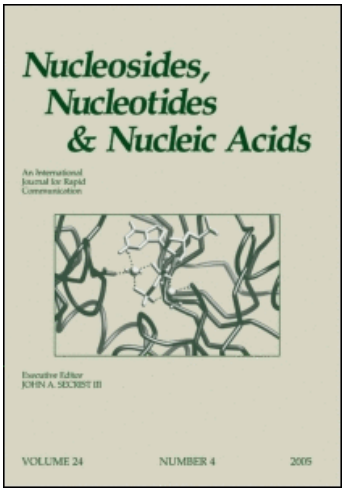

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α-Trifluoromethyl-β-Ureido-Propionic Acid (F_3MUPA): A New Metabolite of Trifluridine (F_3TdR)

Manju Tandon[a]; Piyush Kumar[a]; Leonard I. Wiebe[a]
[a] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada

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α-TRIFLUOROMETHYL-ß-UREIDO-PROPIONIC ACID (F_3MUPA): A NEW METABOLITE OF TRIFLURIDINE (F_3TdR)

Keywords: 5-Trifluro-2'-deoxythymidine, F_3TdR, *in vivo* metabolism, F_3MUPA, α-trifluoromethyl-ß-ureido-propionic acid, ^{19}F NMR spectroscopy

Manju Tandon, Piyush Kumar and Leonard I. Wiebe *

Faculty of Pharmacy and Pharmaceutical Sciences,
University of Alberta, Edmonton, Alberta, Canada T6G 2N8

ABSTRACT

The metabolism of 5-trifluro-2'-deoxythymidine (trifluridine; F_3TdR) in male BALB/c mice bearing EMT-6 tumors has been investigated using ^{19}F NMR spectroscopy. We previously (Tandon *et al*, 1992) reported the detection and identification of 5,6-dihydro-5-trifluorothymine (DHF_3T, 3) and 5,6-dihydroxy-5-trifluorothymine ($DOHF_3T$) as new metabolites of F_3TdR in mice urine. Further exploration of the metabolism of trifluridine has led to the identification of α-trifluoromethyl-ß-ureido-propionic acid (F_3MUPA, 4) as a previously unreported metabolite in the urine of F_3TdR treated mice. Authentic F_3MUPA was obtained by synthesis via an established route. A comparison of chemical shift and the H-F coupling constant of an authentic sample, with the ^{19}F signal from urine, indicated the presence of F_3MUPA in murine urine. Mixing crude urine with authentic F_3MUPA resulted in the enhancement of the corresponding fluorine signal without affecting or introducing others, thereby confirming the presence of F_3MUPA as a urinary metabolite.

INTRODUCTION

5-Trifluoro-2'-deoxythymidine (trifluridine; F_3TdR, 1), a pyrimidine nucleoside, is incorporated into DNA of replicating mammalian cells. It is used clinically for its antiviral properties, especially in the treatment of ophthalmic herpetic infections (Carmine *et al*, 1982). Its antiviral action is due in part to inhibition of thymidylate synthetase (Reyer *et al*, 1964). Earlier studies with trifluridine in tumor bearing mice (Heidelberger *et al*, 1965) and in human cancer patients (Dexter *et al*, 1972) indicated that trifluridine is degraded to 5-carboxy-uracil, fluoride, and 5-trifluorothymine (F_3T, 2). The degradation

to 5-carboxy-uracil and fluoride has been attributed to its instability in alkaline conditions (Neslter and Garret, 1968) rather than to enzymatic defluorination.

Recently new fluorine-containing metabolites of F_3TdR in murine urine have been reported (Tandon *et al*, 1992). With ^{19}F NMR spectroscopy, it was possible to detect 5,6-dihydro-5-triflurothymine (DHF_3T, 3), 5,6-dihydroxy-5-trifluorothymine ($DOHF_3T$) and other as yet unidentified fluorinated metabolites, confirming that contrary to earlier reports by Heidelberger *et al* (1972), F_3TdR undergoes catabolism in a manner similar to that of other 5-substituted pyrimidine nucleosides and pyrimidine bases like thymidine, thymine and 5-fluorouracil (5-FU).

We now report the presence of α-trifluoromethyl-ß-ureidopropionic (F_3MUPA, 4), a new metabolite of F_3TdR in the urine of F_3TdR-treated mice. F_3MUPA was identified in crude urine by ^{19}F NMR spectroscopy. α-Trifluoromethyl-ß-alanine 2(F_3MBA; 8), a possible metabolite of F_3TdR has been synthesized, but its presence in murine urine could not be detected in these experiments.

