BO_3	0.056 g
$(NH_4)_6MoO_2 \cdot 4H_2O$	0.01 g
$ZnSO_4 \cdot 7H_2O$	0.2 g

stage seed. It was incubated as the first stage seed for 72 hours. For the production of pneumocandins, 250-ml unbaffled Erlenmeyer flasks with 40 ml of production medium were inoculated with 2 ml of the second stage seed. Flasks were shaken at 220 rpm (5 cm throw) and 25°C for 14 days.

Harvest and Analysis

Following incubation and after the determination of the final pH, the contents of the flasks were homogenized in a Tissumizer (Tekmar Co., Cincinnati, OH). Twenty five ml of the homogenate were extracted with the equal volume of methyl ethyl ketone. The solvent was evaporated to dryness and the residue was dissolved in 2.5 ml of methanol. The methanol extract was analyzed by reverse phase HPLC. The conditions used for this purpose were as follows: Whatman Partisil 5, ODS-3, analytical column; acetonitrile-water (44:56) at 0.75 ml/minute and column temperature of 40°C. Peaks were detected at 210 nm.

If needed, fractions were collected from the HPLC in 96-well microtiter plate with a Gilson FC 203 microfraction collector (Gilson Medical Electronics Inc., Middleton, WI). The HPLC solvent was evaporated in Savant Speed-Vac concentrator (Savant Instruments Inc., Farmingdale, NY). To each well 0.2 ml of Potato-Dextrose broth (Difco) inoculated with *C. albicans* which had been grown for 24 hours in the same medium was added. The plates were incubated at 37°C overnight and observed for growth. Bioactive fractions inhibited the growth of *C. albicans*.

Preparation of Spore Suspension

The culture was grown on Potato-Dextrose agar (Difco) medium in Petri plates at 25°C for 3 weeks. Ten ml of 0.3 M Tris buffer pH 7.0 was added to the plate and spores were scraped off with a sterile cotton swab. The spore suspension was filtered through glass wool to remove the mycelial pieces. *Z. arboricola* produces spores in clusters which contain anywhere from 1 to 30 spores and therefore, following procedure was devised to remove the multispore clusters. The filtered suspension was vigorously agitated on a vortex mixer and centrifuged successively at 600, 700, 800 and 3,000 rpm in a Beckman TJ-6 centrifuge (Beckman Instruments Inc., Palo Alto, CA). The pellets after the first three centrifugations, which upon microscopic examination were shown to contain the multispore clusters, were discarded. The pellet from the last centrifugation was resuspended in 3 ml of 0.3 M Tris buffer pH 7.0 and was used for mutagenic treatment.

Mutation Procedure

The spore suspension contained in a 15-ml polypropylene centrifuge tube was treated with 0.05 to 0.10 mg NMU per ml for 20 minutes at room temperature or with 1 mg of NTG per ml for 30 minutes at room temperature. In all cases the tubes were shaken at 300 rpm. The mutagen was removed by centrifugation at 3,000 rpm in a TJ-6 centrifuge (Beckman Instruments Inc. Palo Alto, CA) followed by 2 washes with 0.3 M Tris buffer pH 7.0. The pellet was resuspended in the same buffer and plated for isolated colonies on Potato-Dextrose agar. The plates were incubated for 2 weeks at 25°C and the isolated colonies were transferred to slants of the same medium. The slants were incubated for 10~14 days at 25°C and tested for the production of pneumocandins. Alternatively, growing mycelium was treated with 7.5 μg NTG per ml in the seed medium. For this purpose NTG was added at the beginning of the incubation and the flasks (250-ml Erlenmeyer) were shaken for 120 hours. The cellular population was plated on Potato-Dextrose agar (Difco) for confluent growth and allowed to sporulate. The spore suspension was prepared

at previously described, diluted and plated for the isolation of single colonies. These were transferred to slants and tested for the production of pneumocandins as described above.

Results

Isolation of Mutants

In the preliminary studies the effect of different concentrations of NMU and NTG on the survival of *Z. arboricola* spores was determined. The fungus was substantially more sensitive to NMU than NTG. A 90% loss in viability was observed in 5 minutes at 0.05 mg NMU per ml, which increased to 99% at 0.1 mg/ml as compared to a 50% reduction in survivors in 5 minutes at 1.0 mg NTG per ml. At concentrations of NTG lower than 1.0 mg/ml little or no loss in viability was observed. We isolated two classes of mutants from the mutagen treated spores; one consisting of those which produce more pneumocandin A_0 than the parent (superior mutants) and the other containing mutants with different product distribution as compared to the parent (compositional mutants). One superior mutant and two compositional mutants from the survivors of NMU treatment as well as two compositional mutants from the population treated with NTG were found (Fig. 2).

