MILBEMYCINS, A NEW FAMILY OF MACROLIDE ANTIBIOTICS

FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL PROPERTIES OF MILBEMYCINS D, E, F, G AND H

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Strain Au-3, a mutant of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*, obtained with ultraviolet irradiation, was a high-yield strain of milbemycins D, E, F, G and H. Fermentation studies on the strains were conducted in shake flasks and 30-liter jar fermentors. Isolation of the metabolites was performed by adsorption on resinous adsorbent followed by elution with aqueous MeOH and separated by silica gel column chromatography. Milbemycin D was obtained as colorless needles after recrystallization and milbemycins E, F, G and H were purified to homogeneity by column chromatography. Physico-chemical characterization revealed that milbemycins D, E, F, G and H were new antibiotics possessing the 16-membered macrocyclic lactone with a 6,6-membered spiroketal ring system.

Streptomyces hygroscopicus subsp. *aureolacrimosus* produced 13 milbemycins with insecticidal and acaricidal activity[1]; all the milbemycins have the 16-membered macrolide structure[2,3]. The strain also produced a small amount of five new milbemycins D, E, F, G and H[4]. Strain Au-3, a mutant of *S. hygroscopicus* subsp. *aureolacrimosus*, produced these new milbemycins mainly together with milbemycins α_1, α_2, α_3, α_4, α_9, α_{10}, β_1 and β_2.

In this paper, we describe the fermentation, isolation and physico-chemical properties of milbemycins D, E, F, G and H. Biological properties and structure elucidation of the antibiotics will be reported elsewhere.

Materials and Methods

Microorganism

S. hygroscopicus subsp. *aureolacrimosus* and its mutant, strain Au-3, were maintained on YM slant agar (0.4% glucose, 0.4% yeast extract, 1% malt extract and 2% agar, pH 7.2).

Fermentation

Fermentation studies were carried out in shake flasks and 30-liter jar fermentors. The seed cultures of the parent strain and strain Au-3 were prepared by cultivation at 28°C for 2 days on a rotary shaker (220 rpm, 7 cm) in a 500-ml Erlenmeyer flask containing 100 ml of a medium with the following composition: 1% sucrose, 0.35% Polypepton and 0.05% K_2HPO_4. One milliliter of a good preculture was then inoculated into a 100-ml Erlenmeyer flask containing 20 ml of a fermentation medium (BM-2) with the following composition: 6% glucose, 1% soybean meal, 1% skim milk, 0.5% corn starch and 0.05% $CaCO_3$. The pH of the medium was adjusted to 7.2 before sterilizing at 120°C for 25 minutes. Fermentations with two strains were also conducted in aerated and stirred 30-liter jar fermentors. Twenty liters of the BM-2 medium was sterilized in the fermentor by heating to 120°C for 25 minutes. One liter

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of a good preculture grown in two 2-liter Erlenmeyer flasks incubated at 28°C for 2 days was transferred to a jar fermentor. Fermentation was carried out under the following conditions: temperature 28°C, aeration 10 liters/minute, agitation 150~250 rpm and internal pressure 0.5 kg/cm^2.

HPLC Assay

The system developed for the quantitative analyses of milbemycins employed a Waters Associates μBondapak C_{18} reverse-phase column (0.4×30 cm), a solvent system of acetonitrile - water (75: 25, v/v), and a flow rate of 2 ml/minute. A Waters Associates model 6000A pump and model U6K loop sample injector were used. The column effluent was monitored by a Jasco model UVIDEC-100 ultraviolet monitor set at 240 nm. The monitor output was coupled to a Yokogawa Electric Works type 3056 chart recorder for visual observation and a Shimadzu Chromatopac C-R1A digital computing integrator for quantitative results. To determine the amount of milbemycins produced in the culture broth, 3 ml of the broth was mixed with 7 ml of acetone and shaken for 5 minutes and 10 μl of the supernatant solution was injected into the column. The peak areas on the HPLC pattern were compared with those of a standard extract which had been calibrated from the HPLC pattern of purified milbemycin components.

Isolation

The extraction, isolation and purification procedures of each milbemycin were almost identical to those mentioned in the previous paper[1]. Adsorbent resin HP-20 and silica gel (Art. 7734) for column chromatography were purchased from Mitsubishi-Kasei Co., Ltd. and E. Merck Co., Ltd., respectively. Lobar column Si 60 and RP-8 were also purchased from E. Merck Co., Ltd.

