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α-TRIFLUOROMETHYL-β-ALANYL GLYCINE (F_3MBAG): A NOVEL MAMMALIAN METABOLITE OF TRIFLURIDINE (F_3TdR)

MANJU TANDON, PIYUSH KUMAR and LEONARD I. WIEBE*

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

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Abstract—^{19}F NMR spectroscopy has been used to study further the metabolism of 5-trifluoromethyl-2′-deoxyuridine (trifluridine; F_3TdR). The synthesis and characterization of α-trifluoromethyl-β-alanyl glycine (F_3MBAG), a putative new metabolite of F_3TdR, are now reported. This study describes *ex vivo* and *in vivo* detection of F_3MBAG and other previously reported metabolites of trifluridine, using ^{19}F NMR spectroscopy, in male BALB/C mice bearing EMT-6 tumors. A parallel ^{19}F NMR spectroscopic study was also performed on rats dosed with F_3TdR, to observe the qualitative pattern of F_3TdR metabolism in another species. Unexpectedly, 5-trifluoromethyl-5,6-dihydroxyuracil (DOHF_3T), α-trifluoromethyl-β-ureidopropionic acid (F_3MUPA) and fluoride, which result from the metabolic degradation of F_3TdR and which were detected in various biological samples from mice dosed with F_3TdR, could not be identified in rat urine or in homogenized tissue extracts. The presence of these metabolites in intact tissues is uncertain since in this study ^{19}F NMR spectroscopy of these samples always displayed a broad resonance "hump" across the range of chemical shifts that would encompass these metabolites. No clear explanation for the loss of spectroscopic resolution in this region has been rationalized. *N*-Carboxy-α-trifluoromethyl-β-alanine (F_3MBA-CO_2), α-trifluoromethyl-β-alanyl alanine (F_3MBAA) and *N*-acetyl-α-trifluoromethyl-β-alanine (Ac-F_3MBA) were synthesized and characterized, but were not detected as metabolites in any of the biological specimens examined.

Key words: 5-trifluoromethyl-2′-deoxyuridine; fluoronucleosides; α-trifluoromethyl-β-alanyl glycine; metabolism; ^{19}F NMR

Recent advances in the technology of NMR spectroscopy have made it possible to study the biodistribution and metabolism of xenobiotics *in vivo* and *in situ*, using technology that is non-invasive and that frequently provides comprehensive information. In previous communications [1, 2], we reported the detection and characterization of DHF_3T† (**3**), DOHF_3T (**4**), and F_3MUPA (**5**) as new metabolites of F_3TdR (**1**), using ^{19}F NMR spectroscopic techniques. These metabolites were identified in the urine of male BALB/C mice bearing EMT-6 tumors. Assignment of chemical structure to these metabolites involved the synthesis and characterization of several possible metabolites of trifluridine, mixing these synthetic "metabolites" with biological samples (e.g. urine, blood, liver, tumor and their methanolic extracts) and observing intensification of NMR signals at specific chemical shifts and with specific coupling constants. In this way, it was possible to assign structures to metabolites that eluded isolation and characterization by more traditional methods [1–3].

We now report the synthesis (Scheme 1) and characterization of F_3MBAG (**6**), and identify it as a new metabolite of F_3TdR. Other metabolites previously reported to be present in murine urine [1, 2] are now reported to be present in a number of body tissues in both mice and rats.

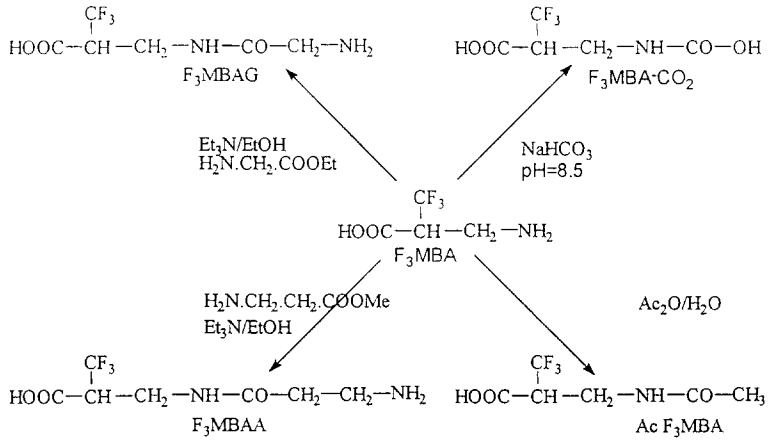
MATERIALS AND METHODS

Chemicals. F_3TdR (**1**) and F_3T (**2**) were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glycine ethyl ester hydrochloride was obtained from J. T. Baker Canada (Toronto, Canada) and chromium acetate was purchased from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All solvents were reagent grade. Possible metabolites were synthesized according to Schemes 1 and 2, as described in the following paragraphs.

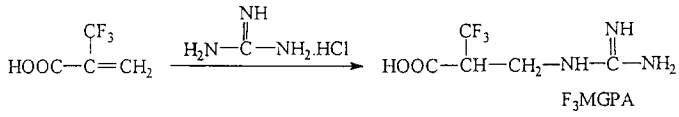
Chromatography. New synthetic products were purified on silica gel (Merck 774, mesh size 100–200 μm) columns, using several eluting solvents to provide an appropriate polarity range. The eluent was selected in consideration of the polarity of the mixture to be separated, as described for each reaction mixture. The products so obtained were

* Corresponding author. Tel. (403) 492-5905/2967; FAX (403) 492-8241.