The production of pneumocandin A_0 by MF5415 was 2.5-fold higher than the parent ATCC 20868, while that of B_0 and A_3 was increased 7- and 4-fold, respectively (Table 2). The product profiles of mutants, ATCC 20957, 20958 and 20988 are shown in Figs. 3 and 4. Pneumocandin B_0 and pneumocandin A_4 produced by ATCC 20957 and 20988, respectively, were also synthesized by the parent, albeit in low amounts,[4] however, pneumocandin A_2 produced by ATCC 20958, was a new compound and like the other pneumocandins it had both anti-candida and anti-pneumocystis activities (Figs. 3 and 4). It should be noted that mutants ATCC 20958 and 20988 had essentially lost their ability to synthesize pneumocandin A_0. ATCC 74030, isolated from ATCC 20957 by mutagenesis of growing mycelium had substantially reduced levels of A_0 (Table 3).

Development of Media for the Production of Minors

A medium was developed which supported the production of minors originally isolated from the solid medium fermentation. It was reported earlier that two liquid media designated as PBM and PB9 supported good production of A_0.[1] A screen of different carbon sources in PB9 medium led to the identification of D-mannitol as an optimum one and to the design of a medium specified as medium A.[5] In our hands, although this medium gave high yields of A_0, it did not support the production of other

Fig. 2. Genealogy of *Z. arboricola* mutants.

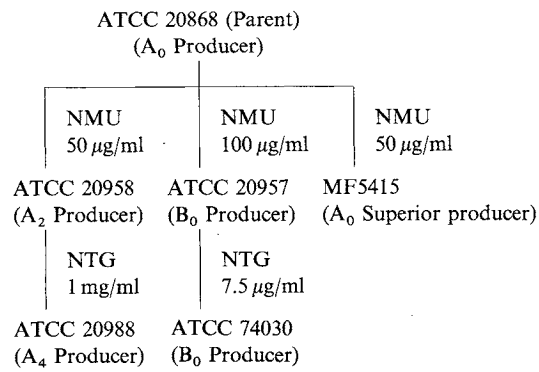


Table 2. Production of pneumocandins by the superior producer MF5415.

Culture	Pneumocandin B_0 (μg/ml)	Pneumocandin A_0 (μg/ml)	Pneumocandin A_3 (μg/ml)
ATCC 20868	18	75	15
MF5415	119	180	64

Fig. 3. Product profiles of ATCC 20868, 20957 and 20958.

Production medium used was S6. No correction has been made for the time lag between the UV detector and the fraction collector. Panel A: ATCC 20868, panel B: ATCC 20957, panel C: ATCC 20958.

■ Indicates the bioactive fractions.

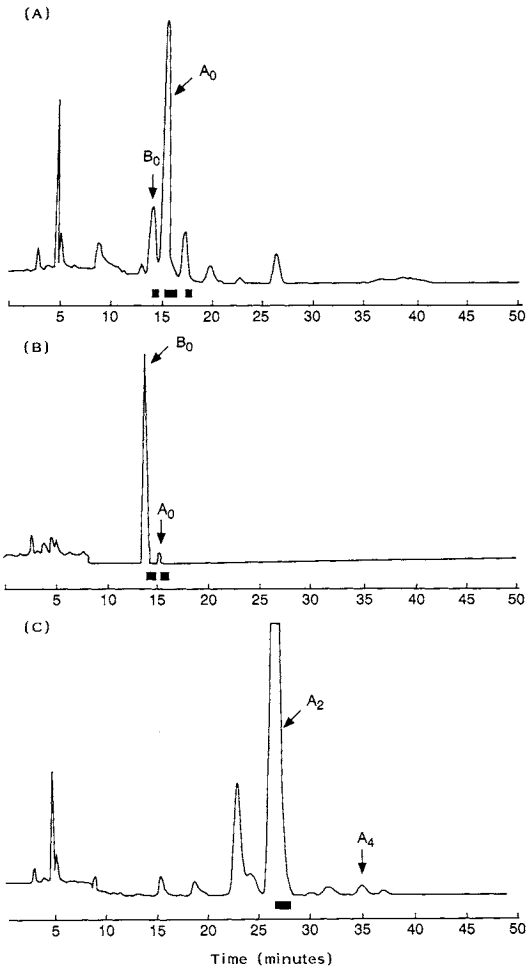


Fig. 4. Product profiles of ATCC 20958 and 20988.

