Biochemical Pharmacology, Vol. 44, No. 11, pp. 2223–2228, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

DETECTION OF NEW METABOLITES OF TRIFLURIDINE (F_3TdR) USING ^{19}F NMR SPECTROSCOPY

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(*Received* 29 *April* 1992; *accepted* 6 *August* 1992)

Abstract—The metabolism of 5-trifluoromethyl-2′-deoxyuridine (trifluridine, F_3TdR) in male BALB/C mice has been studied by ^{19}F NMR spectroscopy. Contrary to previous reports, a number of fluorinated metabolites were observed in urine, whole livers and blood samples taken from mice after i.p. injection of F_3TdR. The present study describes the identification of two new metabolites in mouse urine using the ^{19}F NMR technique. The NMR of crude urine showed the presence of F_3TdR, 5-trifluorothymine (F_3T), the newly-identified metabolites, 5-trifluoromethyl-5,6-dihydrouracil (DHF_3T) and 5-trifluoromethyl-5,6-dihydroxyuracil ($DOHF_3T$), and several new, as yet unidentified fluorinated metabolites. These two new metabolites were characterized by comparison to authentic compounds prepared synthetically from F_3T.

5-Trifluoromethyl-2′-deoxyuridine (trifluridine, F_3TdR†; see compound **1** in Scheme 1) a known antiviral agent, is also incorporated into the DNA of human bone marrow cells grown in culture and has been shown to enhance radiosensitivity [1, 2]. The corresponding nucleotide, 5-trifluoromethyl-2′-deoxyuridine-5′-phosphate (F_3TdRP), inhibits the enzyme thymidylate synthetase. F_3TdR is reported to be active against *Herpes simplex keratitis* in rabbit cornea [3], and it has greater anti-tumor activity than 5-fluoro-2′-deoxyuridine (FUdR) against adenocarcinoma 755 in BDF_1 mice [4]. Earlier studies in tumor-bearing mice [5] and in human cancer patients [6] suggested that the metabolism and catabolism of F_3TdR were very simple; these studies of urinary excretion, tissue distribution and metabolism of ^{14}C-labeled F_3TdR showed that trifluridine was cleaved at the nucleosidic bond to form the free pyrimidine base, 5-trifluorothymine (F_3T; **2**), and that further catabolism to 5-carboxyuracil (**3**) occurred, with the concomitant release of fluoride. Some unmetabolized F_3TdR was also detected. Recently, Rand *et al.* [7] used ^{19}F NMR spectroscopy to observe the incorporation of F_3TdR in *Herpes simplex* virus (HSV) infected mice, but they did not attempt to differentiate between injected **1**, **2** and F_3TdR phosphates, nor did they report the presence of other metabolites that might have been present at lower concentrations. The defluorinated compound **3** and 5-carboxy-2′-deoxyuridine **4**, both of which are catabolites reported by Heidelberger and Anderson [4], are believed to be formed via alkaline hydrolysis of **2** and **1**, respectively, rather than via enzymatic metabolism [8]. With the recent advances in the technology of NMR, it is now possible to study the biodistribution and metabolism of F_3TdR in greater detail using ^{19}F NMR techniques.

We now report the presence of newly identified metabolites of F_3TdR which were detected in murine urine using a Bruker AM-300 and CXP-100 spectrometers. It was observed that F_3TdR undergoes extensive metabolism, since a number of fluorinated metabolites were detected in whole liver, blood and urine samples. Two of the metabolites detected in urine were synthesized to confirm their chemical identity.

MATERIALS AND METHODS

Chemicals. F_3TdR (**1**), F_3T (**2**) and sodium trifluoroacetate were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Chromium acetate was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.).

Chromatography. The samples were initially purified by preparative thin-layer chromatography (P-TLC) on silica gel 60F-254 plates (20 × 20 cm, 0.25 mm layer thickness; Merck, U.S.A.) using a multiple development technique with chloroform:methanol (93:7, v/v) as the developing solvent. Impure fractions were further purified on a Whatman reverse phase preparative high pressure liquid chromatography (P-HPLC) column (Partisil 10 ODS-3) using water:methanol (80:20, v/v) as the eluent.

