[Chem. Pharm. Bull. 28(6)1795—1803(1980)]

Identification of Metabolites of 1-(Tetrahydro-2-furanyl)-5-fluorouracil (FT-207) formed *in Vitro* by Rat Liver Microsomes

TERUYOSHI MARUNAKA, YOSHINORI MINAMI, YUKIHIKO UMENO, AKIO YASUDA, TOSHIYUKI SATO,[1a] and SETSURO FUJII[1b]

Research Laboratory, Taiho Pharmaceutical Co., Ltd,[1a] Institute for Protein Research, Osaka University[1b]

(Received December 22, 1979)

Four metabolites of the antitumor agent 1-(tetrahydro-2-furanyl)-5-fluorouracil, formed *in vitro* by rat liver microsomes, were isolated by thin-layer chromatography or high-performance liquid chromatography. On the basis of mass spectrometry, ^{1}H-NMR spectral analysis, and comparison with authentic samples, these metabolites were identified as 1-(*trans*-4-hydroxytetrahydro-2-furanyl)-5-fluorouracil, 1-(*cis*-4-hydroxytetrahydro-2-furanyl)-5-fluorouracil, 1-(*trans*-3-hydroxytetrahydro-2-furanyl)-5-fluorouracil and 1-(4,5-dehydrotetrahydro-2-furanyl)-5-fluorouracil. These metabolites were also found in the plasma and urine of rats after administration of 1-(tetrahydro-2-furanyl)-5-fluorouracil.

Keywords——1-(tetrahydro-2-furanyl)-5-fluorouracil; antitumor drug; isolation and identification of metabolites; thin-layer chromatography; high-performance liquid chromatography; mass spectrometry; ^{1}H-NMR spectrometry

1-(Tetrahydro-2-furanyl)-5-fluorouracil (FT), named FT-207 or ftorafur, is a derviative of 5-fluorouracil (5-FU) which is widely used clinically as an antitumor agent.

FT is known to be metabolized to an active substance, 5-FU, by a drug-metabolizing enzyme in rat or mouse liver microsomes.[2] In this work we demonstrated the formation of metabolites with the 5-FU skeleton by rat liver microsomes *in vitro*, and isolated four such metabolites by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) for identification. We also found these metabolites in the plasma and urine of rats after administration of FT.

Some of these metabolites have been reported.[3,4] Two hydroxylated metabolites, a dehydrated metabolite and a metabolite with an altered tetrahydrofuran moiety were isolated from the urine of rats and rabbits or the plasma and urine of humans and identified by Wu *et al.*[3] However, their structures have not been fully confirmed. Two hydroxylated metabolites were isolated from human urine and identified by Benvenuto *et al.*[4]

We examined the mass spectra and proton nuclear magnetic resonance (^{1}H-NMR) spectra of the four isolated metabolites, analyzed their structures, and compared the data with those for authentic samples. Three forms of hydroxylated-FT and a dehydrated-FT were identified, and the positions of their hydroxyl group and double bond as well as the configuration of the hydroxyl group relative to the 5-FU skeleton were determined.

This paper reports these structures.

---

1) Location: *a) Hiraishi, Ebisuno, Kawauchi-cho, Tokushimashi, Tokushima, 771-01, Japan*; *b) 5311 Yamada, Suitashi, Osaka, 565, Japan.*

2) H. Fujita, M. Sugiyama, and K. Kimura, Ninth International Congress on Chemotheraphy, London 1975; S. Ohira, S. Maezawa, K. Watanabe, K. Kitada, and T. Saito, Ninth International Congress on Chemotheraphy, London 1975; H. Toide, H. Akiyoshi, Y. Minato, H. Okuda, and S. Fujii, *Gann*, **68**, 553 (1977).

3) A.T. Wu, J.L. Au, and W. Sadée, *Cancer Res.*, **38**, 210 (1978); J.L. Au, A.T. Wu, M.A. Friedman, and W. Sadée, *Cancer Treat. Rept.*, **63**, 343 (1978).

4) J.A. Benvenuto, K. Lu, S.W. Hall, R.S. Benjamin, and T.L. Loo, *Cancer Res.*, **38**, 3867 (1978); J.A. Benvenuto, J.G. Liehr, T. Winker, D. Farquhar, P.M. Caprioli, and T.L. Loo, *Cancer Res.*, **39**, 3199 (1979).

Experimental

Chemicals——FT, 1-(*trans*-3-hydroxytetrahydro-2-furanyl)-5-fluorouracil (*trans*-3′-OH-FT), 1-(*cis*-3-hydroxytetrahydro-2-furanyl)-5-fluorouracil (*cis*-3′-OH-FT), 1-(4,5-dehydrotetrahydro-2-furanyl)-5-fluorouracil (4′,5′-dehydro-FT), 1-(3,4-dehydrotetrahydro-2-furanyl)-5-fluorouracil (3′,4′-dehydro-FT) and 1-(5-methoxytetrahydro-2-furanyl)-5-fluorouracil (5′-OMe-FT) were synthesized at the Research Laboratory, Taiho Pharmaceutical Co., Tokushima, Japan.[5] 5-FU, $NADP^+$ and glucose 6-phosphate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and glucose 6-phosphate dehydrogenase was obtained from Biochemicals Inc., Milwaukee, Wis., U.S.A. Phenyltrimethylammonium hydroxide (PTAH) (20—25% methanol solution, Tokyo Kasei Co., Tokyo, Japan) was used as 2% solution in methanol. Other chemicals used were all analytical grade or chromatographic grade materials (Wako Pure Chemical Co., Osaka, Japan).

