The Mode of Action of UK-2A and UK-3A, Novel Antifungal Antibiotics from *Streptomyces sp.* 517-02

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(Received for publication July 3, 1997)

UK-2A and UK-3A are structural relatives of antimycins, which were isolated as antifungal antibiotics with little cytotoxicity that demonstrated inhibition of respiratory activity. They halve the cellular respiration of yeast within 4~5 minutes and the intracellular ATP content within 2~5 minutes. Moreover, they inhibited the yeast mitochondrial respiration using β-hydroxybutyrate and succinate as a respiratory substrate, but no inhibition was observed using ascorbate-reduced tetramethyl *p*-phenylenediamine as the substrate. The site of respiratory inhibition of UK-2A and UK-3A was thought to be the cytochrome bc_1 complex in the mitochondrial electron transport chain of yeast cells. They also inhibited the mitochondrial respiration of rat liver. It has been suggested that intact animal cells might have some system to defend themselves from the actions of UK-2A and UK-3A.

UK-2A~D and UK-3A were isolated as antifungal antibiotics from the acetone extracts of mycelia of *Streptomyces*. sp 517-02[1,2]. UK-2A~D and UK-3A were very similar in structure to antimycin antibiotics[2,3]. The former's activity was as strong as that of antimycin A, while that of the latter was not so potent. Interestingly, UK-2A~D and UK-3A demonstrated very weak cytotoxicity compared to antimycin A. Antimycins are well-known not only as antibiotics but also as insecticides, miticides, *etc.*[4]. They are used as specific mitochondrial respiratory chain inhibitors only in laboratory studies because of their broad toxicity.

This paper describes the mode of action of UK-2A and UK-3A compared with that of antimycin A_3.

Materials and Methods

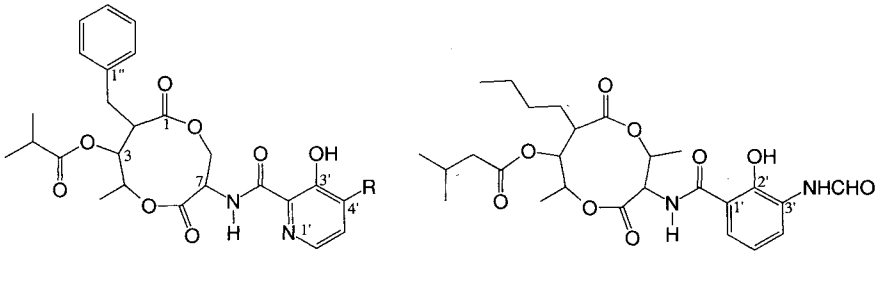
Chemicals

Antimycin A_3 and luciferin-luciferase were obtained from Sigma Chemical Co. Radioactive chemicals were purchased from New England Nuclear. Others were of commercial grade.

Incorporation of Radioactive Precursors Into Cellular Components

Saccharomyces cerevisiae IFO 0203, the most susceptive organism among all the tested yeasts (MICs[1,2] of UK-2A, UK-3A and antimycin A_3 were 0.05, 1.56 and 0.025 μg/ml, respectively), was cultured in a semisynthetic medium[5] (per liter; 20 g lactate, 0.5 g glucose, 3 g Bacto-yeast extract, 1 g KH_2PO_4, 1 g NH_4Cl, 0.5 g $CaCl_2 \cdot 2H_2O$, 0.5 g NaCl and 0.6 g $MgCl_2 \cdot 6H_2O$, ad-

Fig. 1. Structures of UK-2A, UK-3A and antimycin A_3.



UK-2A : R= -OCH_3
UK-3A : R= -H

Antimycin A_3

justed to pH 5.5 with 1 N NaOH) with shaking at 25°C for 24 hours. The growing cells were harvested by centrifugation, washed and suspended to give approximately 10^6 cells/ml in the prewarmed fresh medium. After 30-minutes incubation with shaking at 25°C, the radioactive precursors, UK-2A, UK-3A and antimycin A_3 were added to the cell suspension. At appropriate intervals, 200 μl of the suspension was taken into 200 μl of ice cold 10% trichloroacetic acid (TCA) and kept on ice for 1 hour. The TCA precipitates were collected on a glass fiber filter (Whatman GF/C) and radioactivities on the dried filters were counted by a Beckman liquid scintillation counter with a 5 ml toluene-based scintillation cocktail consisting of 0.3% 1,4-bis-2-(5-phenyloxazolyl)-benzene and 1% 2,5-diphenyloxazole.