NMR spectrometry. Experiments were carried out on Bruker AM-300 (7.05 T, 282.38 MHz) and CXP-100 (2.35 T, 94.26 MHz, 40 cm bore) spectrometers. A three turn, 20 mm diameter, horizontal coil was used with the CXP-100 spectrometer. The magnet was shimmed on water in the case of the Bruker AM-300 spectrometer, while the CXP-100 spectrometer magnet was shimmed on fluorine. ^{19}F NMR data were acquired over a spectral width of

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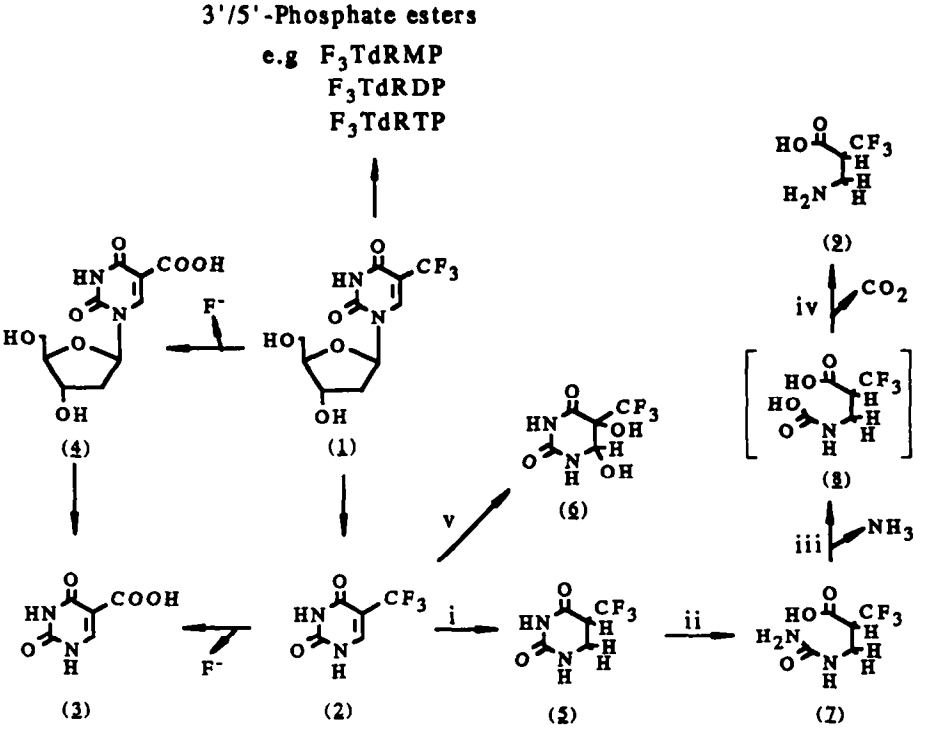
† Abbreviations: F_3TdR, 5-trifluoromethyl-2′-deoxyuridine; F_3T, 5-trifluorothymine; DHF_3T, 5-trifluoromethyl-5,6-dihydrouracil; $DOHF_3T$, 5-trifluoromethyl-5,6-dihydroxyuracil; F_3TdRP, 5-trifluoromethyl-2′-deoxyuridine-5′-phosphate; FUdR, 5-fluoro-2′-deoxyuridine; F_3TBA, α-trifluoromethyl-β-alanine; and F_3TUPA, trifluoromethyluriedopropionic acid.



i = Pyrimidine reductase, ii = Pyrimidinase, iii = Pyrimidine deaminase, iv = β−Ureidopropionase, v = Multiple function oxidase

Scheme 1.

70.0 ppm using 90° and 180° pulses, together with a relaxation delay of 1 sec. The time of acquisition varied with the amount of metabolite present in the sample; up to 41 k scans were used for crude extracts. Exponential line broadening (0–20 Hz) was imposed on the data prior to Fourier transformation. The samples were dissolved in deuterium oxide. An aqueous solution of sodium trifluoroacetate (0.2 M; 5 μL) was used as an internal standard, and an aqueous solution of chromium acetate (0.1 M; 90 μL) was added to enhance the signal/noise (S/N) ratio. The resolution limit was observed between 2.5 to 20 Hz. The proton magnetic resonance (PMR) spectra (300 and 100 MHz for the two spectrometers, respectively) were taken in deuterium oxide solution over a spectral width of 10.0 ppm. Tetramethyl silane was taken as an internal standard and the chemical shifts for protons and carbons are reported in δ ppm.