Preparation of Rat Liver Microsomes——Male Donryu rats (160—180 g) were starved for 24 hr, then their livers were isolated, washed free from blood with ice-cooled physiological saline, minced and homogenized with four volumes of 0.01 M phosphate buffer (pH 7.4) containing 1.15% KCl. The liver homogenate was centrifuged at $10000 \times g$ for 15 min to separate mitochondria, nuclei and debris. The supernatant was recentrifuged at $78000 \times g$ for 90 min. The supernatant was discarded and the remaining microsomal pellet was suspended in the same buffer then recentrifuged at $78000 \times g$ for 90 min. The microsomal pellet thus obtained was suspended in 0.1 M phosphate buffer (pH 7.4). The processes described above were all carried out at 0—4°.

Biotransformation of FT with Rat Liver Microsomes——A mixture containing 5 mM FT, 0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, 12 units of glucose 6-phosphate dehydrogenase, 5 mM $MgSO_4$, 0.15 M KCl, 0.1 mM EDTA and rat liver microsomal suspension (20 mg of protein) in a total volume of 4 ml in 0.1 M phosphate buffer was incubated at 37° for 30 min. The incubation mixture was frozen in a dry ice–acetone bath, thawed and centrifuged to separate protein. The supernatant was used for the separation of metabolites. The protein concentration of the enzyme solution was measured by the method of Lowry *et al.*[6]

Collection of Plasma and Urine——Plasma was collected from male Donryu rats 2—6 hr after oral dosage of 2 mM FT (400 mg/kg). Urine was collected from the rats 0—8 hr after oral administration of 0.5 mM FT (100 mg/kg) daily for a week. The plasma and urine (concentrated to 1/10—1/20 its original volume in a lyophilizer) were used for the separation of metabolites.

Isolation of Metabolites of FT——The supernatant of the reaction system of FT and rat liver microsomes, and the plasma and concentrated urine of rats treated with FT were each adjusted to pH 6.0 with 0.5 M NaH_2PO_4 solution, and extracted with 20 volumes of ethyl acetate. The ethyl acetate layer was evaporated to dryness under nitrogen gas at 40°. The residue was dissolved in a small amount of methanol and subjected to column chromatography (12.5 cm × 0.6 cm I.D., Kieselgel 60, 70—230 mesh, Merck, Darmstadt, W. Germany), eluting with 10 ml of acetonitrile–chloroform–water (40: 4: 1). The eluate was evaporated to dryness under nitrogen gas. The residue was dissolved in 100 μl of methanol, and subjected to TLC (Kieselgel HF_{254}, Type 60, Merck), developing with solvent (I), ethyl acetate–ether–acetic acid (20: 20: 1). Fractions containing materials with ultraviolet (UV) absorption and *Rf* values of 0.27 (FM-1), 0.30 (FM-2), 0.38 (FM-3, 5-FU), 0.65 (FT) and 0.80 (FM-4) were extracted with methanol. The methanol extract of the *Rf* 0.38 fraction was further separated into fractions with *Rf* values of 0.38 (FM-3) and 0.28 (5-FU) by rechromatography using developing solvent (II), chloroform–methanol–25% aqueous ammonia (75: 25: 1).

The metabolites were also separated by reversed-phase HPLC. The ethyl acetate extract dissolved in methanol after column chromatography was injected into the liquid chromatograph under the following conditions: μ-Bondapak C_{18} (30 cm × 4 mm I.D.) (Waters Assoc., Milford, Mass., U.S.A.); mobile phase, 0.01 M sodium acetate buffer (pH 4.0)–methanol (95: 5); flow rate, 2.0 ml/min; detector, UV-254 nm constant wavelength. Peak fractions with retention times of 2.5 min (5-FU), 5.3 min (FM-1), 7.4 min (FM-2), 7.5 min (FM-3), 15.8 min (FT) and 18.6 (FM-4) were isolated (Fig. 1). FM-2 and FM-3 had almost the same retention times and could not be separated, so they were separated by TLC using developing solvent (I) or (II).

Instruments——A Shimadzu LC-3 high-performance liquid chromatograph (Kyoto, Japan) was used for the separation of FT and its metabolites. Measurement of mass spectra by direct inlet or gas chromatographic inlet was carried out using a JEOL JMS-O1SG-2 mass spectrometer connected with a JEOL JGC-20KP gas chromatograph, equipped with an electron impact (EI)/field desorption (FD) ion source and a JEOL JMS-D300 mass spectrometer fitted with an EI/chemical ionization (CI) ion source (Tokyo, Japan). EI mass spectra (direct inlet) were measured with 70 eV ionization energy, 200 μA ionization current and 7.0 kV acceleration voltage: CI mass spectra were measured with 200 eV ionization energy, 300 μA ionization current and 3.0 kV acceleration voltage: FD mass spectra were measured with −6.0 kV cathode voltage and

5) M. Yasumoto, I. Yamawaki, T. Marunaka, and S. Hashimoto, *J. Med. Chem.*, **21**, 738 (1978); M. Yasumoto, N. Ueda, and S. Hashimoto, Meeting of the Chugoku-Shikoku Branch of the Pharmaceutical Society of Japan, Takamatsu, Oct. 1979; J, Zémilićka, R. Gasser, J.V. Freisler, and J.P. Horwitz, *J. Am. Chem. Soc.*, **94**, 3213 (1972).

6) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

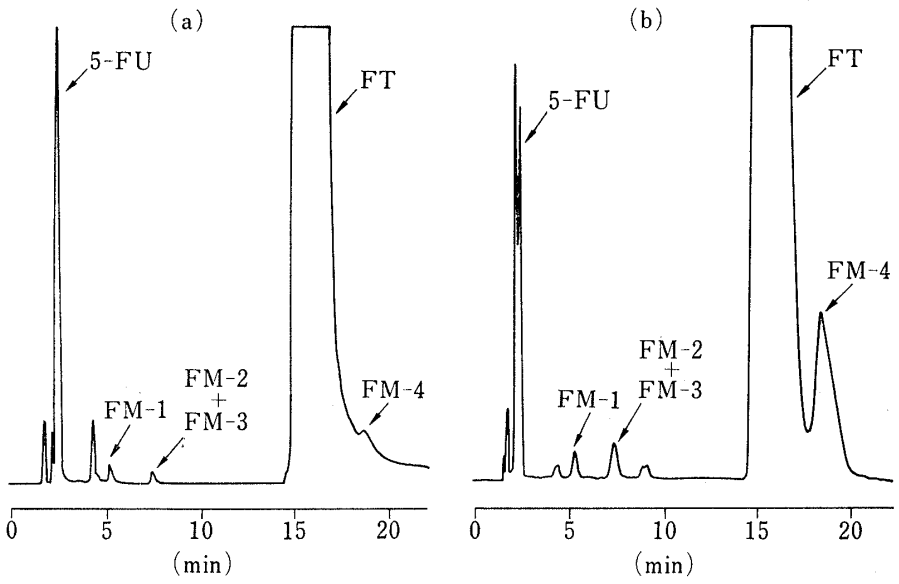

Fig. 1. High-Performance Liquid Chromatograms showing the Separation of FT and Its Metabolites (a) extracted from Rat Liver Microsomal Suspension after Incubation with 5 mM FT for 10 min at 37° and (b) from Rat Plasma and Urine

7.0 kV acceleration voltage; EI mass spectra using a gas chromatographic inlet (GC–MS) were measured using a coiled glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on Chromosorb W AW (80—100 mesh) (Gaschro Kogyo, Tokyo, Japan) at injector, column and detector temperatures of 240, 200 and 200°, respectively, a flow rate of helium carrier gas of 30 ml/min, and an ionization energy of 24 eV. High resolution mass spectra were measured by a photographic plate method and the formula of each fragment ion was calculated.

^{1}H-NMR spectra were measured using a JEOL JNM-FX 90Q nuclear magnetic resonance spectrometer coupled with an FA 100 data system and equipped with a Fourier transform device, using acetone-d_6 as a solvent and tetramethylsilane (TMS) as an internal standard.

Results

In previous studies,[3,4] chloroform–methanol (70: 30) and chloroform–methanol–25% aqueous ammonia (75: 25: 1) were used as developing solvents for TLC. However, FT, 5-FU and the four metabolites could not be completely separated with these solvents. Therefore we used developing solvents (I) and (II) for separation and purification of these compounds.

The four metabolites of FT formed by incubation with rat liver microsomes were shown to be identical with four metabolites in rat plasma and urine after administration of FT by comparison of their *Rf* values on TLC and retention times on HPLC as well as by mass spectrometry and ^{1}H-NMR spectral analysis.

CI mass spectra using isobutane as a reactant gas gave a protonated molecular ion peak of *m/e* 217 for FM-1, FM-2 and FM-3, and one of *m/e* 199 for FM-4. FD mass spectra gave a molecular ion peak (M^+) of *m/e* 216 for FM-1, FM-2 and FM-3, and one of *m/e* 198 for FM-4. These results show that FM-1, FM-2 and FM-3 have a molecular weight of 216 and the formula $C_8H_9FN_2O_4$, that FM-4 has a molecular weight of 198 and the formula $C_8H_7FN_2O_3$, that FM-1, FM-2 and FM-3 have one more oxygen than FT, and that FM-4 has 2 fewer hydrogens.

In the EI (direct inlet) mass spectra of FM-1, FM-2 and FM-3, we observed an $(M\text{-}H_2O)^+$ ion of *m/e* 198 ($C_8H_7FN_2O_3$), suggesting the existence of a hydroxyl group, fragment ions of *m/e* 130 ($C_4H_3FN_2O_2$) and 131 ($C_4H_4FN_2O_2$), suggesting the presence of the 5-FU skeleton, and a base peak of *m/e* 87 ($C_4H_7O_2$), corresponding to a tetrahydrofuran ring with one hydroxyl group, as shown in Fig. 2. On the other hand, it was shown that FM-4 formed a 5-FU ion