Measurement of Intracellular ATP Content

S. cerevisiae IFO 0203 was cultured, harvested and suspended in the semisynthetic medium as described above. After 10-minutes incubation with shaking at 25°C, UK-2A, UK-3A and antimycin A_3 were added to the cell suspension. At appropriate intervals, 100 μl of the suspension was taken into an equal volume of ice cold acetone-formic acid (1 : 1), and sonicated for 3 minutes. The above mixtures were evaporated *in vacuo* and resuspended in 100 μl of distilled water. To this sample was added 20 μl of a reaction mixture prepared by dissolving 2 mg of luciferin-luciferase in 1 ml of sterile 0.1 mM AMP. Light emission was counted in a Berthold biolumat LB9500C for 1 minute according to the ATP bioluminescence assay. The signal was constant for at least 3 minutes due to the inhibition of the initial rate of the reaction by excess AMP. Under these conditions, a range of 10^{-9} M to 10^{-7} M ATP could be determined.

Mitochondrial Preparation from *S. cerevisiae*

S. cerevisiae IFO 0203 was cultured in the semisynthetic medium with shaking at 25°C for 24 hours. The cells were collected by centrifugation at 3000 × *g* for 5 minutes at room temperature and washed with distilled water. The cells were resuspended in 0.1 M Tris-SO_4 (pH 9.3) and 10 mM dithiothreitol. After 10-minutes incubating at 30°C, they were collected by centrifugation, washed once with a solution of 1.2 M sorbitol, 20 mM potassium phosphate, pH 7.4 (spheroplasting buffer), and then resuspended to give approximately 0.1 g cells/ml in spheroplasting buffer to which Zymolyase 20T (KIRIN Brewery Co., Ltd.) had been added (2.5 mg/g cells). After gentle shaking for 45 minutes at 30°C, the spheroplasts were collected by centrifugation and washed 3 times with spheroplasting buffer without Zymolyase. They were resuspended in mitochondrial isolation buffer (MIB, composed of 0.6 M mannitol, 20 mM HEPES-KOH (pH 7.4) and 0.5 mM phenylmethylsulfonyl fluoride) to a final concentration of 0.5 g original cells/ml. They were broken in a Dounce homogenizer with 15 strokes using the B pestle. The homogenate was centrifuged at 3000 × *g* for 5 minutes at 4°C. The supernatant was centrifuged at 9500 × *g* for 10 minutes at 4°C. The pellet from this spin was resuspended in MIB to a final concentration of 2.0 g protein/ml. This crude mitochondrial fraction was used for the oxygen uptake assay.

Mitochondrial Preparation from Rat Liver

Mitochondria were isolated from the livers of adult male Wister rats in a medium containing 250 mM sucrose and 2 mM Tris-HCl (pH 7.4) as discribed by MYERS and SLATER[6]. Protein content in the mitochondrial preparation was measured by the method of BRADFORD[7] after addition of an equal volume of 5% Triton X-100, bovine serum albumin being used as a standard.

O_2 Consumption Activity of *S. cerevisiae* and Rat Liver Mitochondria

Mitochondrial respiration using 10 mM succinate as the respiratory substrate was measured with a Yanagimoto PO-100A oxygen electrode at 25°C. The final mitochondrial protein concentration in the reaction mixture (composed of 0.6 M mannitol, 20 mM HEPES-KOH, 10 mM potassium phosphate, 2 mM $MgCl_2$ and 1 mM EDTA, adjusted to pH 7.4 with 1 N KOH) was 0.7 mg/ml. After an addition of 2,4-dinitrophenol (DNP, uncoupler) at 40 μM to stimulate respiration, the respiratory rate was measured for 2 minutes. Subsequently, the inhibitors were added to the reaction mixture, and the rate was measured for another 3 minutes. Respiratory rates were calculated from the oxygen electrode tracing.