Fermentation broth (100 liters) of strain Au-3 was adjusted to pH 3.0 and filtered with Celite. The cake (15 kg) was extracted twice with 30 liters of methanol (MeOH); to 60 liters of the extract, 50 liters of water was added. The resulting aqueous MeOH was extracted twice with 50 liters of *n*-hexane. The *n*-hexane was evaporated under reduced pressure to give the residue, which was dissolved in 2 liters of MeOH. To this solution 2 liters of water was added. The resulting solution was applied to a 10-liter Diaion HP-20 column. The adsorbate was washed with 30 liters of 60% aqueous MeOH, and eluted with 20 liters of 95% aqueous MeOH. Fractions containing milbemycins were collected and 10 liters of water was added to 12 liters of the fractions. The about 50% aqueous MeOH solution was extracted with 30 liters of *n*-hexane. The extracts were evaporated under reduced pressure to obtain 120 g of oily substance. The residue was dissolved in 200 ml of *n*-hexane and chromatographed on a column (3 liters) of silica gel. The column was eluted with a mixture of *n*-hexane and acetone (95: 5) to obtain successively, fraction I (3.5 liters), II (3 liters) and III (2.3 liters). The eluate fractions were monitored by thin-layer chromatography (TLC)[1]. The column was then eluted with *n*-hexane - acetone (80: 20) to obtain fraction IV (1.5 liters).

Fraction I, containing milbemycins G and H was evaporated under reduced pressure to obtain 1.55 g of the oily substance. The residue thus obtained was dissolved in 3 ml of *n*-hexane and applied to a 50 ml silica gel column, which was eluted with *n*-hexane - ethyl acetate (95: 5) to separate milbemycins G and H. Fractions including milbemycin were collected on the basis of TLC and HPLC analyses and the solvent was removed under reduced pressure. The solids thus obtained were reprecipitated from *n*-hexane - ethyl acetate (20: 1) yielding 0.4 g of milbemycin G and 0.3 g of milbemycin H as amorphous powder, respectively.

Fraction II, containing milbemycin D was evaporated under reduced pressure to obtain 23 g of crude milbemycin D. The crude powder was dissolved in 50 ml of MeOH and applied to a 800 ml Sephadex LH-20 column. The column was eluted with MeOH to obtain fractions containing milbemycin D. The fractions were collected and evaporated under reduced pressure to obtain 22 g of crystalline powder. Colorless needles (18.5 g) of milbemycin D were obtained after recrystallization from a mixture of *n*-hexane and ethyl acetate (20: 1).

Fraction III, containing milbemycins α_3, β_1, D and E was evaporated under reduced pressure to obtain 5.27 g of the oily substance. The residue thus obtained was dissolved in 2 ml of MeOH and applied to a Lobar column RP-8, which was eluted with MeOH - water (85: 15). The fractions containing milbemycins D and E were collected on the basis of TLC analysis and the solvent was removed under reduced pressure. The solid thus obtained was dissolved in 2 ml of *n*-hexane and applied to a Lobar

column Si 60, which was eluted with *n*-hexane - ethyl acetate (7: 3) to separate milbemycin E from D. The fractions containing milbemycin E were pooled on the basis of TLC analysis and the solvent was removed under reduced pressure. The solid thus obtained was reprecipitated from a mixture of *n*-hexane and ethyl acetate (20: 1) to yield milbemycin E (1.4 g) as an amorphous powder.

Fraction IV, containing milbemycin F was evaporated under reduced pressure. Nine hundred milligrams of yellow syrup thus obtained was dissolved in 5 ml of *n*-hexane and applied to a 90 ml silica gel column, which was eluted with a mixture of *n*-hexane and acetone (80: 20). Fractions containing milbemycin F were collected on the basis of TLC analysis and the solvent was removed under reduced pressure. The yellowish syrup thus obtained was dissolved in 20 ml of ethanol (EtOH) and decolorized with 400 mg of active carbon and reprecipitated from a mixture of *n*-hexane and ethyl acetate (20: 1) to yield milbemycin F (0.6 g) as an amorphous powder.