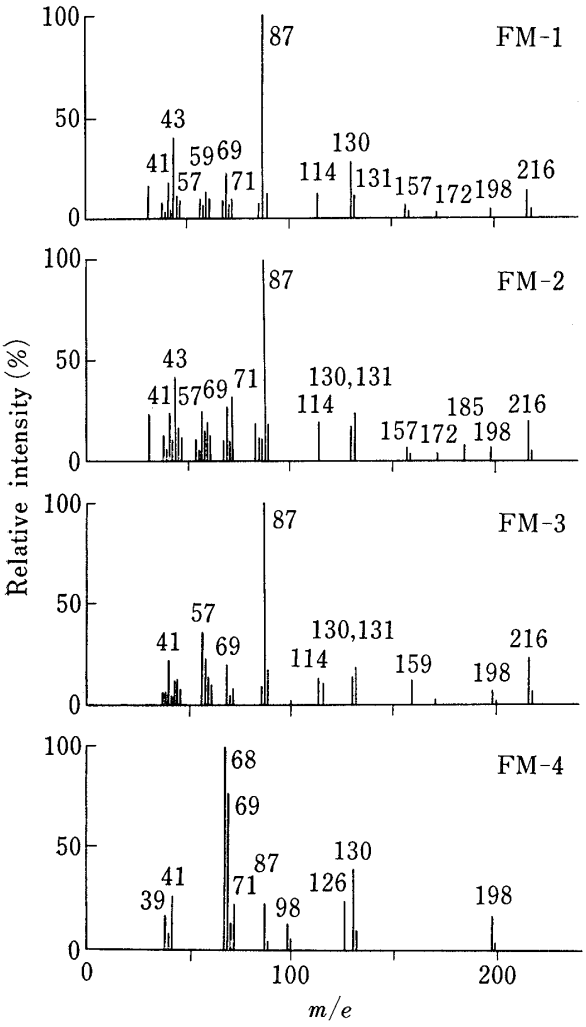

Fig. 2. EI Mass Spectra of FT Metabolites extracted from Rat Liver Microsomal Suspension, and from Rat Plasma and Urine

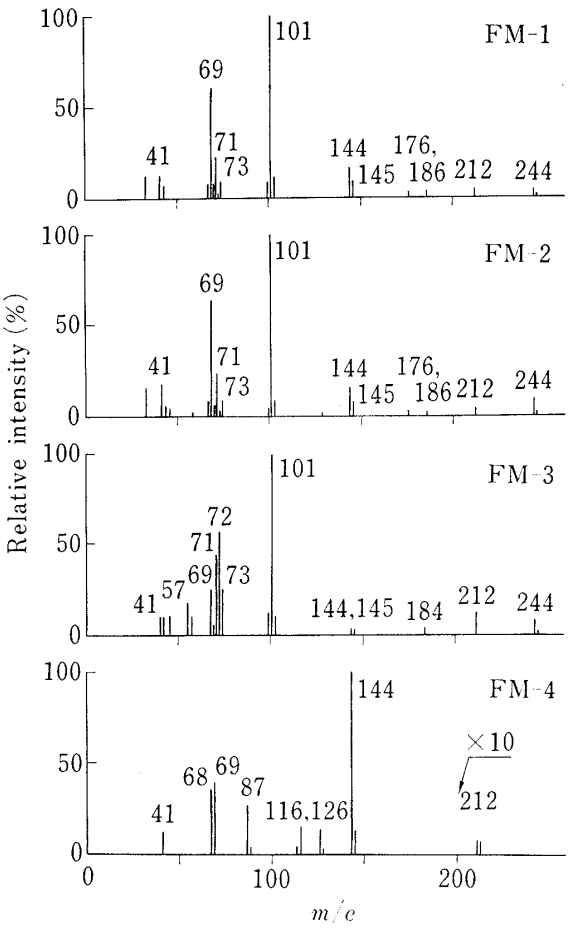

Fig. 3. EI Mass Spectra of Permethylated Derivatives of FT Metabolites extracted from Rat Liver Microsomal Suspension, and from Rat Plasma and Urine

of *m/e* 130, and a base peak of *m/e* 68 (C_4H_4O) or a fragment ion of *m/e* 69 (C_4H_5O), corresponding to a tetrahydrofuran ring but with 2 or 3 fewer hydrogens.

The EI mass spectra of the permethylated derivatives of the four metabolites were next measured by GC–MS using a methanolic solution of PTAH as an on-cloumn methylating agent. These mass spectra are shown in Fig. 3, and the high resolution mass spectral data are shown in Table I. The retention times of FM-1, FM-2, FM-3, FM-4, FT and 5-FU on GC–MS were 7.0, 7.2, 5.0, 3.0, 4.0 and 1.1 min, respectively. The shift of the M^+ ion of *m/e* 216 to 244 showed that methylation had occurred at 2 sites on permethylation of FM-1, FM-2 and FM-3. The shifts of the 5-FU ion of *m/e* 130 and 131 to *m/e* 144 and 145, and that of the fragment ion of *m/e* 87 to *m/e* 101 showed that methylation had occurred at the N-position of the 5-FU skeleton and at a hydroxyl group in the fragment ion of *m/e* 87. Further, fragment ions of *m/e* 212 and 69 were formed by loss of CH_3OH from the M^+ ion and the fragment ion of *m/e* 101, respectively. The mass spectra of the permethylated derivatives of FM-1 and FM-2 were very similar, but that of FM-3 differed from these in the relative intensity of the fragment ions of *m/e* 69—73, indicating a tetrahydrofuran ring moiety, The shift of the M^+ ion of *m/e* 216 observed at very low relative intensity showed that methylation of FM-4 had occurred at one site. The shift of the 5-FU ion of *m/e* 130 to the base peak of *m/e* 144 indicated that the methyl-