Studies on the Inhibition Site in the Electron Transport Chain

Yeast mitochondrial respiration fully stimulated by DNP at 40 μM was measured using the following substrates; 20 mM β-hydroxybutyrate (β-HB, NAD^+-linked substrate), 20 mM succinate (FAD-linked substrate), and a mixture of 10 mM tetramethyl-*p*-phenylenediamine (TMPD, an ascorbate-reducible redox carrier that transfers electrons directly to cytochrome *c*) and 20 mM ascorbate. The respiratory rate was measured as described above.

Results

Macromolecule Synthesis

The effects of UK-2A and UK-3A on DNA, RNA, protein and polysaccharide synthesis in *S. cerevisiae* cells were examined (Fig. 2). In the control cultures, the incorporation of radioactive precursors into the acid-insoluble fractions started instantaneously, and the counts of each fraction increased almost linearly up to 60 minutes after the onset of incubation. UK-2A at 0.05 μg/ml inhibited the incorporation of all species of precursors by about 50% compared with the control. UK-3A at 1.0 μg/ml and antimycin A_3 at 0.05 μg/ml showed more remarkable but incomplete inhibition. No significant difference was observed among the inhibition of the synthesis of DNA, RNA, protein and polysaccharide by these inhibitors.

Intracellular ATP Content

UK-2A at 0.2 μg/ml, UK-3A at 1.0 μg/ml and antimycin A_3 at 0.2 μg/ml halved the intracellular ATP content of *S. cerevisiae* within 2.0, 5.0, and 1.5 minutes, respectively (Fig. 3). There was less possibility that these compounds induced leakage of intracellular ATP outside the cells, because no increase of 260 nm-absorbing materials in supernatant was observed (data not shown). Reductions of ATP contents may relate to the cellular respiratory inhibition. In fact, the respiratory activity was completely inhibited after 30 minutes exposure to UK-2A, UK-3A and antimycin A_3 as previously reported[1,2]. However, intracellular ATP content remained about 25~40% of the control. This may have resulted from the glycolytic ATP production utilizing a small amount of glucose in the medium.

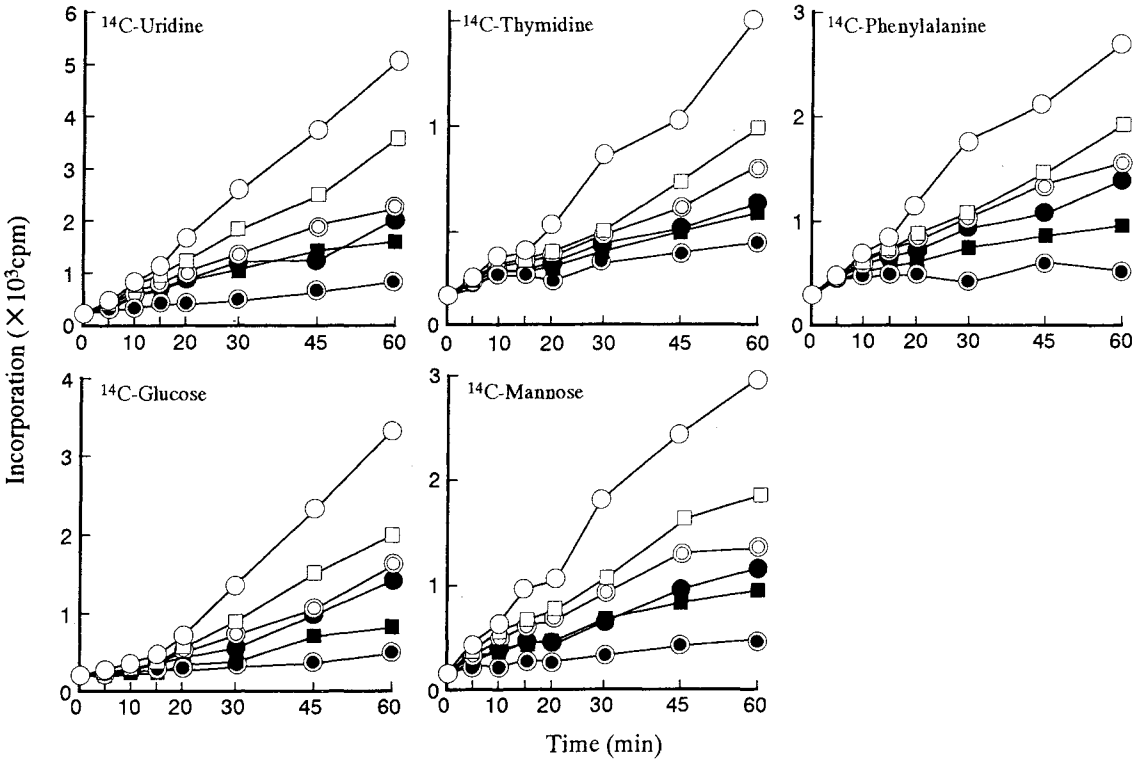
Effects of UK-2A and UK-3A on O_2 Consumption in Yeast Mitochondria