† Abbreviations: DHF_3T, 5-trifluoromethyl-5,6-dihydrouracil; F_3TdR, trifluridine, 5-trifluoromethyl-2′-deoxyuridine; F_3T, 5-trifluoromethyl uracil; F_3MBAG, α-trifluoromethyl-β-alanyl glycine; DOHF_3T, 5-trifluoromethyl-5,6-dihydroxyuracil; F_3MUPA, α-trifluoromethyl-β-ureidopropionic acid; F_3MBA, α-trifluoromethyl-β-alanine; F_3MBAA, α-trifluoromethyl-β-alanyl alanine; F_3MBA-CO_2, *N*-carboxy-α-trifluoromethyl-β-alanine; Ac-F_3MBA, *N*-acetyl-α-trifluoromethyl-β-alanine; F_3MGPA, α-trifluoromethyl-β-guanidopropionic acid; *s*, singlet; *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet; and P-TLC, preparative thin-layer chromatography.



Scheme 1.



Scheme 2.

purified further by P-TLC and analyzed by TLC on silica gel 60F-254 plates (20 × 20 cm, 0.25 to 1.0 mm thickness; Merck, U.S.A.) using chloroform–methanol–water (65 : 35 : 5, by vol.) as the developing solvent.

NMR spectroscopy. A Bruker AM-300 (7.05 Tesla, 282.38 MHz) and a Bruker CXP-100 (2.35 Tesla, 94.26 MHz, 40 cm bore) spectrometer were used for ^{19}F NMR spectroscopy *ex vivo*. Fluorine spectra of blood, whole liver and tumor specimens were acquired on the CXP-100 spectrometer using a 20 mm diameter, three turn horizontal coil. The *in vivo, in situ* experiment was performed on the CXP-100 spectrometer, fitted with a 6-cm single turn circular copper (coated with silver) surface coil. ^{1}H (300 MHz) and ^{19}F spectra for synthesized compounds, blood, urine and extracts of liver and tumor were acquired using the Bruker AM-300 instrument. Pulse length calibration for fluorine, using both 90° and 180° pulses, was done before acquisition of the spectrum. The ^{19}F NMR data were acquired over a spectral width of 70.0 ppm (between +20.0 and −50.0 ppm). The number of scans varied from 2.5 k to 73 k, depending on the type and size of biological sample and the amount of metabolite present in it. A relaxation delay of 1 sec was employed during the acquisition. An aqueous solution of chromium acetate (90 μL; 0.1 M) was used as a relaxation agent to enhance the S/N ratio. Exponential line broadening of 0 to 20 Hz was imposed on the data prior to Fourier transformation, in response to the concentration of the metabolites present in the sample. An aqueous solution of sodium trifluoracetate (5 μL; 0.2 M) was used as an internal standard during acquisition of ^{19}F NMR spectra, while ^{1}H and ^{13}C NMR spectra were locked on deuterium (deuterium oxide was used as a solvent in cases of samples containing blood, urine, liver and tumor extracts), which appeared at δ 4.68 ppm (^{1}H NMR spectra) with respect to tetramethyl silane (TMS). The resolution limits for ^{1}H, ^{13}C and ^{19}F were observed between 0.5–1.1, 2–3 and 1.3–20 Hz, respectively. The chemical shifts are reported in δ ppm.

Synthesis of α-trifluoromethyl-β-alanyl glycine (F3MBAG; **6**). Ethyl glycinate (88 mg; 0.63 mM), α-trifluoromethyl-β-alanine F_3MBA (99 mg; 0.63 mM) [2] and triethyl amine (88 mg; 0.87 mM) were dissolved in anhydrous ethyl alcohol (20 mL) and stirred at 70° (oil bath) under an inert (N_2) atmosphere. After 24 hr, TLC of the reaction mixture showed complete disappearance of F_3MBA. The solvent was removed *in vacuo*, and the residue was subjected to silica gel column chromatography. An elution with chloroform : methanol : water (65 : 35 : 5, by vol.) gave pure F_3MBAG (13 mg; 9.5%) as a hygroscopic solid; ^{1}H NMR (D_2O) δ 3.28–3.42 (*m*, 3H, –C$\underline{H}$–C$\underline{H}_2$–NH), 4.11 (broad *d*, 2H, C$\underline{H}_2$); ^{19}F NMR ($\underline{D}_2O$) δ 8.55 (*d*, $J_{H,F}$ = 8.9 Hz, CF_3); chemical ionization for $C_6H_9F_3N_2O_3$ (M^+ as NH^+_4), relative abundance 0.4%.

Synthesis of α-trifluoromethyl-β-guanidopropionic

acid (F_3MGPA; **7**). Guanidine hydrocholoride (100 mg; 1.05 mM) was dissolved in water (2 mL), and the pH of the solution was raised to 11 by dropwise addition of aqueous sodium hydroxide (2%, w/v). At this time, 3,3,3-trifluoromethyl acrylic acid (146 mg; 1.0 mM) was added to the reaction mixture, and the temperature of the reaction was set at 90°. After stirring the mixture at this temperature for 16 hr, the TLC showed complete conversion of reactants to product. Excess solvent was evaporated, and the residue was subjected to silica gel column chromatography. Using chloroform : methanol : water (65 : 35 : 3, by vol.) as eluent, pure F_3MGPA (110 mg; 55%) was obtained from the column as a gummy solid; ^{1}H NMR (D_2O) δ 3.16 (t, $J_{CH,CH2}$ = 6.0 Hz of q, $J_{F,CH}$ = 9.0 Hz, 1H, CH) and 3.88 (d, $J_{CH,CH2}$ = 6.0 Hz, 2H, CH_2); ^{19}F NMR (D_2O) δ 9.26 (d, $J_{H,F}$ = 9.1 Hz, CF_3). +^{ve}FAB for $C_5H_8F_3N_3O_2$ (as M^+), relative abundance 1.85%.