Animal studies. The animals were dosed daily, to provide sufficient metabolites for chemical identification. F_3TdR (250 mg/kg in water for injection; 0.2 mL) was administered intraperitonially (i.p.) to five male BALB/C mice (22–25 g), without visible side effects. Urine was collected from animals housed individually in 400 mL beakers lined with Whatman No. 1 filter paper; the animals received normal food and water *ad lib.* The filter papers were collected at 3 and 24 hr after every daily injection. On day 5, blood samples were collected by cardiac puncture 30 min after injection of F_3TdR from mice euthanized with carbon dioxide. Livers were dissected out upon necropsy, and all blood and liver samples were frozen at −15° for storage until further use.

Urine was extracted from the filter papers with 60% (v/v) methanol in water; the extracts were lyophilized and the dried residues were dissolved in 20% (v/v) dichloromethane in methanol. These solutions were pooled and filtered, and the filtrate was again freeze dried. A ^{19}F NMR spectrum of this crude metabolite residue, dissolved in D_2O, was acquired to confirm the presence of ^{19}F-containing species. Ten ^{19}F resonances, listed in Table 1, were detected. Purification of this crude urine extract was performed by P-TLC. Chromatographic fractions visualized under UV light, other than those present in the control urine (urine of mice not injected with F_3TdR), were separated. These fractions were

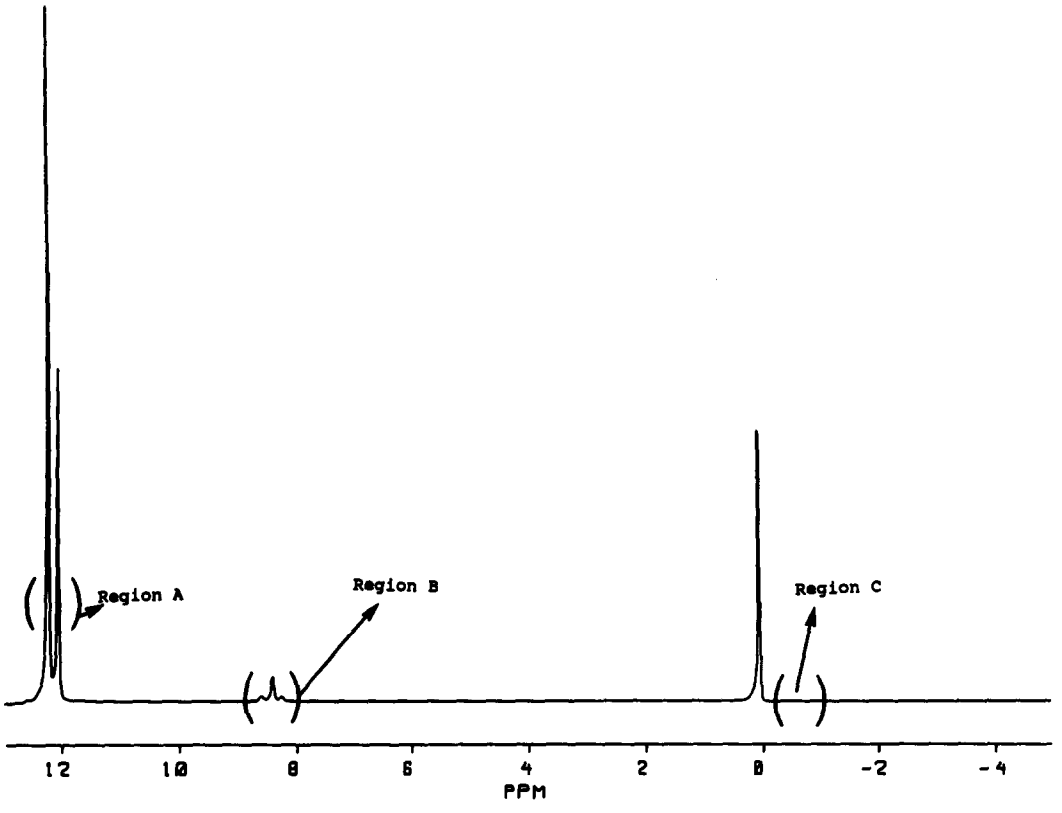


Fig. 1. ^{19}F NMR spectrum of crude urine from mice dosed with F_3TdR.

further subjected to P-HPLC purification, and their ^{19}F chemical shifts were compared with the spectra of authentic samples that had been purchased or synthesized. ^{19}F NMR spectra of crude urine were also recorded before and after the addition of these fully characterized chemicals for comparison and further confirmation of structure assignments of metabolites present in the extract.