Analytical Procedure

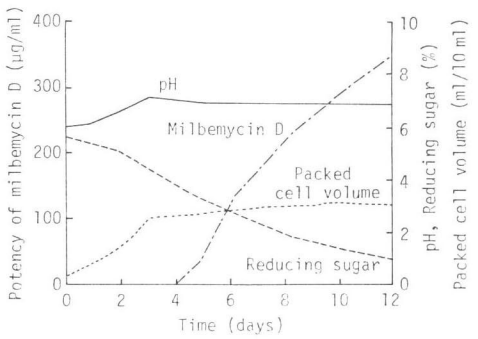
UV absorption spectra were obtained in EtOH with a Hitachi 200-20 spectrophotometer. IR absorption spectra were measured using a Jasco IRA-2 spectrometer. ^{1}H NMR spectra were measured at 100 MHz with a Varian HA-100 using TMS as internal reference.

Results and Discussion

Fermentation

Fermentations of the parent strain and its mutant strain Au-3 were conducted in 100-ml Erlenmeyer flasks containing 20 ml of the BM-2 medium. After 12 days cultivation, the production of milbemycins D, E, F, G and H by the parent strain reached 21, 10, 8, 5 and 2.5 μg/ml and those of strain Au-3 reached 360, 70, 25, 22 and 8 μg/ml, respectively. Fermentation studies on strain Au-3 were conducted in 30-liter jar fermentors containing 20 liters of the BM-2 medium. The packed cell volume in the culture broth increased in the first 3 days and was constant thereafter (Fig. 1). The antibiotics were first detected in the broth after 5 days cultivation and increased at a linear rate for the next 7 days. The HPLC pattern of the culture broth after 12 days cultivation showed that strain Au-3 produced milbemycins D, E, F, G and H with the production of milbemycins α_1, α_2, α_3, α_4, α_9, α_{10}, β_1 and β_2 (Fig. 2). The yields of milbemycins D, E, F, G and H reached 350, 65, 23, 18 and 8 μg/ml at 12 days cultivation, respectively. However, milbemycins α_5, α_6, α_7 and α_8 reported in the previous paper[1] were not detected in the culture broth. The results described above suggest that strain Au-3 was a high-yielding strain of milbemycins D, E, F, G and H, and was blocked in the biosynthetic pathway of milbemycins α_5, α_6, α_7 and α_8, to all of which 2-methyl hexanoyloxy group was attached at the carbon 23 position.

Fig. 1. Time course of milbemycin D production by strain Au-3 in a 30-liter jar fermentor.



Isolation

The isolation and separation procedures for milbemycins D, E, F, G and H are outlined in Figs. 3 and 4. Milbemycin D was obtained as colorless needles after recrystallization from a mixture of *n*-hexane and ethyl acetate (20: 1). Milbemycins E, F, G and H were obtained as amorphous powders with homogeneity as shown by silica gel and Lobar column chromatography.

Fig. 2. HPLC pattern of 12-day culture broth of strain Au-3.

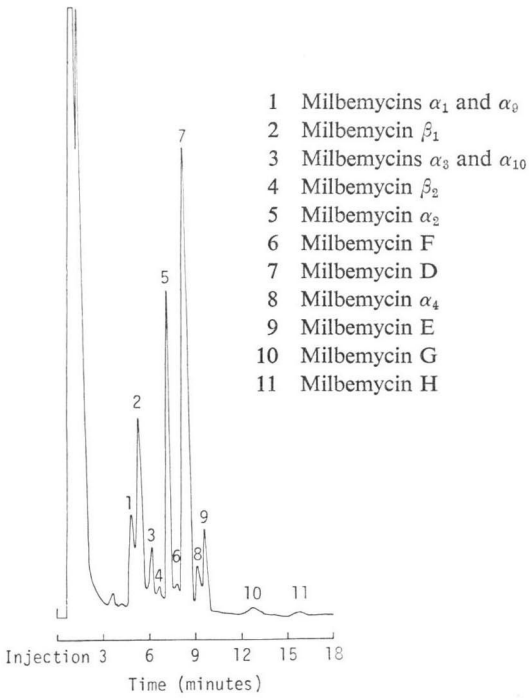


Fig. 3. Extraction and isolation of milbemycins D, E, F, G and H.