Synthesis of N-*carboxy-α-trifluoromethyl-β-alanine (F_3MBA-CO_2;* **8**). F_3MBA (25 mg; 0.16 mM) was added to an aqueous solution of sodium bicarbonate (0.16 N; 1 mL). The pH of the solution mixture was 8.5 at this time. The reaction mixture was stirred at 25° for 4 hr. A TLC examination of the reaction mixture at this time showed complete disappearance of F_3MBA and formation of the product. Dowex 1 × 8-50 ion exchange resin was added to this solution, raising the pH to 7. Removal of the resin by filtration and evaporation of water left the crude product and residue, which was purified on a P-TLC plate using chloroform : methanol : water (65 : 35 : 10, by vol.) to yield 5 mg (15%) of pure **8** as a sticky solid; ^{1}H NMR (D_2O) δ 3.40–3.60 (m, 3H, C$\underline{H}$–C$\underline{H}_2$); ^{19}F NMR (D_2O) δ 8.27 (d, $J_{H,F}$ = 8.8 Hz, CF_3).

Synthesis of α-trifluoromethyl-β-alanyl alanine (F_3MBAA; **9**). A mixture of F_3MBA (25 mg; 0.16 mM) and β-alanine methyl ester hydrochloride (22 mg; 0.16 mmol) was dissolved in anhydrous ethanol (2 mL). Triethylamine (89 μL; 0.64 mmol) was added dropwise, and the reaction mixture was stirred at 60° under complete exclusion of moisture. The progress of the reaction was monitored by TLC, which showed complete disappearance of starting materials after 24 hr. Ethanol was evaporated, and the crude product was subjected to column chromatography using a gradient elution with chloroform : methanol : water (65 : 35 : 10, by vol.) to recover 8.4 mg (20%) of **9**, as a gummy solid ^{1}H NMR (D_2O) δ 2.69 (t, $J_{CH2,CH2}$ = 6.2 Hz, 2H, CH_2 NH_2), 3.19 (t, $J_{CH2,CH2}$ = 6.2 Hz, 2H, CO CH_2), 3.3–3.4 (m, 3H, 1H of C$\underline{H}$–CF_3 and 2H of CH–C$\underline{H}_2$) ppm; ^{19}F (D_2O) δ 8.57 (d, $J_{H,F}$ = 9.3 Hz, CF_3); chemical ionization for $C_7H_{11}F_3N_2O_3$ (228.18 as M^{+1}), relative intensity 0.5%.

Synthesis of N-*acetyl-α-trifluoromethyl-β-alanine Ac-F_3MBA;* **10**). Acetic anhydride (20 μL; 0.21 mM) was added to an aqueous solution (1 mL) of F_3MBA (27 mg; 0.17 mM), and the mixture was stirred at 25° until F_3MBA completely disappeared (TLC) in the reaction mixture. The contents were evaporated *in vacuo* and the crude product was recovered from a silica gel column using chloroform : methanol : water (65 : 35 : 5, by vol.) as eluent. Evaporation of the fractions containing the product yielded pure **10** (15 mg; 44%) as a viscous syrup; ^{1}H NMR (D_2O) δ 2.06 (s, 3H CO$\underline{CH}_3$) and 3.16–3.56 (m, 3H, –C$\underline{H}$–C$\underline{H}_2$–); ^{19}F NMR (D_2O) δ 8.02 (d, $J_{H,F}$ = 8.5 Hz, CF_3) ppm.

Animal studies. Ex vivo studies were performed in five male BALB/C mice (20–25 g; Health Sciences Laboratory Animal Services, University of Alberta). A suspension of EMT-6 tumor cells grown in culture (0.1 mL containing 10^6 cells) was injected subcutaneously into the right thigh of recipient mice. The tumors grew to the desired size (0.5 to 1 g) in 10 days. Trifluridine (250 mg/kg in water for injection) was administered i.p., daily for 5 consecutive days, in order to establish a metabolic pool sufficient to allow detection. The mice received normal food and water *ad lib.* during the experiment. There were no visible signs of toxic side-effects at this dose. Urine and blood samples were collected as described previously [1]. On day 5, 30 min after injection of F_3TdR and immediately upon CO_2 euthanasia, the tumors and livers were dissected out and stored at −15° until further use. Methanolic extracts of tumors and livers were prepared by homogenizing the solid tissues in methanol, followed by centrifugation at 1000 *g* for 10 min, after which the supernatant was carefully decanted for ^{19}F NMR acquisition.

In vivo NMR studies were performed on two male Sprague–Dawley rats (120–130 g). The rats were dosed with single i.p. injections of an aqueous solution of trifluridine (350 mg/kg) and immediately anesthetized with Ketalean (0.1 mL/100 g) and Rompun (Xylazine, 0.05 mL/100 g); Ketalean and Rompun were injected separately into opposite thighs. The rats were mounted on a specifically designed gantry fitted with a circular surface coil (ϕ = 6 cm) and then placed into the cavity of the magnet of the CXP-100 spectrometer, to acquire 2.5 k scans. The rats were removed from the assembly upon completion of spectroscopy. Blood was collected from the rats by cardiac puncture, immediately after they had been euthanized by carbon dioxide. Liver was recovered during necropsy, for spectroscopic study of the intact liver or liver extract using the sample preparation methods for murine specimens as described, above.