Table I. High Resolution Mass Spectral Data for Permethylated Derivatives of FT Metabolites extracted from Rat Liver Microsomal Suspension

Metabolite	*m/e*	Elemental composition	Observed mass	Calculated mass
FM-1	244	$C_{10}H_{13}FN_2O_4$	244.0853	244.0859
	212	$C_9H_9FN_2O_3$	212.0599	212.0597
	144	$C_5H_5FN_2O_2$	144.0333	144.0335
	101	$C_5H_9O_2$	101.0601	101.0603
	71	C_4H_7O	71.0497	71.0497
	69	C_4H_5O	69.0342	69.0340
FM-2	244	$C_{10}H_{13}FN_2O_4$	244.0852	244.0859
	212	$C_9H_9FN_2O_3$	212.0597	212.0597
	144	$C_5H_5FN_2O_2$	144.0334	144.0335
	101	$C_5H_9O_2$	101.0607	101.0603
	71	C_4H_7O	71.0501	71.0497
	69	C_4H_5O	69.0342	69.0340
FM-3	244	$C_{10}H_{13}FN_2O_4$	244.0862	244.0859
	212	$C_9H_9FN_2O_3$	212.0592	212.0597
	144	$C_5H_5FN_2O_2$	144.0336	144.0335
	101	$C_5H_9O_2$	101.0601	101.0603
	72	C_4H_8O	72.0571	72.0575
	71	C_4H_7O	71.0501	71.0497
FM-4	212	$C_9H_9FN_2O_3$	212.0600	212.0597
	144	$C_5H_5FN_2O_2$	144.0340	144.0335
	126	$C_5H_6N_2O_2$	126.0373	126.0371
	116	$C_4H_5FN_2O$	116.0391	116.0386
	87	C_3H_2FNO	87.0123	87.0120
	69	C_4H_5O	69.0345	69.0340
	68	C_4H_4O	68.0266	68.0262

ation of FM-4 had occurred at the N-position of the 5-FU skeleton. The fragment ions of *m/e* 68 and 69 showed no shift after permethylation.

From the results of mass spectrometry described above and the findings that FT has a molecular weight of 200 and the formula $C_8H_9FN_2O_3$, and that the base peak in the EI mass spectrum is *m/e* 71, indicating a tetrahydrofuran ring,[7] it is concluded that FM-1, FM-2 and FM-3 are metabolites containing a hydroxyl group on the tetrahydrofuran ring of FT, that FM-1 and FM-2 have very similar structures, and that FM-4 is a metabolite with a double bond in the tetrahydrofuran ring of FT.

The ^{1}H-NMR spectra were next examined to determine the position of the hydroxyl group and double bond.

The ^{1}H-NMR spectra showed that FM-1, FM-2, FM-3 and FM-4 each had an anomeric proton at C-2′. The signals of the anomeric proton at C-2′ of each metabolite are shown in Fig. 4. The chemical shifts and coupling constants (J) of the proton at C-6 and the anomeric proton at C-2′ of FT and the four metabolites are shown in Table II.

The anomeric protons at C-2′ of FM-1 and FM-2 (δ 6.23 and 6.15 ppm) appeared as a pseudotriplet-doublet and a doublet-pseudotriplet, respectively. These signals seemed to be due to splitting into a doublet-doublet by coupling with two vicinal protons (J_{vic}, 6.9—6.4 Hz for FM-1, 7.9—2.2 Hz for FM-2) and further splitting into a doublet by long-range coupling with fluorine at C-5 ($J_{H2'-F}$, 1.8 Hz for FM-1 and FM-2). Thus, both FM-1 and FM-2 have two protons at C-3′, and these two protons were assigned to a multiplet signal of δ 2.21 and 2.35 ppm for FM-1 and δ *ca.* 2.0 (doverlapping with a solvent signal) and 2.57 ppm for FM-2. These coupling forms were also confirmed by the spin-decoupling method.

7) T. Marunaka, Y. Umeno, Y. Minami, and T. Shibata, *Biomed. Mass Spectrom.*, "accepted".

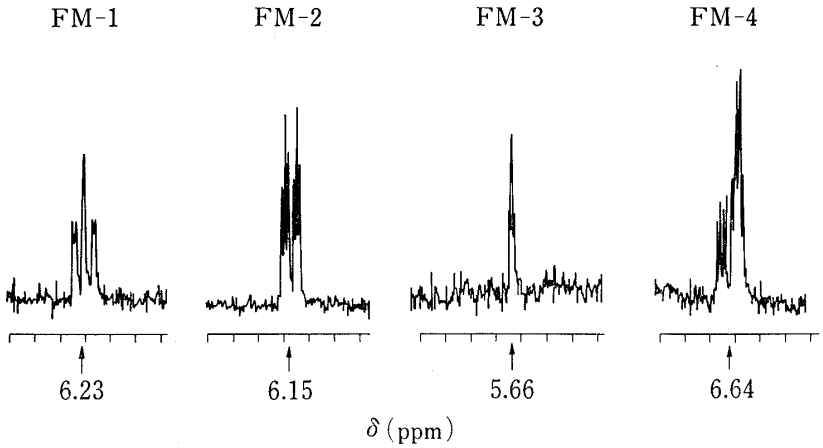

Fig. 4. Comparison of the Anomeric Proton (C-2′) Signals in the ^{1}H-NMR Spectra of FT Metabolites in Acetone-d_6 (TMS Internal Standard) after Extraction from Rat Liver Microsomal Suspension, and from Rat Plasma and Urine

The anomeric proton signal of FM-4 includes a C-5′ proton signal (δ 6.57 ppm, *q*).