MATERIALS AND METHODS

Chemicals: F_3TdR and sodium trifluoracetate were purchased from Sigma Chemical Co. (St. Louis, USA). Chromium acetate was obtained from Aldrich Chemical Co. (Milwaukee, USA), and 3,3,3-trifluoropropene was purchased from PCR Inc. (Gainesville, USA).

NMR Spectroscopy: The ^{19}F spectroscopic experiments were carried out on a Bruker AM-300 (7.05 T, 282.38 MHz) and Bruker CXP-100 (2.35 T, 94.26 MHz, 40 cm bore) spectrometers. The CXP-100 spectrometer magnet was shimmed on fluorine, while the AM-300 spectrometer magnet was shimmed on hydrogen. A 20 mm diameter, three

turn horizontal coil was used with CXP-100 spectrometer, for ^{19}F spectroscopy of crude urine and intact and homogenized tissue samples. ^{19}F NMR data were acquired over a spectral width of 70.0 ppm (between +20.0 and -50.0 ppm) using 90° and 180° pulses. A relaxation delay of 1 sec. was employed while acquiring the spectra. Aqueous solution of chromium acetate (90 μL, 0.1M) was used as a relaxation agent to enhance the S/N ratio. The time of acquisition depended on the concentration of metabolite present in the sample; up to 42 k scans were acquired for crude extracts.

The hydrogen magnetic resonance (^1H NMR) spectra were acquired in deuterium oxide solution, over a spectral width of 10.0 ppm and the spectra were locked on deuterium. The signal for D_2O appeared at δ 4.68 ppm with respect to tetramethylsilane and was used as internal standard to assign chemical shift of the desired signal. The resolution limit for ^1H spectra was between 0.5-1.2 Hz.

^{19}F spectra were recorded with respect to sodium trifluoroacetate (5μL, 0.2M). The samples (0.5 mL volume) were prepared in deuterium oxide. The resolution limit for ^{19}F spectra was between 25-20 Hz.

^{13}C NMR resonances were assigned using J modulation spin echo technique (Jmod), where methylene and quarternary resonances appear below the baseline and methine peaks are plotted above the baseline to determine the number of attached hdyrogen. In the case of ^{13}C spectra, the observed resolution limit was 2-3 Hz.

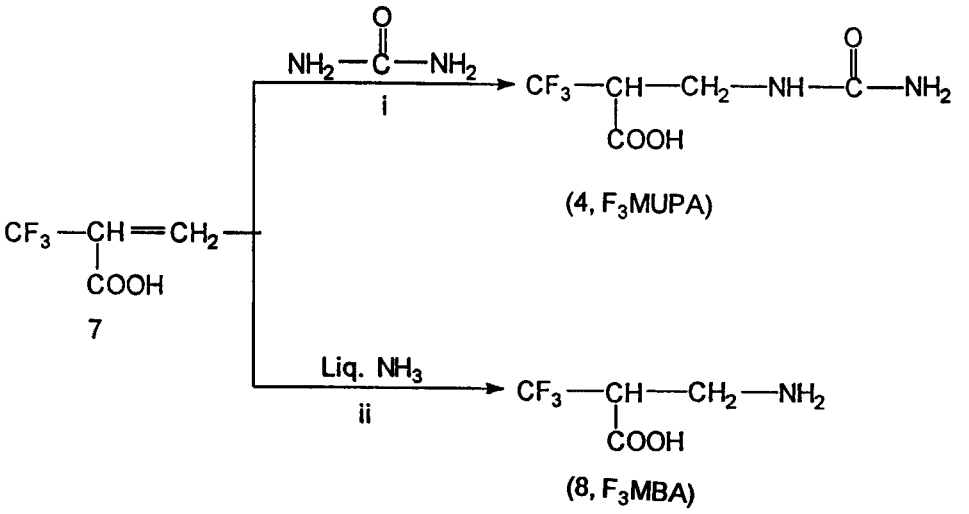
Animal Studies: Five male BALB/c mice (20-25 g) were housed individually in 400 mL beakers lined with Whatman no. 1 filter paper. The animals received normal food and water ad lib during the course of the experiment. Trifluridine (250 mg/kg in water for

injection) was administered by intraperitoneal (i.p.) injection. The animals were given single injections daily for five days in order to build up sufficient metabolite. No visible toxic effects were observed during the experiment. Urine, absorbed on filter paper, was collected at 3 h and 24 h after each daily injection.