All animal studies were conducted according to regulations of the Canadian Council on Animal Care, according to protocols approved by the Health Sciences Animal Research Ethics Committee of the University of Alberta.

RESULTS

The ^{19}F NMR spectrum (Fig. 1) of a liver taken from a F_3TdR-treated mouse was characterized by two broad signals between δ 1.9 and 10.7 ppm. The virtual absence of the F_3TdR resonance (δ 12.2) indicates that F_3TdR, despite being extensively metabolized, was not stored in the liver. Improved spectral resolution obtained from the methanolic liver extract facilitated the detection of DHF_3T (δ 8.21; $J_{H,F}$ = 9.0 Hz), F_3MUPA (δ 8.37; $J_{H,F}$ = 9.3 Hz) and a novel metabolite subsequently identified as F_3MBAG (δ 8.54; $J_{H,F}$ = 8.6 Hz), along with F_3T (δ 12.08) and a small amount of unchanged

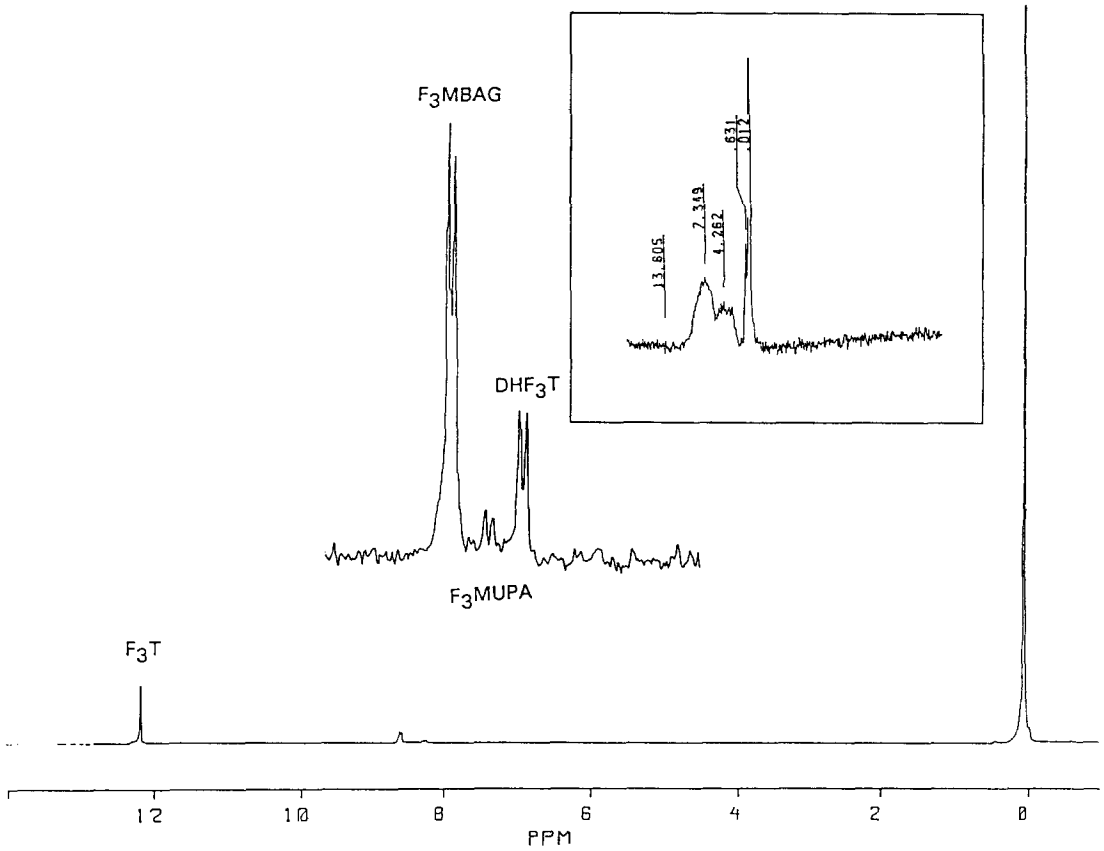


Fig. 1. ^{19}F NMR spectrum of intact mouse liver (inset) and its methanolic extract.

F_3TdR (δ 12.20). Table 1 provides a summary of the ^{19}F NMR properties of all metabolites observed and identified by ^{19}F NMR spectroscopy.

Table 1. Summary of ^{19}F NMR chemical shifts of authentic compounds, synthesized as possible metabolites of F_3TdR, and their respective coupling constants

Authentic compounds	^{19}F Chemical shifts (δ ppm)*	Coupling constants ($J_{H,F}$) (Hz)
DHF_3T	8.19 (*d*)†	8.4
$DOHF_3T$	−0.53 (*s*)‡	
F_3MUPA	8.35 (*d*)	9.1
F_3MBAG	8.54 (*d*)	8.9
F_3TdR	12.20 (*s*)	
F_3T	12.02 (*s*)	
F_3MBA	8.02 (*d*)	8.5
F_3MGPA	9.26 (*d*)	9.1
F^-	−47.28 (*s*)	
$F_3MBA\text{-}CO_2$	8.27 (*d*)	8.8
F_3MBAA	8.57 (*d*)	9.3
$Ac\text{-}F_3MBA$	8.02 (*d*)	8.5

* Chemical shifts are reported with respect to CF_3COONa (0.2 M; 5 mL) as an internal standard.

† The resonance was observed as a doublet (*d*).

‡ The resonance was observed as a singlet (*s*).