UK-2A and UK-3A inhibited respiration not only at the cell level but also at the mitochondrial level. The molar concentrations of inhibitors (I_{50}) needed to halve the DNP-stimulated respiratory rate of yeast mitochondria are shown in Table 1. The log of the reciprocal of I_{50}, pI_{50}, is used here as the index of inhibitory potency.

The potency of UK-2A was similar to that of antimycin A_3. UK-3A showed about a hundredth of the potency of UK-2A and antimycin A_3. Therefore, the methoxy group attached to C-4′ in pyridine ring of UK-2A seems to play a significant role in the inhibitory action.

Fig. 2. Effects of UK-2A, UK-3A and antimycin A_3 on incorporation of radioactive macromolecular precursors into acid-insoluble fraction of *Saccharomyces cerevisiae* IFO 0203 cells.

○: Control, ◎: UK-2A at 0.05 μg/ml, ◉: UK-2A at 0.2 μg/ml, □: UK-3A at 0.25 μg/ml, ■: UK-3A at 1.0 μg/ml, ●: antimycin A_3 at 0.05 μg/ml.



Inhibition Site of UK-2A and UK-3A in the Electron Transport Chain

As shown in Fig. 4, UK-2A at 1.0 μM and UK-3A at 10 μM inhibited the DNP-stimulated respiration using both β-HB and succinate as respiratory substrates. The inhibition was not recovered by the subsequent addition of succinate or β-HB but was recovered by that of ascorbate-reduced TMPD as the substrate.

In view of the structures of UK-2A and UK-3A, it is considered that these compounds do not have the ability to withdraw the electrons directly from $CoQH_2$. All these suggested that the action site of UK-2A and UK-3A is between $CoQH_2$ oxidation and cytochrome *c* reduction, that is, the cytochrome bc_1 complex, similar to that of antimycins.

Effects of UK-2A and UK-3A on O_2 Consumption in Rat Liver Mitochondria

UK-2A and UK-3A also inhibited respiration in rat liver mitochondria. The molar concentration for 50% inhibition (I_{50}) of rat liver mitochondria respiration was measured. As shown in Table 1, the activities of UK-2A and UK-3A were about a fifth and a fiftieth of that of antimycin A_3. We previously reported that UK-2A and UK-3A demonstrated about a ten-thousandth cytotoxicity compared to antimycin A[1,2]. It may be that the plasma membrane of animal cells is hardly permeable by UK-2A and UK-3A.

Time-dependent Change in Respiratory Inhibition by UK-2A and UK-3A

It is of interest that the respiratory inhibition by UK-2A and UK-3A in rat liver mitochondria decreased with time (Fig. 5(A), (B)). A conformational change in the cytochrome bc_1 complex caused by antimycin A

Fig. 3. Effects of UK-2A, UK-3A and antimycin A_3 on intracellular ATP content in *Saccharomyces cerevisiae* IFO 0203 cells.

◎: UK-2A at 0.2 μg/ml, ○: UK-3A at 1.0 μg/ml, ●: antimycin A_3 at 0.2 μg/ml.