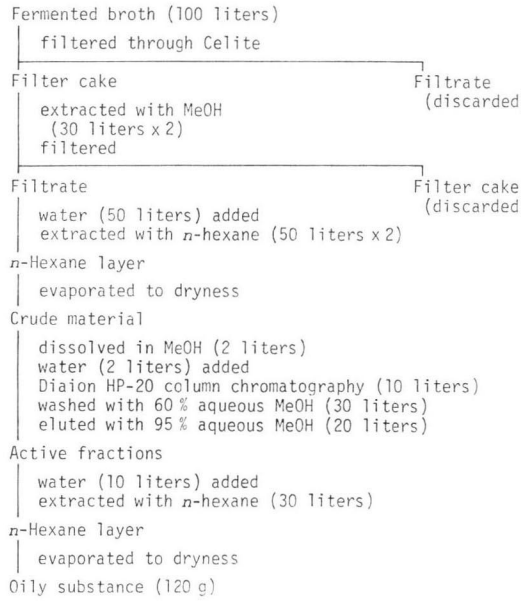
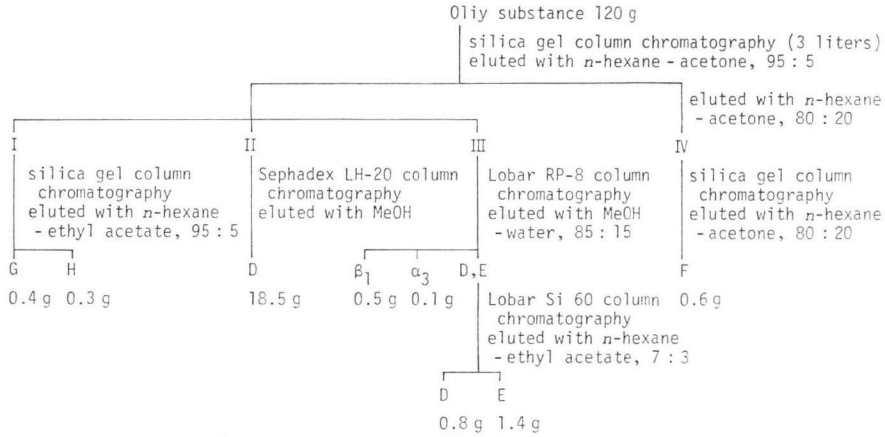


Fig. 4. Separation of milbemycins.



Physico-chemical Properties

Physico-chemical data of milbemycins D, E, F, G and H are listed in Table 1. Milbemycins D, E, F, G and H are easily soluble in common organic solvents, such as benzene, chloroform, acetone, ethanol and methanol. They are sparingly soluble in water.

The UV spectra of milbemycins D, E and G indicated the presence of a conjugated diene system by the comparison with those of milbemycins α_1, β_1 and α_2, respectively, and the UV spectrum of milbemycin F suggested the presence of a conjugated diene system and pyrrole by the comparison with that of mil-

Table 1. Physico-chemical properties of milbemycins D, E, F, G and H.

Milbemycin	D	E	F	G	H
Nature	Needles	Amorphous powder	Amorphous powder	Amorphous powder	Amorphous powder
mp (°C)	186~188	—	—	—	—
Mol. Form.	$C_{33}H_{48}O_7$	$C_{34}H_{52}O_7$	$C_{33}H_{51}NO_9$	$C_{34}H_{50}O_7$	$C_{33}H_{48}O_6$
MW. (mass)	556	572	665	570	540
UV λ_{max}^{EtOH} nm (ε)	238 (sh) 244 (31000) 253 (sh)	241 (26000)	238 (sh) 245 (24100) 253 (21400) 266 (sh)	238 (sh) 244 (30500) 253 (sh)	237 (25200)
IR ν_{max} (cm^{-1})	3450, 1710	3490, 1715	3320, 1730, 1710	3450, 1710	3490, 1715, 1685
$[\alpha]_D^{27}$*	+107°	+157°	—	+108°	+60°
Rf**	0.46	0.61	0.22	0.86	0.88

* *c* 0.25, acetone.
** Silica gel 60 F-254 plates (Merck), developed in dioxane - CCl_4, 15: 85.
—; not measured
sh; shoulder.

bemycin α_9. The UV spectrum of milbemycin H displayed the presence of a conjugated diene system and/or an α,β-unsaturated ketone (Table 1).