The ^{19}F NMR spectrum of an intact murine tumor displayed a broad signal ranging from δ 4.0 to 12.5 ppm, whereas the spectrum of its methanolic extract showed the presence of only traces of F_3MUPA (δ 8.34; $J_{H,F}$ = 9.4) and DHF_3T (δ 8.21; $J_{H,F}$ = 8.4 Hz). Although F_3TdR and F_3T were barely detectable in solid tumor tissue, they were readily detected in the tumor extract (Fig. 2).

^{19}F NMR spectroscopy of murine blood showed the presence of only F_3MUPA, F_3T and some unchanged F_3TdR; no other metabolites were detected in these samples.

An *in vivo, in situ* ^{19}F NMR spectrum of the hepatic region of F_3TdR-treated rats, using a surface coil, is shown in Fig. 3. These *in situ* spectra characteristically showed an unresolved broad resonance band between δ 6.0 and 11.0 ppm and a similar, smaller "hump" between δ 3.5 and 6.0 ppm. The parent drug (F_3TdR) and the free base metabolite (F_3T) were hardly detectable in these studies.

The *ex vivo* ^{19}F NMR spectrum of rat liver exhibited two broad humps between δ 5.0 and 12.5 ppm. Noticeable broadening of the trifluoracetic acid (TFA) signal could indicate the presence of a

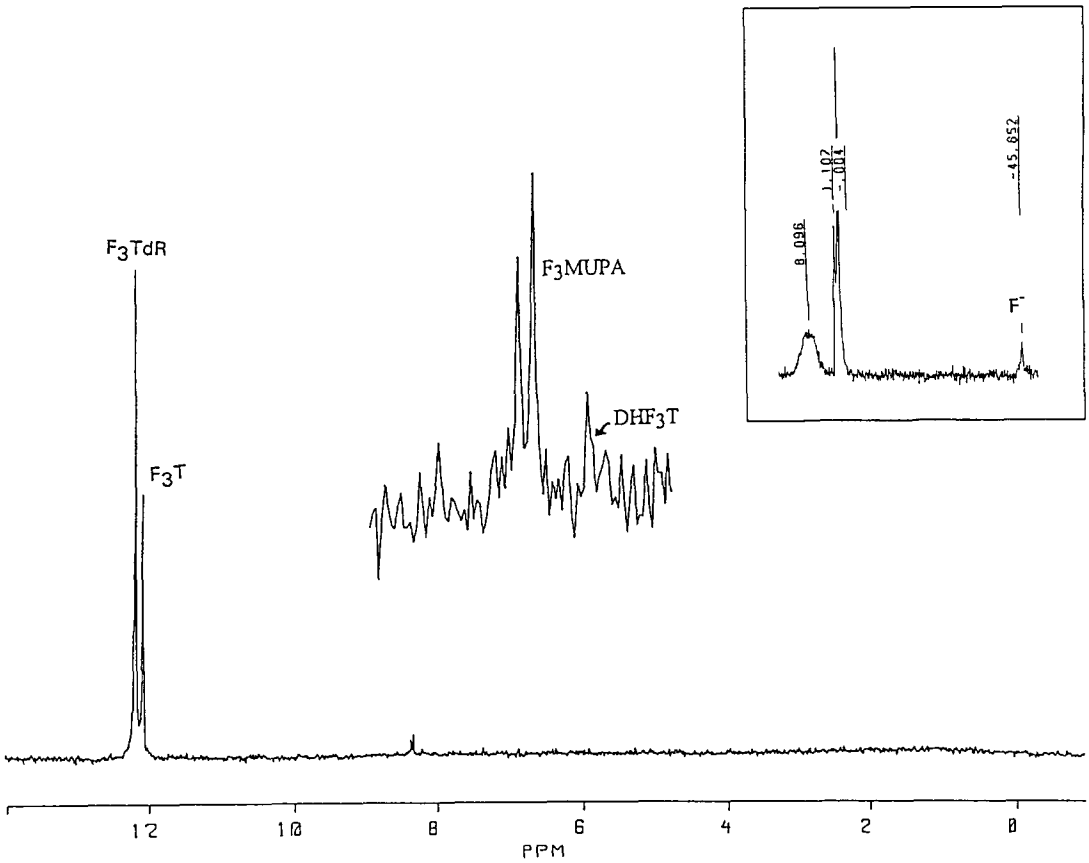


Fig. 2. ^{19}F NMR spectrum of intact mouse tumor (inset) and its methanolic extract.

fluorinated metabolite with a chemical shift very close to that of TFA. The resonance signal for authentic $DOHF_3T$ (δ −0.59 ppm) [1] could have produced this broadening effect. The signal for F_3T could also be seen in this spectrum (δ 11.97 ppm). The ^{19}F NMR spectrum of the methanolic extract of rat liver (Fig. 4) indicated the presence of DHF_3T (δ 8.20; $J_{H,F} = 9.0$ Hz), F_3MBAG (δ 8.54; $J_{H,F} = 8.8$ Hz), F_3T (δ 11.97) and a small amount of F_3TdR, but there was no indication that $DOHF_3T$ or other metabolites were present. F_3MUPA, which was present in all murine specimens, was not present in the blood or liver extracts of rats. ^{19}F NMR spectra of rat blood and urine showed only those resonances attributable to F_3TdR and F_3T. A summary of the metabolites detected in various tissues from mice and rats is presented in Table 2.

