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Short Communication

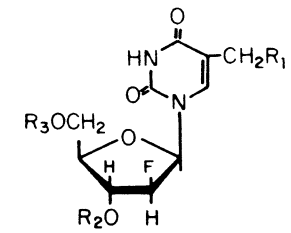
Structures of Metabolites Isolated from Urine of Mice Treated with the Antiviral Agent, 1-(2-Fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil

1-(2-Fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (*structure 1a*, fig. 1), which inhibits the *in vitro* replication of herpes simplex virus types 1 and 2 by more than 90% in concentrations of 0.04 μM (1–3), greatly prolongs the survival of herpes-infected mice (1). FMAU[1] is also chemotherapeutically effective against lines of murine leukemias which have acquired resistance to 1-β-D-arabinofuranosylcytosine (4). We report here the structures of two metabolites isolated from mouse urine.

Male mice were treated iv or po with 2-[^{14}C]FMAU (5) at a dose of 10 mg/512 μCi/kg. The specific activity and radiochemical purity of the drug before dilution were 12.4 Ci/mmol and 99.5%, respectively. Plasma was obtained at either 0.5 or 6 hr after drug administration and was deproteinized (6) prior to HPLC analysis. Urine was collected for 24 hr. A Spectra-Physics model 8000B HPLC instrument with a model 8400 UV detector set at a wavelength of 266 nm was used with a 25 × 4.6 cm Whatman Partisil ODS-3 column. Radioactivity of the eluant was determined in a Packard Tri-Carb model 3775 liquid scintillation spectrometer. The data obtained from HPLC of the samples are summarized in table 1.

Fig. 2 shows an HPLC separation of the radioactive components in urine from mice treated with both 2-[^{14}C]FMAU (10 mg/512 μCi/kg) and [*methyl*-^{3}H]thymidine (New England Nuclear, radiochemical purity of 97.3%) (10 mg/2 mCi/kg). Thymidine *in vivo* is degraded to thymine, dihydrothymine, and β-ureidoisobutyric acid (7) (each of which contains both the 5-methyl group and C-2 of the original thymidine). We surmised that these three products were present in the urine of these mice and were eluting with retention times between 3 and 12 min (thymine was retained for 11.5 min). The absence of ^{14}C radioactive peaks at these times suggested that the same three compounds were not being formed from FMAU by initial cleavage of the glycosyl bond. Since, in addition, the ^{14}C-containing peaks eluted at retention times characteristic of nucleosides for the mobile phase used, we postulated that these compounds contained intact glycosyl bonds.

To obtain sufficient material for structure determination of the metabolites, mice were treated iv with 2-[^{14}C]FMAU, 501 μCi/365 mg/kg. The 24-hr urine collection contained 93% of the radioactivity administered. HPLC analysis of the urine showed 97.2, 1.5, 0.2, and 0.6% of the recovered radioactivity as FMAU and as metabolites A, B, and C, respectively. Fractionation of the urine by HPLC yielded 0.98 mg of metabolite A and 0.3 mg of metabolite C.



1a) R_1, R_2, R_3 = H
1b) R_1 = OH; R_2, R_3 = H
1c) R_1 = H; R_2 and R_3 = H and

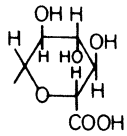


FIG. 1. *Structure of FMAU (1a), proposed structure for metabolite A (1b), and proposed partial structure for metabolite C (1c).*

The UV spectrum of metabolite A determined with a Cary 15 spectrophotometer had maxima and minima in 0.5 N HCl at 262 λ_{max} ($\epsilon = 9.4 \times 10^3$), 198 λ_{max} ($\epsilon = 9.9 \times 10^3$), and 223 λ_{min} ($\epsilon = 2.6 \times 10^3$); in H_2O, 262 λ_{max} ($\epsilon = 9.6 \times 10^3$), 208 λ_{max} ($\epsilon = 9.5 \times 10^3$), and 233 λ_{min} ($\epsilon = 2.6 \times 10^3$); and in 0.5 N NaOH, 263 λ_{max} ($\epsilon = 7.0 \times 10^3$), and 243 λ_{min} ($\epsilon = 4.6 \times 10^3$). Chemical ionization mass spectra of FMAU with a DuPont 21-492 coupled to a VG 2035 data system showed an abundant peak at *m/z* 261 $(M + 1)^+$ (100%) and a peak at *m/z* 127 (7.3%), corresponding to thymine. The spectrum of metabolite A had an $(M + 1)^+$ peak at *m/z* 277 (100%). This corresponded to FMAU with an extra oxygen. In addition, there were peaks at *m/z* 259 (46.3%, corresponding to loss of water) and at *m/z* 143 (3.6%, corresponding to thymidine + oxygen). There was no peak at *m/z* 127. These data are consistent with *structure 1b* in fig. 1. For exact molecular weight determination, the sample was analyzed by Cf-252 fission fragment ionization. The experimental value obtained, which includes one sodium atom, was 299.073, which corresponds to a molecular weight of 276.083. The calculated molecular weight of the compound is 276.078.

The ^{1}H NMR (D_2O) of metabolite A (external TMS standard on a Fourier transform NMR, JEOL JNNPF 100) showed the following: δ 3.67 (2H, d, H-5′), 3.80 (1H, m, H-4′), 4.15 (2H, s, 5-CH_2), 4.28 (1H, double q, H-3′, $J_{3'F} = 18.9$, $J_{2',3'} = 3.1$, $J_{3',4'} = 2.4$ Hz), 5.04 (1H, double q, H-2′, $J_{2',F} = 52.5$, $J_{1',2'} = 4.0$, $J_{2'3'} = 3.1$ Hz), 6.11 (1H double d, H-1′, $J_{1',F} = 14.7$, $J_{1',2'} = 4.0$ Hz), and 7.65 (1H broad s due to allylic couplings, H-6).