Urine was extracted from the filter paper as described by Tandon *et al* (1992). ^{19}F NMR spectra of crude urine samples were acquired both before and after the addition of the authentic samples of suspected metabolites, to confirm the presence of these metabolites in urine.

Chemistry: F_3MUPA and F_3MBA were synthesized as described in Scheme 1. Since the literature (Fuchikami *et al*, 1982) does not provide a detailed characterization of F_3MUPA, complete spectroscopic characterizations for this known compound and for α-trifluoromethyl-ß-alanine (F_3MBA), a novel compound, are now reported.

An ^{19}F NMR spectrum of F_3MUPA showed the presence of a doublet ($J_{H,F}$=9.1 Hz at δ 7.91 ppm) caused by the coupling of an α-proton with fluorine atoms. The spectrum also displayed coupling of fluorine atoms with carbon, which is characteristic and which depends on 1) the extent of π bond formation between carbon and fluorine, 2) the ionic character of C-F bond, and 3) the "*s*" character of the carbon orbital in the C-F bond (Sothers, 1972). A typical ^{13}C NMR spectrum of F_3MUPA in deuterium oxide displayed two quartets, one at δ 52.4 ppm ($J_{P,C}$=24.5 Hz) and the other at δ 125.5 ppm ($J_{P,C}$=279.2 Hz) for C-2 and CF_3 carbons, respectively. These coupling constants are within the range for the type of carbons described (Vander Kelen and Eeckhaut, 1963). The appearance of the signals at δ 37.77 ppm (methylenic carbon) and δ 161.81 ppm (amidic carbon) in ^{13}C spectrum of F_3MUPA confirmed its formation.



Where, i=Dimethyl formamide/ 80°C/ 4 hr.
ii= 25°C/ 8 hr.

Scheme 1

F_3MBA was synthesized by reacting 2-trifluoromethyl acrylic acid and ammonia at 25°C. The proton and fluorine NMR spectra for this compound exhibit dual resonances for protons and for fluorine atoms due to the presence of rotamers. The re-acquisition of these spectra at an elevated temperature (75°C) resulted in coalescence of these resonances, so that only one resonance was observed for the hydrogen and for the fluorine atoms in the elevated temperature spectrum. An ^{19}F NMR spectrum of F_3MBA at 25°C exhibited two doublets (due to rotamers) at δ 8.59 ppm ($J_{H\text{-}F}$=9.4 Hz). The ^{13}C carbon spectrum corresponded to the assigned structure.

SYNTHESIS

α-Trifluoromethyl-ß-ureidopropionic acid (F_3MUPA, 4). A mixture of 2-trifluoromethyl acrylic acid (200 mg; 1.4 mmol) and urea (85 mg; 1.4 mmol) was dissolved in dimethyl formamide (3mL) and heated on an oil bath at 80°C with continuous stirring (Fuchikami

and Ojima, 1982). The solvent was removed under reduced pressure after the reaction was complete. The crude solid residue was subjected to silica gel column chromatography (particle size 60-200 mesh) and eluted with methanol/chloroform (25/75, v/v) to obtain pure 4; yield, 177 mg (62%); mp, 150° (Fuchikami *et al*, 1984); ^{1}H NMR (D_2O) δ 3.50 (d, J_{gem}=14 Hz of d, $J_{2,1}$=6.0 Hz, 1H of $C\underline{H}_2$), 3.42 (d, J_{gem}=14 Hz of d, $J_{2,1}$=6.0 Hz, 1H of $C\underline{H}_2$) and 3.35 (m, 1H, H-1); ^{13}C NMR (D_2O) δ 172.78 ($\underline{C}OOH$), 161.82 ($\underline{C}ONH$), 125.64 (q, $J_{F,C}$=279.2 Hz, $\underline{C}F_3$), 52.45 (q, $J_{F,C}$=24.5 Hz, $\underline{C}H$) and 37.77 ($\underline{C}H_2$); ^{19}F NMR (D_2O) δ 8.36 (d, $J_{H,F}$=9.1 Hz) ppm; chemical ionization for $C_5H_7F_3N_2O_3$ (200.126) M^{+1} (100%) and as NH_4^+ (87.1%).