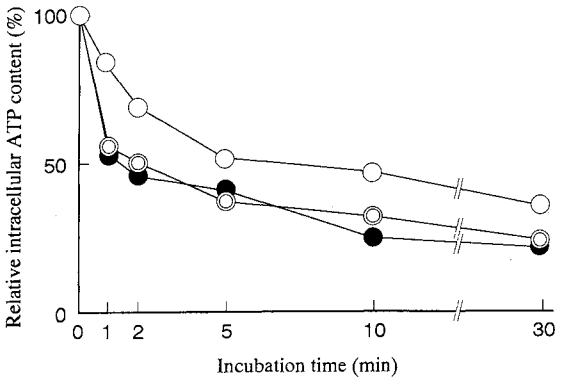
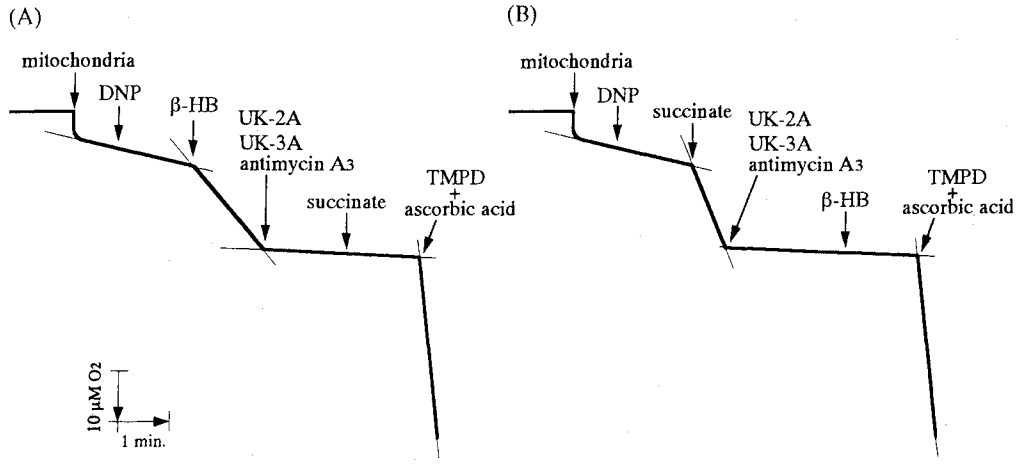


Table 1. Respiratory inhibition of UK-2A, UK-3A and antimycin A_3 in mitochondria from yeast and rat liver.

Mitochondria source	pI_{50}[a]		
	UK-2A	UK-3A	Antimycin A_3
Yeast	8.10	5.96	8.12
Rat liver	6.77	5.89	7.43

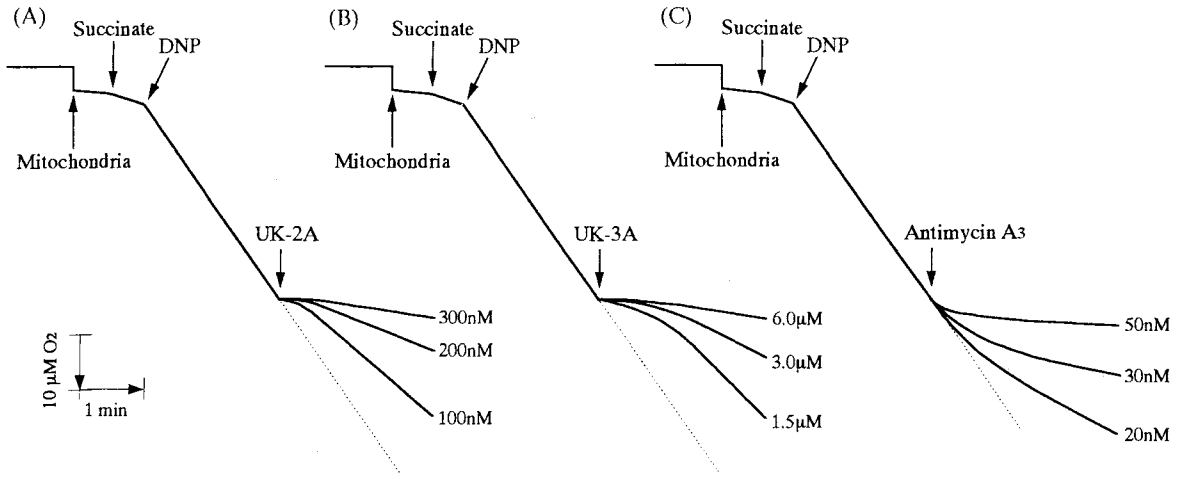
[a] The log of reciprocal of I_{50}. 10^{-6} M of UK-2A, UK-3A and antimycin A_3 equivalent to 5.15, 4.84 and 5.21 μg/ml, respectively.

Fig. 4. Effect of UK-2A, UK-3A or antimycin A_3 on yeast mitochondria respiration fully stimulated by DNP at 40 μM.