Synthesis of 5-trifluoromethyl-5,6-dihydrouracil (DHF$_3$T; 5). Rhodium/alumina catalyst (212 mg) was added to a solution of **2** (100 mg; 0.56 mM) in triple-distilled water (25 mL) and stirred under positive pressure (12 psi) of hydrogen for 24 hr. The catalyst was filtered and the solvent exaporated *in vacuo*. The crude solid was subjected to column chromatography (silica gel particle size 60–200 mesh) and purified using methanol in chloroform (6%, v/v) as eluent to give 45 mg of pure **2**; m.p. 203–205° (dec.) [9]; ^1H NMR (D_2O) δ 3.57 (m, 2H, H-6) and 3.7 (m, 1H, H-5); ^{19}F NMR (D_2O) δ 8.18 (d, $J_{H,F}$ = 8.6 Hz, 3F, CF_3); ^{13}C NMR (D_2O) δ 37.0 (C-6), 45.5 (q, $J_{F,C}$ = 27 Hz, C-5), 125.5 (q, $J_{F,C}$ = 280 Hz, CF_3), 155.3 (C-2) and 166.0 (C-4).

Synthesis of 5-trifluoromethyl-5,6-dihydroxyuracil (DOHF$_3$T; 6). Bromine (155 μL) was added to a solution of **2** (100 mg; 0.55 mM) in triple-distilled water (4.5 mL) and stirred at 25° for 45 min. At this time the TLC showed complete conversion of starting material to the corresponding bromohydrin [10]. Excess bromine was removed by repeated washing of this solution with chloroform (three times 5 mL) and the aqueous solution was purged with nitrogen gas to remove any remaining traces of bromine and chloroform. Freshly prepared silver oxide (1.65 mM) was added to this aqueous solution and the heterogenous solution was stirred overnight (16–18 hr) at 25°. The solid was removed by centrifugation and the supernatant was evaporated under reduced pressure to yield 111 mg of crude product. This was purified on a silica gel column using 6% (v/v) methanol in chloroform as eluent to give 91 mg of pure **6**; m.p. 177–180° (dec.); ^1H NMR (D_2O) δ 4.92 (s, H-6); ^{19}F NMR (D_2O) δ -0.59 (s, CF_3); ^{13}C NMR (D_2O) δ 74.5 (C-6), 75.4 (q, $J_{F,C}$ = 27 Hz, C-5), 124.3 (q, $J_{F,C}$ = 287 Hz, CF_3), 154.5 (C-2) and 167.5 (C-4); chemical ionization for $C_5H_5F_3N_2O_4$ (as $M^+ + NH_3$) relative abundance 21.2%.

RESULTS

A ^{19}F NMR spectrum of a crude urine extract was acquired with 41,786 scans (Fig. 1). This spectrum showed the presence of ten ^{19}F resonances, suggesting the presence of several fluorinated metabolites, as well as the starting material (F_3TdR) itself. This large number of F-containing metabolites is contrary

Table 1. ^{19}F NMR chemical shifts (δ ppm) of crude urine after i.p. injection of F_3TdR into mice

Crude urine ^{19}F chemical shifts (δ ppm)	Authentic compound	Chemical shift (δ ppm) of authentic compound
12.20 (s)	F_3TdR (**1**)	12.19 (s)
12.16 (s)		
12.02 (s)	F_3T (**2**)	12.02 (s)
11.99 (s)		
8.54 (d) ($J_{H,F}$ = 8.5 Hz)		
8.35 (d) ($J_{H,F}$ = 9.1 Hz)		
8.19 (d) ($J_{H,F}$ = 8.4 Hz)	DHF_3T (**5***†)	8.18 (d) ($J_{H,F}$ = 8.4 Hz)
−0.53 (s)	$DOHF_3T$ (**6***†)	0.59 (s)
−0.64 (s)		
−45.20 (s)	F^-	−47.28

Chemical shifts of authentic compounds are also reported.
* s = singlet; d = doublet.
*† New metabolite.