DISCUSSION

The ^{19}F NMR resonances at δ 8.54 represented the single remaining unidentified metabolite of F_3TdR detected by ^{19}F NMR in our earlier studies [1, 2]. This resonance is now assigned to F_3MBAG. The assignment of this dipeptide structure was based largely on supportive experimental data, rather than on its isolation from body fluids or tissues. The α-trifluoromethyl moiety of all of these metabolites has been shown to be very labile, undergoing hydrolysis of C–F bonds to produce defluorinated products. The parent compound (F_3TdR) and the free base (F_3T) formed upon deglycosylation of the nucleoside, are known to be chemically unstable at alkaline pH, with base-catalyzed hydrolysis resulting in rapid and complete defluorination to yield the corresponding 5-carboxy pyrimidine [3]. Similarly, the fluorine-containing catabolites reported previously [1, 2] are sensitive to defluorination at physiological pH, thereby providing a rational explanation for the difficulties experienced in isolating metabolites by conventional techniques [3]. We were also unsuccessful in attempts to isolate many of the fluorine-containing metabolites, although a number of ^{19}F resonances were clearly visible in the spectra of urine, blood and liver of F_3TdR-dosed mice [1].

Assignment of the resonance at δ 8.54 was therefore based on both direct and indirect evidence. Most importantly, chemically synthesized F_3MBAG provided an exact match to the ^{19}F NMR signal present in the spectrum of the biological sample, enhancing only the δ 8.54 signal, with an identical coupling constant ($J_{H,F} = 8.6$ Hz). The related dipeptide F_3MBAA (**9**), Ac-F_3MBA (**8**) and

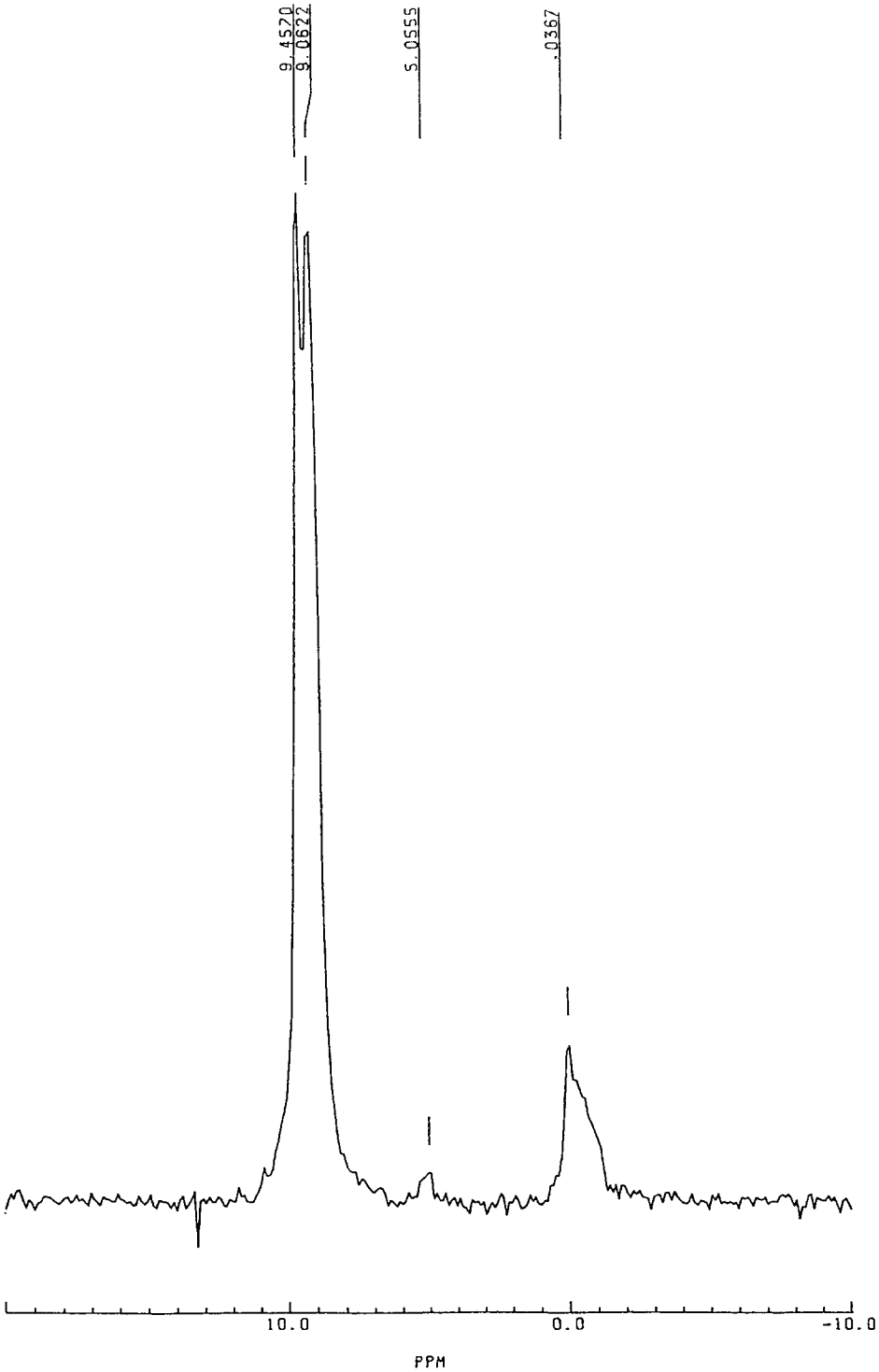


Fig. 3. ^{19}F NMR spectrum of whole rat.

F_3MGPA (7), all of which are potential metabolites, have ^{19}F resonances and coupling constants of δ 8.57 ($J_{H,F}$ = 9.3 Hz), δ 8.02 ($J_{H,F}$ = 8.5 Hz) and δ 9.26 ($J_{H,F}$ = 9.1 Hz), respectively; when added to metabolite-containing urine, there was no enhancement of "metabolite" resonances; rather, new and distinct signals appeared.