β-HB, succinate and ascorbate-reduced TMPD were used as respiratory substrates. Concentrations of UK-2A, UK-3A and antimycin A_3 were 500 nM, 10 μM and 100 nM, respectively.

Fig. 5. Effects of UK-2A, UK-3A and antimycin A_3 on rat liver mitochondria respiration fully stimulated by DNP at 40 μM.



Succinate was used as the respiratory substrate. The dotted lines showed the trace in the absence of an inhibitor.

binding has been reported[8~10]. Further, this change increases its binding affinity[11]. Therefore, a time-dependent increase in the respiratory inhibition by antimycin A_3 was observed (Fig. 5(C)). The decrease with time in the inhibition by UK-2A and UK-3A may be due to a decrease in their binding affinity which is caused by substitution of a picolinic acid moiety for the salicylic acid moiety.

Discussion

The actions of UK-2A and UK-3A on the growth of yeast and fungi seemed to be fungistatic instead of fungicidal like that of antimycin A[1,2]. They inhibited each incorporation of each of radioactive macromolecule precursors, thymidine, uridine, phenylalanine, glucose and mannose, into the TCA insoluble fraction of yeast cells to the same extent. Leakages of 260 nm-absorbing materials and potassium ion by UK-2A at 0.1 μg/ml and UK-3A at 2.0 μg/ml were not observed (data not shown). They halved the cellular respiration of *S. cerevisiae* within 4~5 minutes, and the intracellular ATP content was reduced to half within 2~5 minutes. These results suggested that UK-2A and UK-3A would interfere with energy metabolism as do several respiratory-inhibiting antibiotics[12~14]. Of course, ATP is required for the active uptake of various compounds and for the biosynthesis of macromolecules.

UK-2A and UK-3A efficiently inhibited the respiration of yeast mitochondria ($I_{50}=7.9\times10^{-9}$ M, 2.0×10^{-6} M, respectively); especially, the effectivity of the former was similar that of antimycin A_3 ($I_{50}=7.6\times10^{-9}$ M). UK-2A and UK-3A showed the inhibition of O_2 consumption using β-HB or succinate as the respiratory substrate but did not show this using ascorbate-reduced TMPD as the substrate. It was indicated that, like antimycin A, both UK-2A and UK-3A inhibited electron transport on the cytochrome bc_1 complex.

As previously reported[2], the antifungal activity of UK-3A was a fourth or an eighth of that of UK-2A. In the present study, the inhibitory activity of UK-3A for yeast mitochondrial respiration was about a hundredth of that of UK-2A. The structural difference between UK-2A and UK-3A involves no more than a methoxy group attached to the C-4′ in the picolinic acid moiety. Therefore, this moiety having the methoxy group may play a significant role in the respiratory inhibition and the dilactone ring moiety is not so important. These suggestions agree with a report that the binding of antimycin A to a binding domain at a Qi center of cytochrome *b* is primarily governed by the salicylic acid moiety[15]. Because a 3′-formylamino group on the salicylic acid moiety of antimycin A is essential for tight binding to the domain, lack of the 3′-formylamino group is responsible for a decrease to the thousandth in the activity[16]. However, this degree of decrease is much more than that due to the lack of a 4′-methoxy group on the picolinic acid moiety. It is suggested that the picolinic acid moiety with or without the presence of the methoxy group could fit tightly into the cavity of cytochrome *b*, while the salicylic acid moiety without the formylamino group could not fit so tightly. The difference

in the fitting between the two moieties may be responsible for the time-dependent change in inhibition.

UK-2A and UK-3A showed little cytotoxicity but marked respiratory inhibition in rat liver mitochondria. These compounds can inhibit the respiration and cell growth only when they are spread on the mitochondria. These results suggested that animal cells may have some barriers to prevent UK-2A and UK-3A from diffusing to the mitochondria or some system to inactivate them in the cytoplasm, which do not exist in yeast and fungal cells.

UK-2A and UK-3A have very interesting bioactive properties. Further investigations in comparison with antimycin A are needed to elucidate which portions of their structure are related to the small cytotoxic potency, and what factors protect animal cells from the mitochondrial respiratory inhibition by them. Studies on the detailed action of UK-2A and UK-3A are currently in progress.

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