Table II. Chemical Shifts and Coupling Constants of the C-6 Proton and C-2′ Anomeric Proton of FT and Its Metabolites extracted from Rat Liver Microsomal Suspension and from Rat Plasma and Urine

Metabolite	C-6-H (ppm)	C-2′-H (ppm)	$J_{C\text{-}6\text{-}H-F}$ (Hz)	$J_{C\text{-}2'\text{-}H-C\text{-}3'\text{-}H}$ (Hz)	$J_{C\text{-}2'\text{-}H-F}$ (Hz)	C-2′-H Signal[a]
FT	7.71	5.69	7.0	5.7(4.0)	1.8	*m*
FM-1	7.76	6.23	7.0	6.9(6.4)	1.8	*t–d*
FM-2	8.15	6.15	7.5	7.9(2.2)	1.8	*d–t*
FM-3	7.64	5.66	6.6	1.3	1.8	*t*
FM-4	7.51	6.64	6.6	9.7(4.4)	1.8	*d-q*

a) *d*, doublet; *t*, triplet; *q*, quartet; *m*, multiplet.

FM-1 showed two doublet-doublet signals at δ 3.38 and 4.32 ppm (*J*, 9.2—1.3 Hz, 9.2—4.0 Hz) and FM-2 showed two doublet-doublet signals at δ 3.88 and 4.16 ppm (*J*, 9.7—3.1 Hz, 9.7—1.3 Hz). These protons were concluded to be two protons at C-5′ on the basis of their chemical shifts and their coupling forms with a geminal proton and a vicinal proton.

Based on these results, it was concluded that FM-1 and FM-2 have one proton and a hydroxyl group at C-4′. The multiplet signals of δ 4.61 ppm for FM-1 and δ 4.57 ppm for FM-2 were assigned to the protons at C-4′, and the broad signals of δ *ca.* 4.4 ppm for FM-1 and δ *ca.* 4.6 ppm for FM-2 to the hydroxyl groups at C-4′.

Next, we investigated whether the hydroxyl groups of FM-1 and FM-2 were *cis* or *trans* relative to the 5-FU skeleton.

The signal of the anomeric proton at C-1′ of 1-(2′-deoxy-β-D-ribofuranosyl)-5-FU reported by Cushley *et al.*,[8] in which a hydroxyl group at C-3′ is *trans* to the 5-FU skeleton, showed a coupling similar to that of the anomeric proton at C-2′ of FM-1, while those of the anomeric protons at C-1′ of 1-(2′-deoxy-α-D-ribofuranosyl)-5-FU and 1-(2′-deoxy-β-D-lyxofuranosyl)-5-FU, in which the hydroxyl groups at C-3′ are *cis* to the 5-FU skeleton, showed coupling similar to that of the anomeric proton at C-2′ of FM-2.

It was reported that in the hydroxylated derivatives of tetrahydrofuran, the vicinal protons *trans* to a hydroxyl group are shifted downfield compared to the *cis* vicinal protons, and that

8) R.J. Cushley, I. Wempen, and J.J. Fox, *J. Am. Chem. Soc.*, **90**, 709 (1968).

the J values of the *cis* protons (J_{cis}) with respect to a vicinal proton are larger than those of the *trans* protons (J_{trans}).[9] Furthermore, it was reported that in 1-(2′-deoxy-β-D-ribofuranosyl)-uracil derivatives, the protons at C-2′ *trans* to a hydroxyl group at C-3′ are shifted downfield compared to the *cis* protons, and that J_{cis} between the protons at C-1′ and C-2′, and those at C-2′ and C-3′ was larger than J_{trans}.[10] These chemical shifts and J values are affected by the bases or substituents at C-5′ in cytidine and thymidine nucleosides derivatives.[11] However, FM-1 and FM-2 seem to be unaffected, because these compounds are uracil derivatives, and contain a tetrahydrofuran moiety but no ribose.

Thus, it is concluded that the proton at C-3′ of FM-1, showing J_{vic} 6.4 Hz by coupling with the anomeric proton at C-2′ and appearing at δ 2.35 ppm is *trans* with respect to both the anomeric proton and the hydroxyl group ,and that the other proton at C-3′ (J_{vic} 6.9 Hz, δ 2.21 ppm) is in the reverse relationship, while the proton at C-3′ of FM-2 (J_{vic} 7.9 Hz, δ 2.58 ppm) is *cis* to the anomeric proton and *trans* to the hydroxyl group, and the other proton at C-3′ (J_{vic} 2.2 Hz, δ *ca.* 2.0 ppm) is in the reverse relationship.