In the IR spectra of milbemycins D, E, F, G and H, absorption bands at 3600~3330 and 1710~1707 cm^{-1} were assigned to a hydroxyl and 16-membered lactone groups, respectively. The absorption band due to the lactone ring was observed at a lower frequency than that of the usual lactone group. In the IR spectrum of milbemycin H, an absorption band at 1685 cm^{-1} was also assigned to an α,β-unsaturated carbonyl group (Table 1).

The ^1H NMR spectra of milbemycins D, E, F, G and H are illustrated in Fig. 5. The ^1H NMR

Fig. 5-a. ^1H NMR spectrum of milbemycin D ($CDCl_3$).

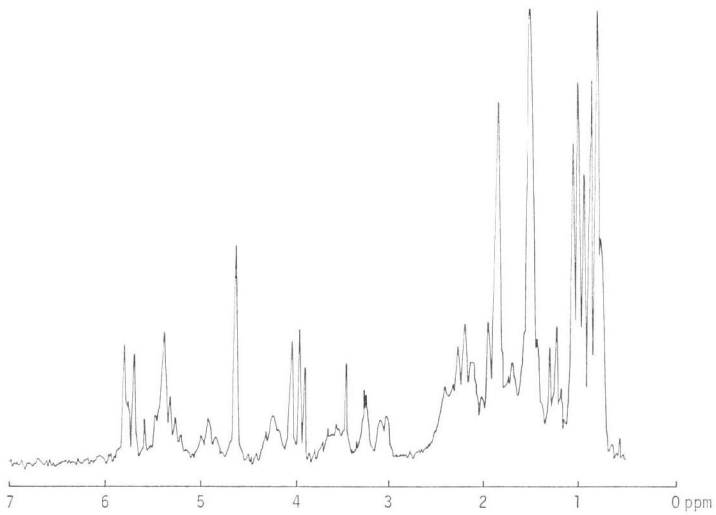


Fig. 5-b. ^1H NMR spectrum of milbemycin E ($CDCl_3$).

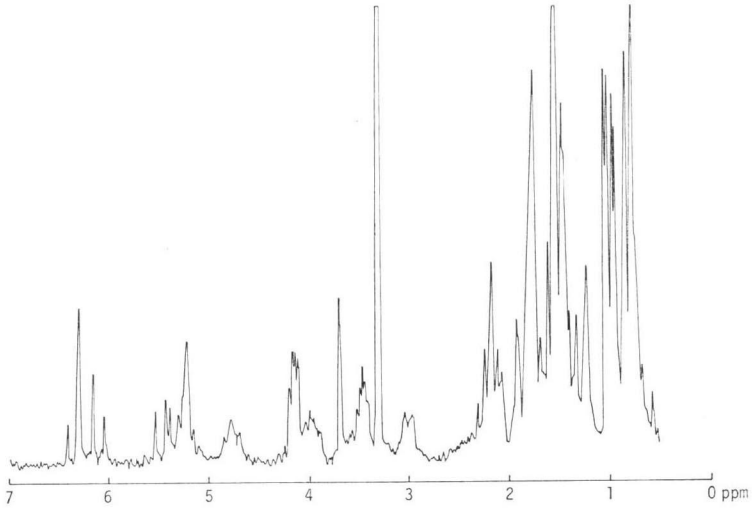
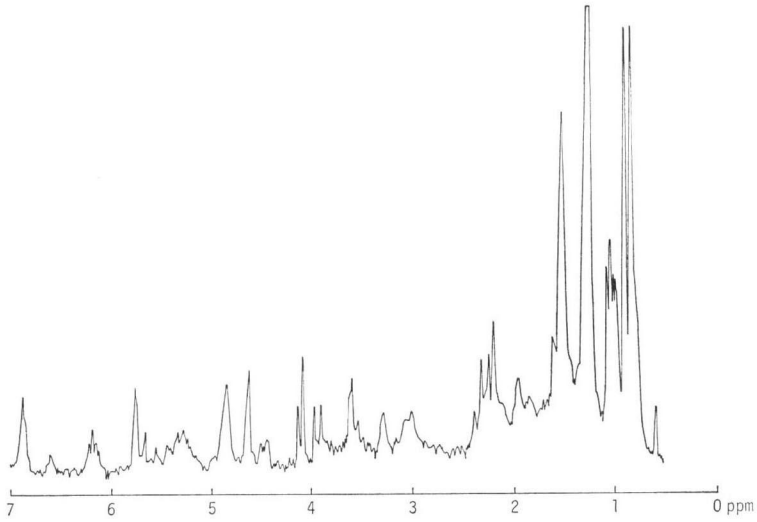