Based on the model of catabolism of 5-fluorouracil (5-FU) and its nucleosides, two other classes of metabolites remained to be excluded or identified

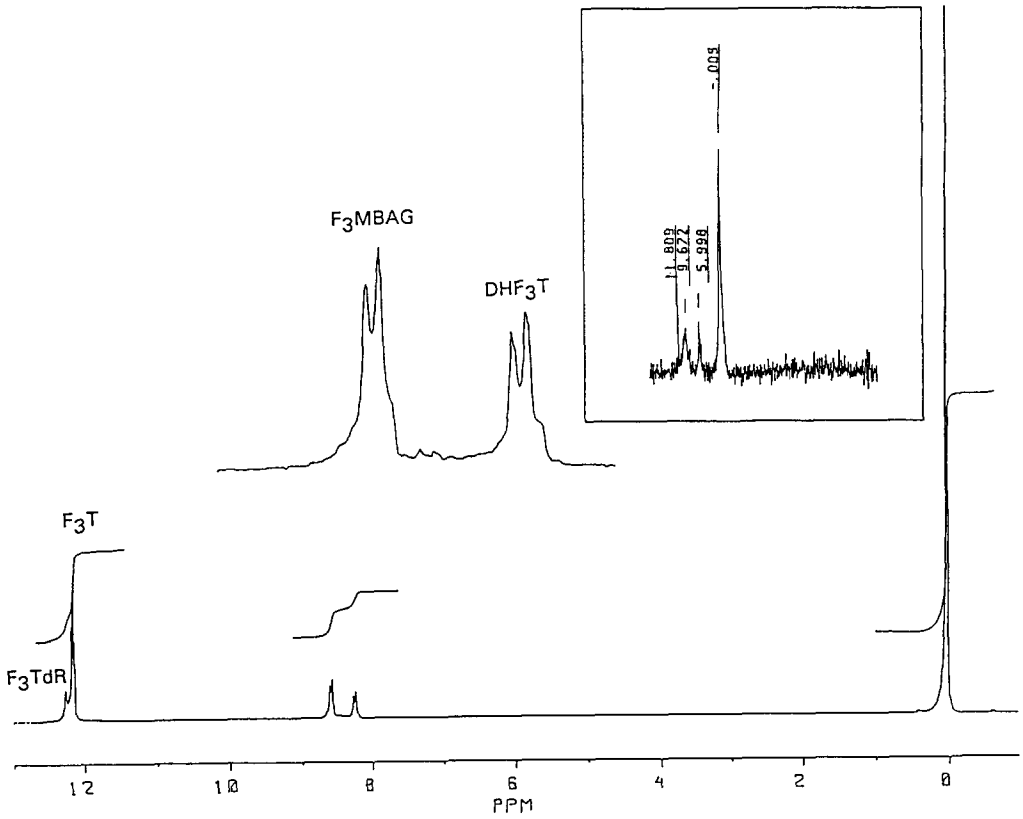


Fig. 4. ^{19}F NMR spectrum of intact rat liver (inset) and its methanolic extract.

as possible metabolites; these are F_3MBA-CO_2 (**8**) and the bile acid conjugates of F_3MBA (F_3MBA-BACs) [4]. All attempts to synthesize the bile acid conjugates led to loss of the fluorine from the α-trifluoromethyl moiety, and to the formation of complex reaction mixtures under TLC examination. Given this instability, it was concluded that the resonance at δ 8.54 was not due to bile acid conjugates, which would at best be very transient species. Although F_3MBA-BACs could possibly be formed under acceptable (enzymatic) conditions, the presence of the unidentified metabolite (δ 8.54) in urine essentially precluded the assignment of the structure to this class of metabolites, as bile acid conjugates are excreted by the hepatobiliary, not the urinary, system. The ^{19}F resonance for F_3MBA-CO_2 occurred at δ 8.27 and was clearly not the unidentified metabolite.

The glycine conjugation of α-substituted-β-alanines has not been reported previously as a biotransformation reaction for xenobiotics. α-Fluoro-β-alanine, a metabolite of 5-FU [5], and β-aminoisobutyric acid [6], the corresponding metabolite of thymine (5-methyluracil), have been studied in detail, without reported evidence for this reaction. Glycine conjugation of other compounds has been known as an important biotransformation reaction for over a century. This reaction occurs primarily in liver; it is ATP dependent and involves the formation of a carboxylic acid thioester of coenzyme A (acyl-CoA), which reacts with glycine under the influence of an acyl-CoA synthetase and glycine *N*-acyl transferase [7]. The reaction rate has been shown to be dependent on substrate and glycine concentration [8] and, consequently, has implications not only for glycine conjugation but for the multi-enzyme glycine cleavage system [9] and its role in homeostasis. In the case of F_3MBA, low substrate concentrations after dosing with F_3TdR imply minimal physiological significance, but the formation of F_3MBAG remains a biochemical curiosity.

Equally important, base-catalyzed defluorination of all of the α-trifluoromethyl carboxylic acid metabolites would give rise to substituted malonic acids which, at least on the basis of quantity and reactivity, are likely to be disruptive to a number of biochemical processes in the body. Studies of the metabolism of 5-carboxyuracil, the hydrolytic by-product/metabolite of F_3T, detected no evidence for the presence of such carboxy-containing ring-scission metabolites [3]. However, the labile nature of the C–F bonds in F_3MUPA, F_3MBAG and other metabolites of F_3TdR mitigates strongly in favor of the existence of a parallel set of metabolites in which $-CF_3$ is replaced by –COOH (Fig. 5). Unfortunately, the ^{19}F NMR technique cannot be applied to the resolution of these aspects of the catabolic biotransformation of F_3TdR.