Production medium used was S6. No correction has been made for the time lag between the UV detector and the fraction collector. Panel A: ATCC 20958, panel B: ATCC 20988.

■ Indicates the bioactive fractions.

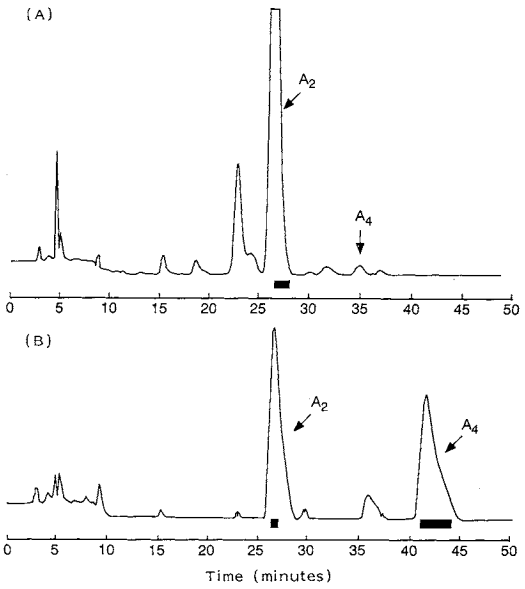


Table 3. Comparison of production of pneumocandins in SP-5 medium by mutants ATCC 20957 and ATCC 74030.

Mutant	Pneumocandin B_0 (μg/ml)	Pneumocandin A_0 (μg/ml)	Ratio B_0/A_0
ATCC 20957	285	30	10
ATCC 74030	241	3	80

minors in detectable concentrations. We attempted to simplify medium A and found good yields of minors in one of the media tested. It was designated as S2 (Table 1). The addition of soybean oil, a presumptive source of precursor(s) for the dimethylmyristic acid moiety, resulted in medium designated as S6. The yields of pneumocandin A_0 in S6 medium were increased 100% over those in S2 medium (Table 4). The HPLC product profiles in S2 and S6 media are shown in Fig. 5. In both media A_0 production was accompanied by that of the minors B_0, A_1, A_3 and A_4. The distribution of pneumocandins in S2 and S6 media was qualitatively similar, however, the yields were higher in S6 medium and, therefore, it was used for the detection of mutants as described above.

Development of Media for Increased Production

Since the production of pneumocandins in S6 medium, while adequate for the screening purposes,

Table 4. Production of pneumocandin A_0 in different media by ATCC 20868.

Medium	Pneumocandin A_0 (μg/ml)	
	7 days	14 days
S2	14	52
S6	14	115

Table 5. Effect of complex nitrogen sources in S6 medium on the production of pneumocandin A_0.

Nitrogen source (10 g/liter)	Pneumocandin A_0 (μg/ml)
Peptonized milk	129
Pharmamedia	173
NZ-Amine type E	52
Promosoy	22
Soybean meal	92
Primary yeast	61
Ardamine PH	13
Solulac	33
Lard water	93
Lexein	23

Fig. 6. Kinetics of production of pneumocandin A_0 by MF5415 in SP-5 and SP-7 media.

□; SP-5, ○; SP-7.

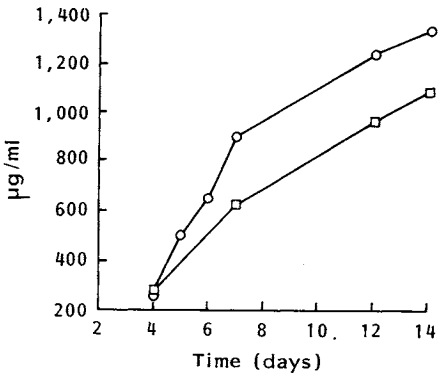


Fig. 5. Product profiles of ATCC 20868 in S2 and S6 media.

No correction has been made for the time lag between the UV detector and the fraction collector. Panel A: medium S2, panel B: medium S6.

■ Indicates the bioactive fractions.

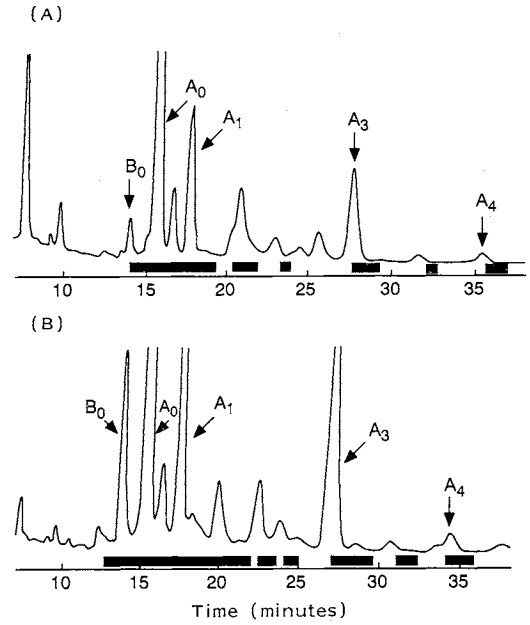


Table 6. Effect of KH_2PO_4 on the production of pneumocandin A_0 in SP-5 medium.