Fig. 5-c. ^1H NMR spectrum of milbemycin F ($CDCl_3$).



spectra of milbemycins D, E, F, G and H showed signals due to an isopropyl group at C-25 position at δ 0.86 (3H, d), 1.05 (3H, d), 1.90 (1H, m) and 3.07 (1H, dd). Detailed analyses of ^1H NMR spectra of milbemycins D, E, F, G and H will be described in the structure elucidation paper. Spectra data indicated that the five new macrolide, milbemycins D, E, F, G and H, possessed a 16-membered macrocyclic lactone structure with a 6,6-membered spiroketal ring system bearing an isopropyl side chain.

Fig. 5-d. ^1H NMR spectrum of milbemycin G ($CDCl_3$).

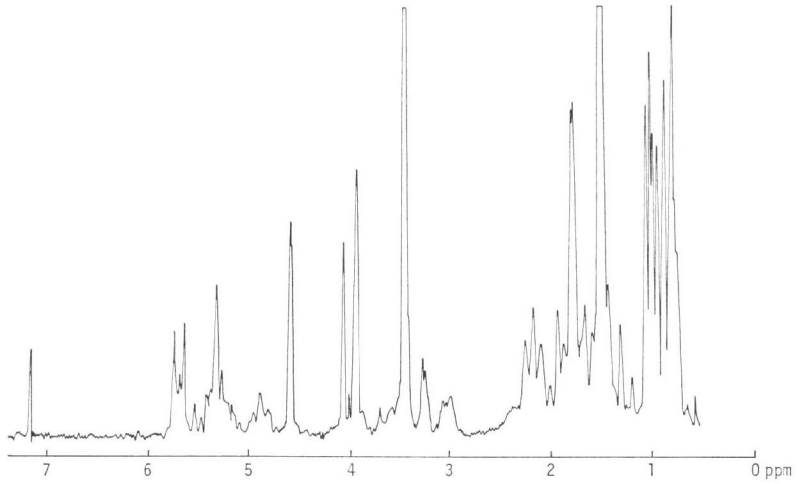
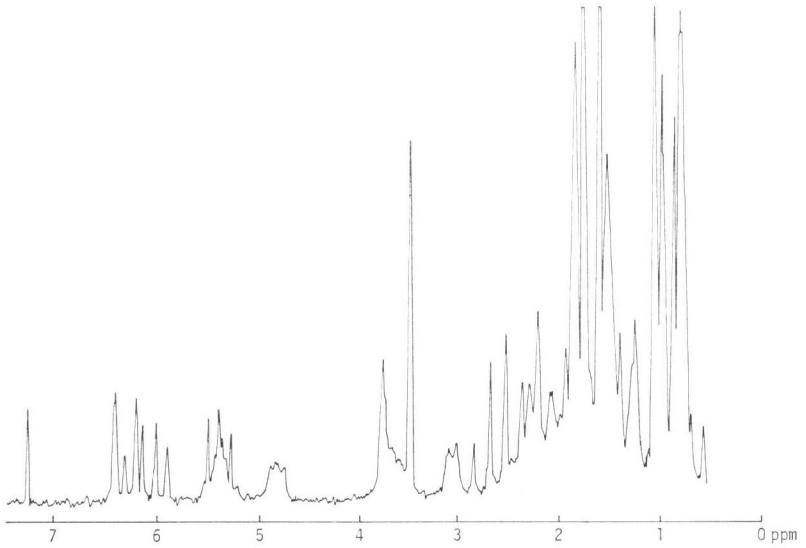


Fig. 5-e. ^1H NMR spectrum of milbemycin H ($CDCl_3$).



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