On the basis of the above results, FM-1 and FM-2 were identified as 1-(*trans*-4-hydroxytetrahydro-2-furanyl)-5-FU (*trans*-4′-OH-FT) and 1-(*cis*-4-hydroxytetrahydro-2-furanyl)-5-FU (*cis*-4′-OH-FT), respectively.

In addition, the bulky hydroxyl group of FM-2 seems to have much more effect than that of FM-1, since the proton at C-6 of FM-2 (δ 8.15 ppm) shifted downfield relative to that of FM-1 (δ 7.76 ppm).

The anomeric proton at C-2′ of FM-3 (δ 5.67 ppm) gave a pseudotriplet signal by long-rang coupling with a fluorine at C-5 ($J_{H2'-F}$ 1.8 Hz) and by coupling with a vicinal proton (J_{vic} 1.3 Hz). Thus, it was concluded that FM-3 has a one proton and a hydroxyl group at C-3′, corresponding to a multiplet signal at δ 4.46 ppm and the broad signal at δ *ca.* 4.7 ppm, respectively. The multiplet signals at δ *ca.* 2.15 ppm (overlapping with a solvent signal) and those at δ 4.15 and 4.37 ppm were assigned to the two protons at C-4′ and C-5′, respectively.

On the basis of similar considerations, the proton at C-3′ of FM-3 was concluded to be *trans* to the anomeric proton, and the hydroxyl group at C-3′ to be *cis*. Thus, FM-3 was identified as *trans*-3′-OH-FT.

The anomeric proton at C-2′ of FM-4 (δ 6.65 ppm) gave a doublet-quartet signal by coupling with two vicinal protons (J_{vic} 9.7—4.4 Hz) and further by long-range coupling with fluorine at C-5. This result indicates the existence of two protons at C-3′ and a double bond in the tetrahydrofuran ring between C-4′ and C-5′. The two protons at C-3′ were assigned to the multiplet signals at δ 2.77 and 3.22 ppm, and those of the protons at C-4′ and C-5′ to a pseudotriplet signal at δ 5.19 ppm and a quartet signal at δ 6.57 ppm, respectively. Thus, FM-4 was identified as 4′,5′-dehydro-FT.

Discussion

Four unknown metabolites of FT formed *in vitro* by rat liver microsomes were identified as *trans*-4′-OH-FT, *cis*-4′-OH-FT, *trans*-3′-OH-FT and 4′,5′-dehydro-FT by mass spectrometry and ^{1}H-NMR spectral analysis (Chart 1). These metabolites were also found in rat plasma and urine after administration of FT.

To determine the structures of the four metabolites, we compared these metabolites with authentic samples of *trans*-3′-OH-FT, *cis*-3′-OH-FT, 4′,5′-dehydro-FT, 3′,4′-dehydro-FT and 5′-OMe-FT. Authentic samples of 2′-OH-FT and 5′-OH-FT could not be obtained

9) J.P. Lambert, J.J. Papay, S.A. Khan, K.A. Kappauf, and E.S. Magyar, *J. Am. Chem. Soc.*, **96**, 6112 (1974).
10) J. Cadet, R. Ducolomb, and C. Taieb, *Tetrahedron Lett.*, **40**, 3455 (1975).
11) B. Fraser-Reid and B. Radatus, *J. Am. Chem. Soc.*, **93**, 6342 (1971); E.M. Nottori, J.B. Lambert and R.L. Letsinger, *J. Am. Chem. Soc.*, **99**, 3486 (1977).

because they are too unstable. Neither *trans*-4′-OH-FT nor *cis*-4′-OH-FT has yet been synthesized. The *Rf* values on TLC, and the mass spectral and ^{1}H-NMR spectral data for the authentic samples are shown in Table III.

Based on these data, FM-3 was concluded to be *trans*-3′-OH-FT and FM-4 to be 4′,5′-dehydro-FT.

Authentic samples of FM-1 and FM-2 could not be obtained, but the existence of a hydroxyl group at C-4′ was deduced from comparative studies.