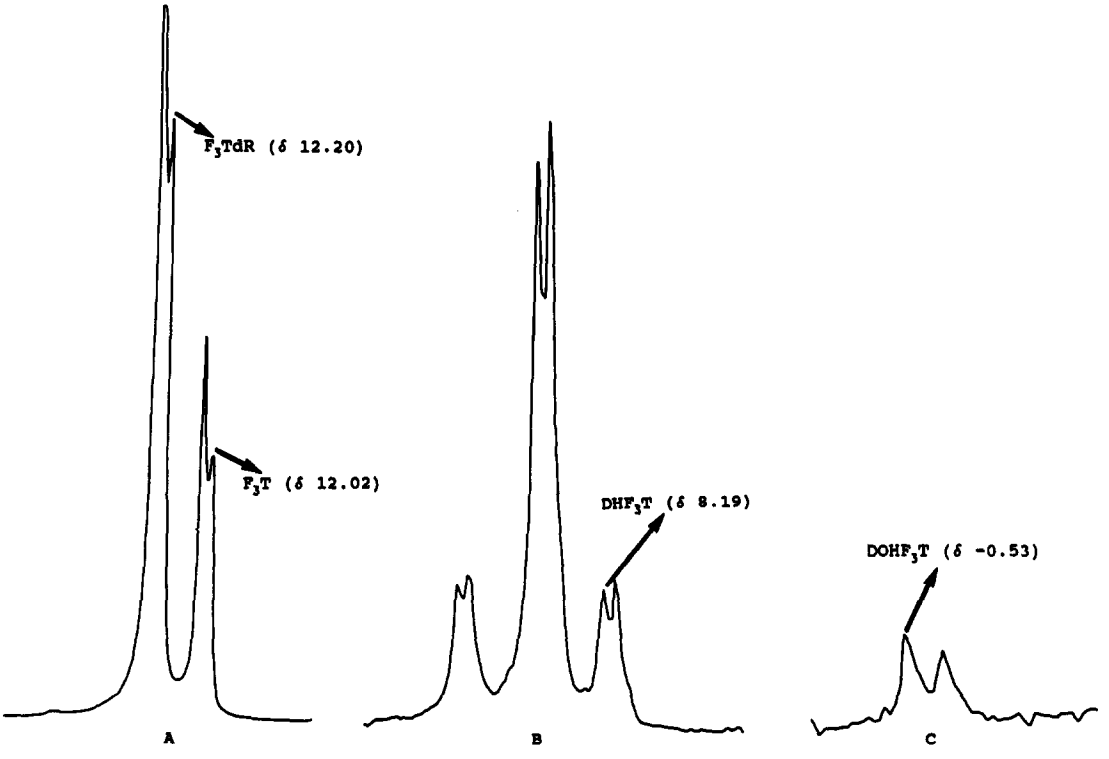


Fig. 2. Expansion of regions A, B and C as shown in Fig. 1.

to studies by Heidelberger and coworkers [5, 6], which reported the presence of only F_3T and F^-, together with some unchanged F_3TdR. Of the signals which were detected in the present study (Table 1), compounds responsible for the ^{19}F chemical shifts (δ ppm) at 12.20 (s), 12.02 (s), 8.19 (d, $J_{H,F}$ = 8.4 Hz), −0.53 (s) and −45.20 (s) have been identified.

The metabolism of trifluridine can theoretically lead to the formation of compounds **5** and **6**. Therefore, these compounds were synthesized to confirm their presence in the crude urine. The reduction of the vinylic bond in 5-trifluoromethyl uracil yielded **5** which moved the signals for H-5 and H-6 protons upfield. The proton at C-5 appeared at δ 3.7 as a multiplet ($J_{F,C}$ = 27 Hz) due to coupling

with fluorines at the CF_3 moiety. The signal for two protons at C-6 also shifted upfield. Compound **6** was synthesized by treating **2** with Br_2/H_2O, followed by its reaction with silver oxide. The proton at C-6 for this compound was observed as a singlet at δ 4.92.