α-Trifluoromethyl-ß-alanine (F_3MBA, 8). A mixture of 2-trifluoromethyl acrylic acid (70 mg, 0.49 mmol) and liquid ammonia (20 mL; liquified at -78°C) was stirred at room temperature. A cold trap was used to stop the escape of ammonia gas. The reaction was complete in 8 h. A stream of dry nitrogen was used to remove excess NH_3 and the solid, so obtained, was pure 8; yield, 69 mg (88%); mp, 144-8 (dec.). ^{1}H NMR (D_2O) δ 3.15-3.40 (m, 3H, $C\underline{H}_2$ and $C\underline{H}$); ^{13}C NMR (D_2O) δ 170.7 ($\underline{C}OOH$), 125.20 (q, $J_{F,C}$=279.6 Hz, $\underline{C}F_3$), 48.40 (q, $J_{F,C}$=24.7 Hz, C-2), 45.50 (C-3); ^{19}F NMR (D_2O) δ 8.59 (d, $J_{H,F}$=9.4 Hz) shows dual resonance due to the presence of rotamers; chemical ionization for $C_4H_6F_3NO_2$ (157.088) M^{+1} (100%) and M^+ and as NH_4^+ (14.7%).

RESULTS

The ^{19}F NMR spectrum of crude urine extract revealed the presence of several fluorinated metabolites, including unchanged F_3TdR itself (Tandon *et al*, 1992). The ^{19}F NMR spectrum of an authentic sample of F_3MUPA exhibits a doublet at δ 8.36 ppm ($J_{H,F}$=9.1 Hz). This compound, when mixed with crude urine, intensified the doublet at δ 8.36 ppm in the crude urine spectrum with no change in the J value (Fig. 1) and without

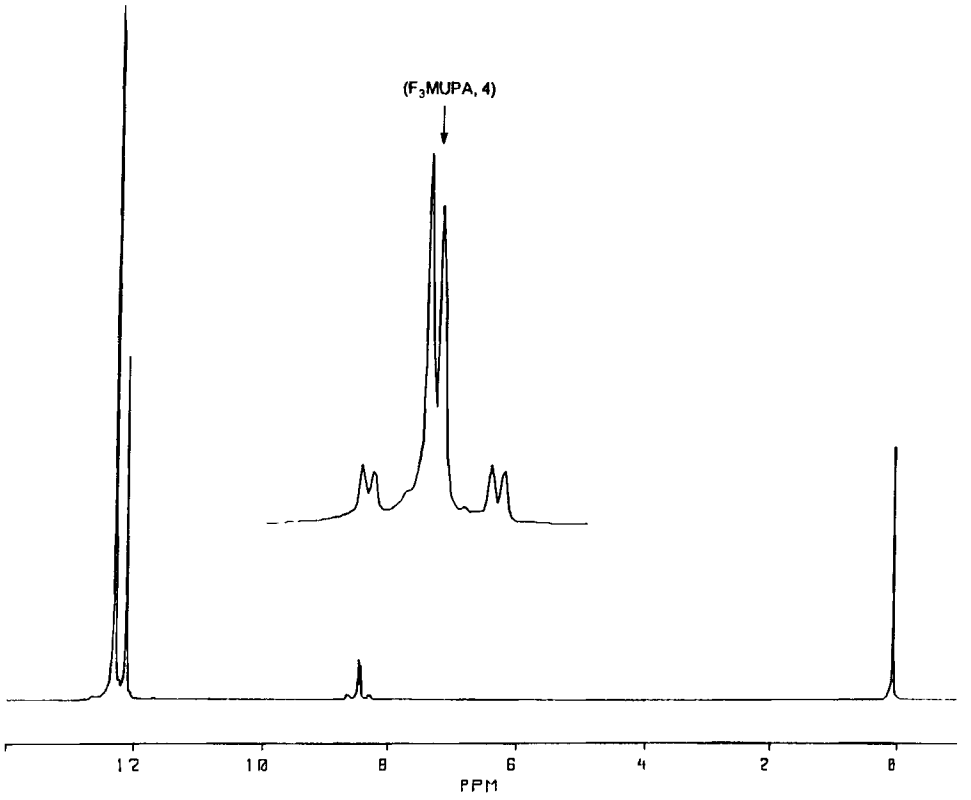


Fig. 1 ^{19}F NMR spectrum of mice urine mixed with F_3MUPA

effect on any other fluorine signal in the NMR spectrum of the crude urine sample. On the other hand, the addition of F_3MBA (δ 8.59) to crude urine sample did not intensify any pre-existing fluorine signal; instead, a new doublet (for this compound) appeared in the NMR specturm (Fig. 2).