KH_2PO_4 (g/liter)	Pneumocandin A_0 (μg/ml)
0	170
1	800
2	1,075
3	1,095
4	1,140
5	1,050
9	1,020

was not high enough for large scale production, studies were undertaken to increase the yields. A number of nitrogen sources were tested in S6 medium as replacement of peptonized milk. As shown in Table 5 the production of pneumocandin A_0 was substantially higher in the medium containing Pharmamedia than that in the medium with peptonized milk (control), while in the medium with either soybean meal or lard water as a nitrogen source, it was comparable to the control (Table 5). Further optimization of concentration of Pharmamedia, D-mannitol and Cerelose as well as the recognition that in this medium glycine, lactic acid and trace elements did not affect the yields, resulted in the formulation of SP-5 medium (Table 1). The production of pneumocandin A_0 by MF5415 was increased 6-fold in this medium over that

in S6 medium (Fig. 6, Table 2). Another 25% improvement in the yields was obtained by increasing the concentrations of D-mannitol and Pharmamedia to 100 g/liter and 35 g/liter, respectively (Fig. 6). In SP-5 medium KH_2PO_4 was essential for good production. The optimum concentration of KH_2PO_4 was 4 g/liter (Table 6).

Discussion

Mutation has been one of the most effective means of improving product yield in fermentations.[6~9] It has been applied also to isolate mutants blocked in the synthesis of a fermentation product and these have been used to study the biosynthesis of that product.[10] It was noted that these mutants sometimes accumulated new products.[11,12] Unwanted coproducts have been eliminated by mutating the producing culture.[13,14] In the light of present understanding of pneumocandin biosynthesis[15,16] the compositional mutants appear to be blocked in the biosynthesis of A_0 and thus have a different product spectrum. Mutant ATCC 20958 produced a new compound, A_2, while mutants ATCC 20957, 20988 and 74030 produced compounds known to have been produced by the wild type culture. Based on the structural similarity, it is possible that this compound (A_2) is an intermediate in the biosynthesis of pneumocandin A_0. In addition all of these mutants produced substantially reduced levels of A_0, which was, in this case, an unwanted product. Therefore, these mutants were useful in a number of ways. They facilitated the isolation of the known and new minors and they will be helpful in the study of the biosynthesis of these compounds as well as of the genetics of *Z. arboricola*.

S2 medium does not contain as many complex carbon and nitrogen sources as medium PBM,[1] PB9[1] or A[5] and that might have made it limiting for the synthesis of A_0, which in turn led to the accumulation of presumed intermediates. The role of soybean oil is not clearly understood. Since it did not affect the spectrum of the minors but their yield, it may be simply acting as another carbon source. This hypothesis is supported by the observation that when the concentration of D-mannitol was increased, soybean oil had no effect on the production (data not shown). Oils have been used in a number of fermentations as a carbon source.[17~20]

The substantial increase in the yields observed with Pharmamedia as a nitrogen source was intriguing. It is possible that both the amino acid composition and the molecular weight distribution of proteins and peptides in the complex nitrogen source are critical for good production. This suggestion is supported by the fact that soybean meal and peptonized milk were better nitrogen sources than Promosoy and NZ-Amine type E, respectively. Furthermore, during the studies to determine the effect of sterilizaton conditions on Pharmamedia, it was observed that the post-sterilization molecular weight distribution of the proteins/peptides was critical to obtain good production (MASUREKAR, unpublished results). The effect of phosphate in SP-5 medium was examined since it is known to adversely affect the production of some secondary metabolites.[21~24] It was interesting that even at high levels of phosphate (9 g/liter) yields were reduced by only 11% from the maximum. This insensitivity to phosphate is similar to that observed by CURDOVA *et al.* (1989) in the avermectin fermentation.[25]

To summarize, the results of the studies described here allowed the production of different naturally occurring pneumocandins in the quantities required for the determination of biological activity. Furthermore, the mutants, which produced these minors, will be useful in biosynthetic and genetic studies.

In addition to the pneumocandins described in this paper, there were other bioactive compounds which are currently being isolated and characterized and will be the subject of a future publication.

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