The ^{19}F NMR resonance at −0.53 ppm in a spectrum of the crude urine was intensified by the addition of Compound **6** to the sample. Similarly, the doublet at 8.19 ppm intensified upon addition of Compound **5** to the crude sample, without further influence on other ^{19}F resonances (Fig. 2). Besides these signals, F_3T (δ 12.02), F^- (δ −45.20) and unchanged F_3TdR (δ 12.20) were also detected in the crude urine. Compounds exhibiting fluorine signals at other chemical shifts are still being characterized and will be reported later. Table 1 also lists the ^{19}F NMR chemical shifts of the authentic samples which were either purchased or synthesized to confirm the chemical structures of the fluorine-containing metabolites. The unidentified metabolites, also found to be present in the NMR spectra of whole livers*, are being investigated further.

DISCUSSION

A comparison of the data in Table 1 shows that the signal at 12.20 ppm in urine can be attributed to F_3TdR. This was further confirmed by isolating F_3TdR from crude urine using P-TLC; the retention time of this chemical isolated from the biological sample was the same as that of an authentic sample of F_3TdR (33.5 min on a C-18 reverse phase column eluted with methanol:water (15:85) at a flow rate of 1.5 mL/min). The ^{19}F NMR signal at 12.02 ppm was identified as F_3T. F_3T was recovered from urine using the same technique as described for F_3TdR, and its retention time on HPLC under similar conditions was 19.2 min, matching the retention time of an authentic sample.

A small signal at −0.53 ppm was tentatively identified as Compound **6**, because its ^{19}F chemical shift was identical to that of an authentic sample of the synthesized material. Moreover, the ^{19}F NMR spectrum of a mixture of synthetic **5** and the crude urine resulted in intensification of the signal at −0.53 ppm. Most possibly an oxidative enzyme, multiple function oxidase, catalyzes the oxidation of the vinylic bond between C-5 and C-6 and results into the metabolic formation of **6** [11]. Small changes in the ^{19}F chemical shifts between authentic sample and extracts in this mixture were attributed to the difference in pH of the solutions of synthetic metabolites and biological sample. The pH values for DHF_3T, $DOHF_3T$ and crude urine samples in deuterated water were found to be 4.41, 6.71 and 5.89, respectively.

The presence of three new doublets at 8.54 ($J_{H,F}$ = 8.5 Hz), 8.35 ($J_{H,F}$ = 9.1 Hz) and 8.19 ($J_{H,F}$ = 8.4 Hz) ppm indicates the reduction of the 5,6 double bond of the pyrimidine moiety. This reduction process, which is possible *in vivo*, introduces one proton each at the C-5 and C-6 positions. The coupling of H-5 with fluorines of CF_3 results in a doublet. Since three doublets of different magnitude were observed in the spectrum of urine, it indicates the presence of three different compounds with a 5,6-dihydro linkage. The signal at 8.19 is attributable to DHF_3T (**5**), and this signal was intensified when an NMR spectrum of a mixture of crude urine and the synthetic DHF_3T was acquired. Other doublets at 8.54 and 8.35 are thought to belong to the products formed upon ring cleavage of the N-3/C-4 amido bond of 5,6-DHF_3T to form trifluoromethyluriedopropionic acid (F_3TUPA; **7**), subsequent deamination to form **8** and/or decarboxylation to form α-trifluoromethyl-β-alanine (F_3TBA; **9**) (Scheme 1). This metabolic step is directed by β-uriedopropionase enzyme [11]. The degradation steps to DHF_3T (**5**) and F_3TUPA (**7**) are mediated by the enzymes dihydropyrimidine dihydrogenase and dihydropyrimidinase, respectively. An analogous catabolic pathway has already been reported for 5-fluoro-2′-deoxyuridine and other pyrimidine bases/nucleosides [12]. The separation and characterization of other metabolites are underway.

Acknowledgements—This work was supported by the Medical Research Council of Canada, Grant MA 9684. The authors also acknowledge the Alberta Cancer Board for its financial support, and the Alberta Heritage Foundation for Medical Research for the use of the NMR facility.

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