Synthetic F_3MBA was found to degrade even under refrigeration, to generate an additional singlet at δ-54.00 ppm in its ^{19}F NMR spectrum. This degradation product was not seen in urine.

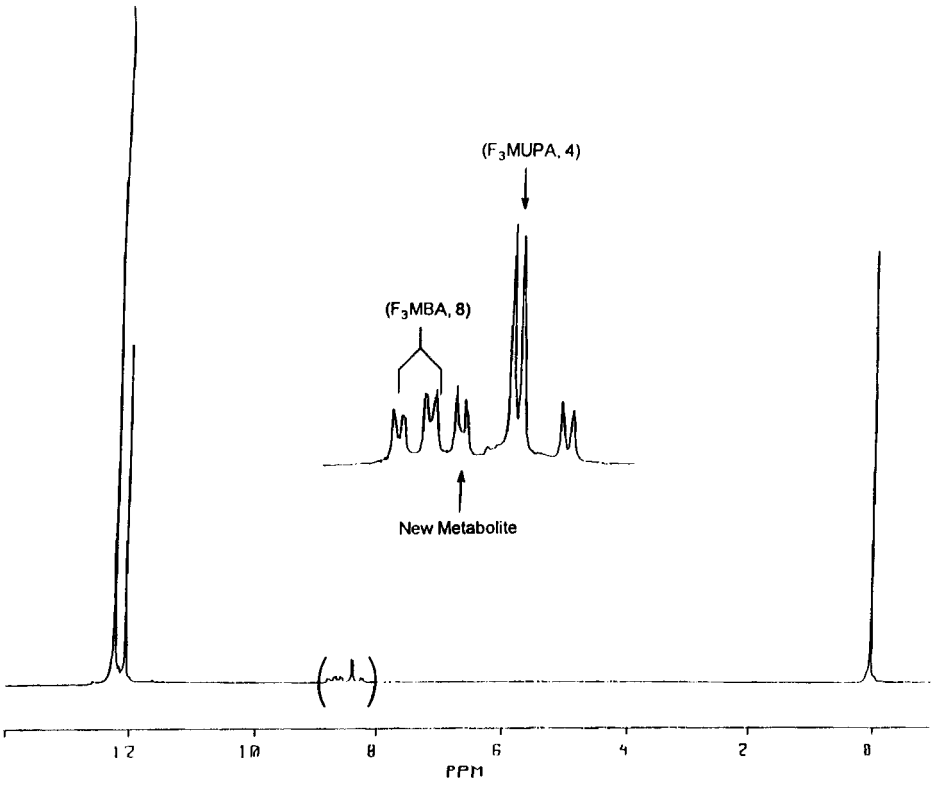


Fig. 2 ^{19}F NMR spectrum of mice urine mixed with F_3MBA

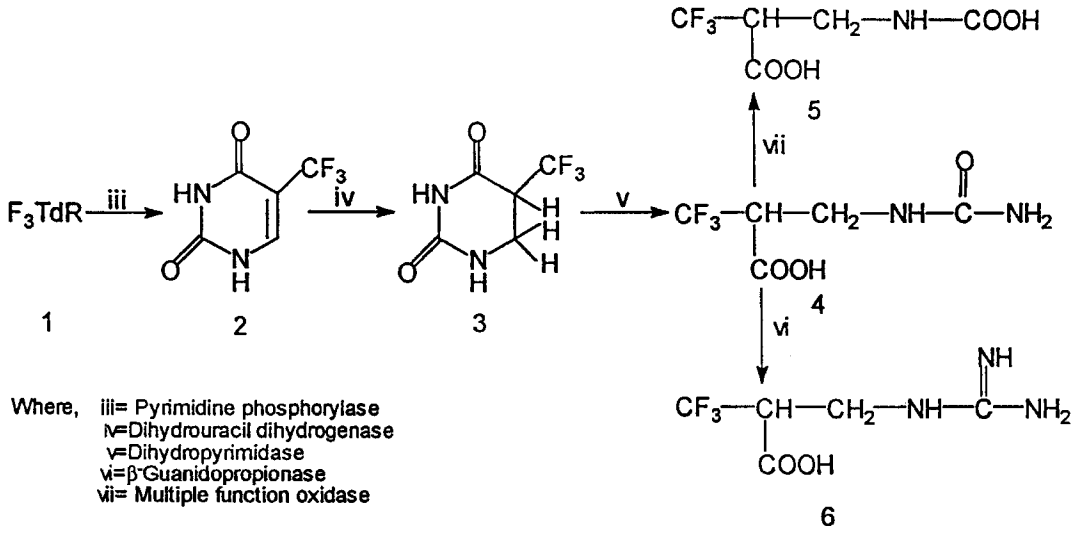
DISCUSSION

The presence of DHF_3T, $DOHF_3T$, F_3T and F_3TdR in murine urine after dosing with F_3TdR has been reported (Tandon *et al*, 1992) (Table 1). Through comparisons with authentic F_3MUPA, the fluorine resonance at δ 8.36 ppm in crude urine has now been identified as F_3MUPA (Fig. 1). This metabolite is most probably derived from DHF_3T, which is formed *in vivo* by reduction of the 5,6-vinylic bond of F_3T. We propose that DHF_3T is unstable, and is cleaved by dihydropyrimidase (Fink *et al*, 1953; Canellakis, 1956; Sommadossi *et al*, 1982 and Sijens *et al*, 1991) at the N-3/C-4 bond, to form F_3MUPA, (Scheme 2) in a manner analogous to that for most dihydropyrimidines.

Table 1. Summary of assigned ^{19}F NMR chemical shift resonances in murine urine after 5 daily doses (250 mg/kg., i.p.) of F_3TdR. Chemical shifts are given as δ ppm, relative to sodium trifluoracetate; s=singlet, d=doublet.

^{19}F Chemical Shift in Urine	Authentic Compound	^{19}F Chemical Shift in D_2O
12.20 (s)	F_3TdR (1)	12.19 (s)
12.02 (s)	F_3T (2)	12.02 (s)
8.35 (d)($J_{H,F}$=9.1 Hz)	F_3MUPA (4)	8.36 (d)($J_{H,F}$=9.1 Hz)*
8.19 (d)($J_{H,F}$=8.4 Hz)	DHF_3T (3)	8.18 (d)($J_{H,F}$=8.4 Hz)
-0.53 (s)	$DOHF_3T$	-0.59 (s)
-45.20 (s)	F^-	-47.28 (s)

* new metabolite



Scheme 2