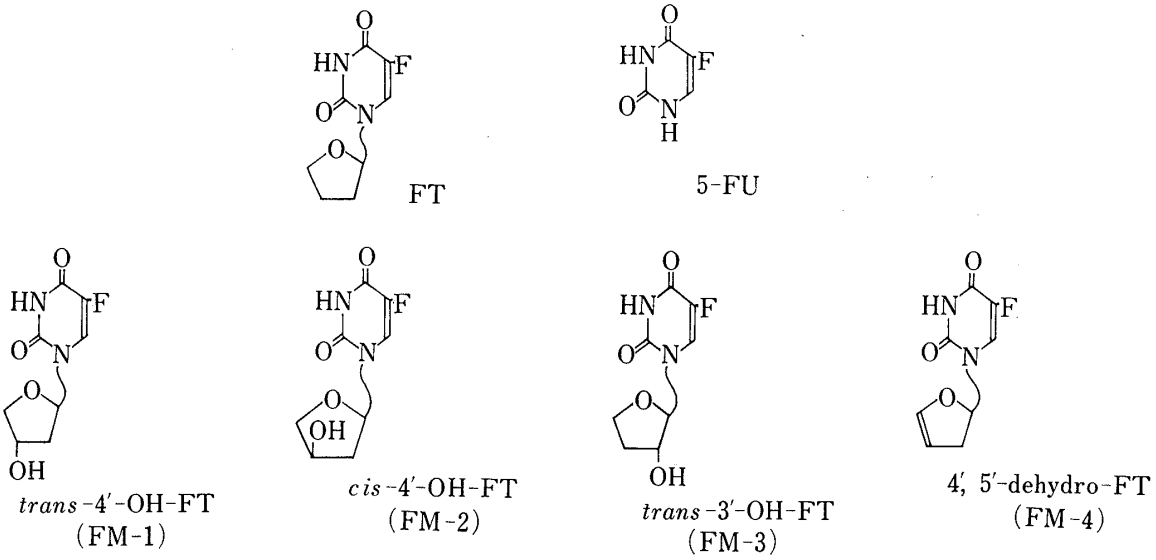

Chart 1. Structures of FT and Its Metabolites extracted from Rat Liver Microsomal Suspension and from Rat Plasma and Urine

Table III. Physicochemical Data for Authentic Samples

Method	*trans*-3′-OH-FT	*cis*-3′-OH-FT	3′,4′-Dehydro-FT	4′,5′-Dehydro-FT	5′-OMe-FT
TLC[a]					
Rf value	0.38	0.43	0.70	0.80	
MS[b]					
M$^+$ [*m/e*]	216 (23)	216 (23)	198 (16)	198 (12)	
M$^+$−FU [*m/e*]	87 (100)	87 (100)	69 (100)	69 (73)[c]	
M$^+$−FU−H_2O [*m/e*]	69 (21)	69 (32)	—	—	
GC–MS (permethylated)[b]					
M$^+$ [*m/e*]	244 (7)	244 (10)		212 (0.7)	244 (5)
M$^+$−FU [*m/e*]	101 (100)	101 (100)	Decomposed	69 (37), 68 (34)[d]	101 (100)
M$^+$−FU−CH_3OH [*m/e*]	69 (25)	69 (17)	to 5-FU	—	69 (50)
Retention time [min]	5.0	5.4		3.0	5.7
NMR (acetone-d_6)					
[C-6H]					
δ [ppm, signal]	7.64, *d*	7.65, *d*	7.43, *d*	7.52, *d*	
J(C-6H-F) [Hz]	6.6	7.0	6.6	6.6	
[C-2′H]					
δ [ppm, signal]	5.67, *t*	5.91, *q*	6.59, *m*	6.65, *d–q*	
J(C-2′H-C-3′H) [Hz]	1.3	3.5	6.2	9.7 (4.4)	
J(C-2′H-F) [Hz]	1.8	1.8	1.8	1.8	

a) Solvent; ethyl acetate: ether: acetic acid (20: 20: 1).
b) Numbers in parentheses show relative intensity.
c) Base peak of *m/e* 68 (C_4H_4O).
d) Base peak of *m/e* 144 ($C_5H_5FN_2O_2$).

On GC-MS of the permethylated derivatives, the retention time of FM-1, identified as *trans*-4′-OH-FT, was shorter than that of FM-2, identified as *cis*-4′-OH-FT, and the retention time of *trans*-3′-OH-FT was shorter than that of *cis*-3′-OH-FT. On comparison of the ^{1}H-NMR spectra of *trans*-3′-OH-FT and *cis*-3′-OH-FT, the anomeric proton at C-2′ *trans* to a hydroxyl group was shifted downfield compared to the *cis* anomeric proton. With regard to J_{vic} between the anomeric proton at C-2′ and a proton at C-3′, J_{cis} was larger than J_{trans}. This relation between the *cis* and *trans* forms seems to be applicable to FM-1 and FM-2.

Thus, by comparison with related authentic samples, FM-1 was concluded to be *trans*-4′-OH-FT, and FM-2 to be *cis*-4′-OH-FT. These conclusions require confirmation by comparison of the compounds with authentic samples.

Another metabolite was found by TLC in an experiment using ^{3}H-labeled-FT. It may be *cis*-3′-OH-FT, but could not be definitely identified because too little material was available for analysis.

FM-1 seems to be identical with metabolite M_2 or MH-1 reported by Wu *et al.*[3] and FM-4 with metabolite MH-4. However, FM-2 differs from metabolite M_1 or MH-2, and we did not isolate metabolite MH-3. FM-2 seems to be identical with metabolite X_1 reported by Benvenuto *et al.*[4] and FM-3 with metabolite X_2.

FT is a mixture of *R* and *S* isomers.[12] We could not determine the ratio of the optical isomers of the four metabolites from their mass spectra and ^{1}H-NMR spectra.

The three hydroxylated metabolites were not converted to 5-FU by incubation in phosphate buffer at 37° for 4 hr. They were converted by rat liver microsomes, but to a lesser extent than FT. The dehydrated metabolite was converted to much the same extent FT. Experiments are now in progress on the mechanism of conversion of FT to 5-FU in relation to the formation of the four metabolites described in this paper, and methods for quantitative determination of these metabolites are under investigation.

12) J.P. Horwitz, J.J. McCormik, K.D. Philips, V.M. Maher, J.R. Otto, D. Kessel, and J. Zémilićka, *Cancer Res.*, **35**, 1301 (1975); M. Yasumoto, A. Moriyama, N. Unemi, S. Hashimoto, and T. Suzue, *J. Med. Chem.*, **20**, 1592 (1977).