Table 2. Summary of metabolites of F_3TdR in various organs of mice and rat as detected by *ex vivo* and *in vivo* ^{19}F NMR spectroscopy

	Mouse						Rat			
Metabolite	Whole liver*	Liver extract (δ)	Whole tumor†	Tumor extract (δ)	Blood (δ)	Urine (δ)	Whole liver‡	Liver extract (δ)	Blood (δ)	Urine (δ)
DHF_3T	+§	+ (8.21)	+	+ (8.21)	−	+ (8.20)	+	+ (8.20)	−	−
$DOHF_3T$	?‖	−	−	−	−	+ (−0.59)	?	−	−	−
F_3MUPA	+	+ (8.37)	+	+ (8.34)	+ (8.35)	+ (8.36)	?	−	−	?
F_3MBAG	+	+ (8.54)	−	−	−	+ (8.54)	+	+ (8.54)	−	−
F_3TdR	+	+ (12.21)	+	+ (12.16)	+ (12.46)	+ (12.19)	+	+ (12.21)	+ (12.51)	+ (12.18)
F_3T	+	+ (12.08)	+	+ (12.07)	+ (12.19)	+ (12.02)	+	+ (12.03)	+ (12.15)	+ (12.0)
F^-	−	−	+ (−45.85)	−	+ (−42.52)	−	−	−	−	−

Chemical shifts are reported as δ (ppm).

* A large resonance at δ 1.90–10.70 was observed in the ^{19}F NMR spectrum of intact mouse liver.

† A hump appeared at δ 4.00–12.50 ppm in ^{19}F NMR spectrum of intact mouse tumor.

‡ ^{19}F NMR spectrum of intact rat liver displayed two resonance humps at δ 5.00–7.50 and 8.00–12.50 ppm.

§ A plus (+) sign indicates the detection of the metabolite and a minus (−) sign indicates the failure to detect the metabolite.

‖ indicates that because a broad hump was observed in the ^{19}F spectrum of intact liver of mouse and rat, it was not possible to establish the presence or absence of this metabolite.

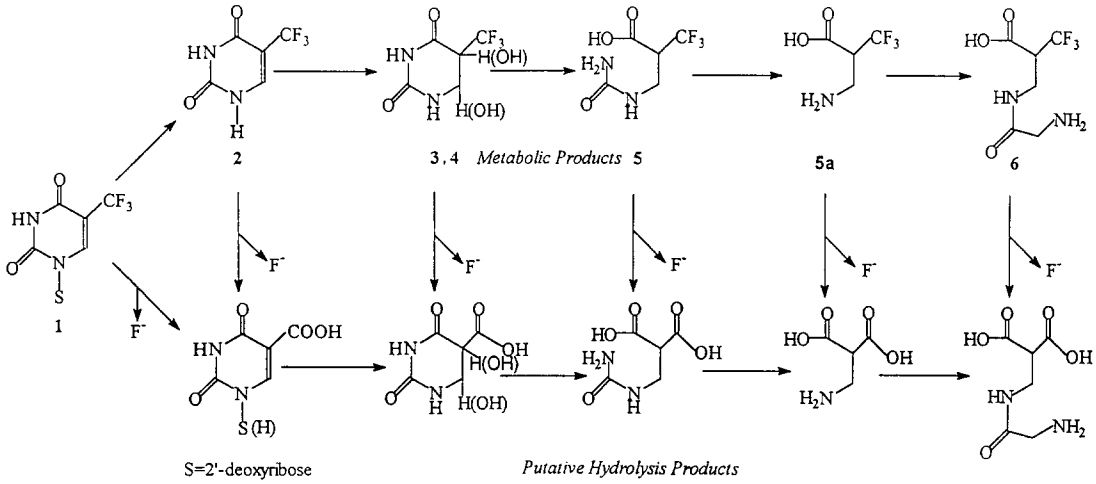


Fig. 5. Schematic representation of metabolic and degradation products of trifluridine.

F_3MBAG and the previously reported metabolites DHF_3T, $DOHF_3T$ and F_3MUPA could not be detected in the blood or urine samples of F_3TdR-dosed rats, although DHF_3T and F_3MBAG were found to be present in the liver extract. In mice, F_3MUPA was present in every biological sample (blood, urine, liver extract and tumor extract), whereas $DOHF_3T$ was found only in urine. DHF_3T was present in all of the samples except blood, but F_3MBAG was detected only in murine liver extracts and urine. The absence of a number of the murine metabolites in rat tissue/fluid specimens is attributed to the differences in dose/dosing protocols (e.g. 1 dose in rats vs 5 doses to mice) rather than to species differences. The multiple dosing protocol was used for the initial murine study in an effort to build up a pool of metabolites *in vivo*, although there was no evidence to support this approach. Given the relatively low quantitative sensitivity of the NMR technique, it was rationalized that multiple doses would raise concentrations of some or all metabolites. In single-dose rat studies, failure to detect several metabolites may be interpreted as supportive of the concept that multiple dosing affects metabolite pools. Small variations in the chemical shifts and/or coupling constants of metabolites can be attributed to the differences in pH of different biological samples. F_3MUPA resonances, for example, range from δ 8.3510 (pH 6.1) to δ 8.3560 (pH 7.0), with coupling constants ($J_{H,F}$) of 8.99 and 9.16 Hz at the respective pH levels.

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