F_3MUPA, when mixed with crude urine intensified the fluorine signal at δ 8.36 ppm (d) confirming its presence in the urine of F_3TdR-dosed mice.

F_3MBA is also a suspected metabolite of F_3TdR, just as α-fluoro-ß-alanine and ß-alanine are metabolites of 5-fluorouracil (Sommadossi *et al*, 1982) and deoxythymidine (Canellakis, 1956), respectively. Hence, authentic F_3MBA was synthesized and mixed with the crude urine. This resulted in the appearance of a new signal (δ 8.59 ppm), and with a different coupling constant ($J_{H,F}$=9.4 Hz for F_3MBA), compared with the unassigned resonance at δ 8.54 ppm ($J_{H,F}$=8.5 Hz) in murine urine (Fig. 2). It is clear that the doublet at δ 8.54 ppm in urine does not belong to F_3MBA. However, it is possible that F_3MUPA can be catabolized to α-trifluoromethyl-ß-guanidopropionic acid (F_3MGPA, 5) and/or α-trifluoromethyl-ß-carboxyamino propionic acid (F_3MCPA, 6). An analogous catabolic pathway for 5-fluoro-2'-deoxyuridine, which has already been reported in literature (Malet-Martino *et al*, 1984), supports this hypothesis.

The failure to detect F_3MBA in urine may be due to its chemical instability, even when stored at neutral pH and under refrigeration. Although this chemical degradation has not been studied in depth, it is unlikely to be responsible for the apparent lack of F_3MBA in urine, since the signal at -54.00 ppm that results upon chemical degradation of F_3MBA was not evident in the ^{19}F NMR spectrum of crude urine. It appears more likely that F_3MBA is either not found in vivo or that if formed, it does not undergo urinary excretion.

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