Metabolite A was synthesized by refluxing a mixture of 5-hydroxymethyluracil (3.5 g, 25 mmol) and $(NH_4)_2SO_4$ (30 mg) in hexamethyldisilazane (50 ml) for 5 hr and then evaporating to dryness *in vacuo*. The residue was treated with a solution of 3-*O*-acetyl-5-*O*-benzoyl-2-fluoro-2-deoxy-2-fluoro-D-arabinosyl bromide (8) in CH_2Cl_2 and the mixture was stirred at room temperature for 4 days. The mixture was then refluxed for 10 hr,

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[1] Abbreviations used are: FMAU, 1-2(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil; FMAUMP, FMAU monophosphate; FMAUDP, FMAU diphosphate; FMAUTP, FMAU triphosphate; D_2O, deuterium oxide; TMS, tetramethylsilane.

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TABLE 1

The per cent recovered radioactivity in plasma and urine collections, comprising FMAU and its metabolites after treatment with 2-[^{14}C]FMAU, 10 mg/kg

Source	Time after Administration	Route of Administration	FMAU	Metabolite A	Metabolite B	Metabolite C
	hr		%	%	%	%
Plasma	0.5	po	94.9	2.2	0.2	2.0
	0.5	po	94.0	2.4	0.2	2.8
	0.5	iv	94.2	2.9	0.2	1.8
	0.5	iv	95.0	2.9	0.2	1.1
	6	po	82.0	15.3	0.2	2.0
	6	po	85.5	9.6	0.1	4.4
	6	iv	78.4	18.7	0.2	1.9
	6	iv	83.9	13.2	0.2	2.2
Urine	24	po	77.4	13.4	1.2	6.4
	24	po	70.2	19.0	1.7	7.1
	24	iv	80.8	11.6	1.2	4.6
	24	iv	86.3	6.9	0.7	4.3

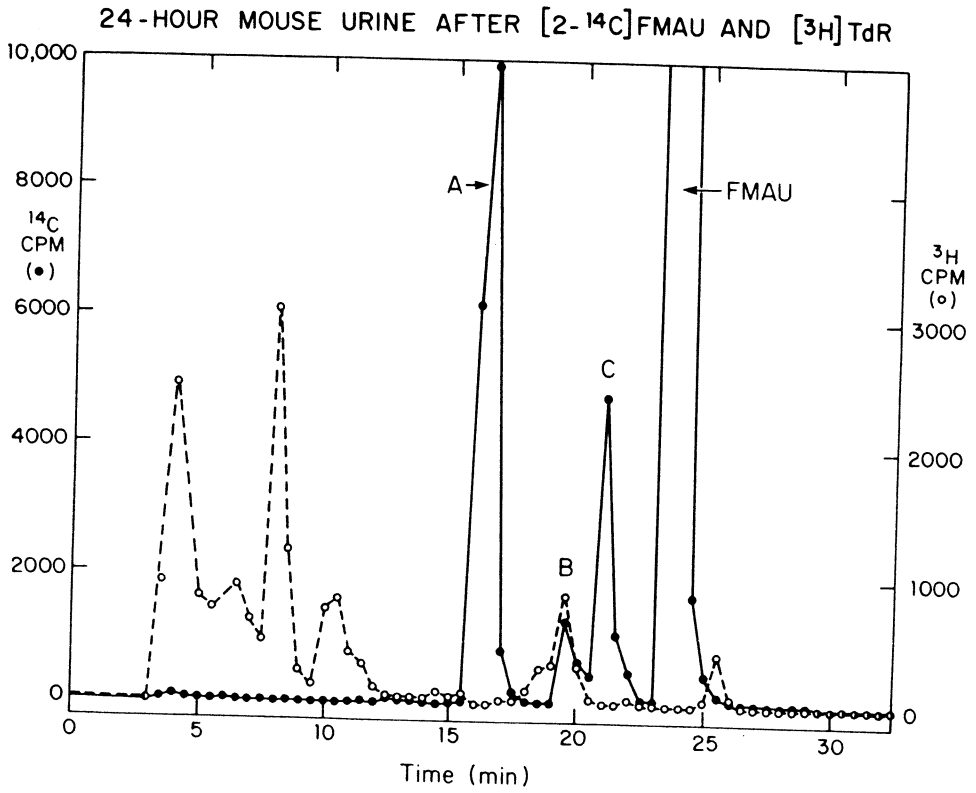

FIG. 2. *Radioactivity profile obtained when urine from mice treated with [2-^{14}C]FMAU, 10 mg/kg, and [methyl-^{3}H]thymidine (TdR), 10 mg/kg, was analyzed by HPLC.*

The compounds were eluted with a gradient of 0–30% methanol in 0.02 M phosphate, pH 3.0, over 30 min. ---, ^{3}H; ——, ^{14}C.

cooled to room temperature, diluted with MeOH (10 ml), and filtered through a Celite pad. The filtrate was concentrated *in vacuo* and the residue was chromatographed on a silica gel G-60 column (26 × 5 cm) using $CHCL_3$-MeOH (40:1, v/v) as the eluent. The fractions containing the major nucleoside component were evaporated to give the crude, protected nucleoside (3.5 g) as a syrup. The syrup was suspended in saturated NH_3/MeOH (50 ml) and the mixture was stirred overnight at room temperature. The solvent was removed by evaporation *in vacuo* and the residue chromatographed on a silica gel column (25 × 5 cm) using $CHCl_3$-MeOH (20:1) as the eluent. The fractions containing the nucleoside were combined and evaporated to dryness *in vacuo*, and the crystalline residue was recrystallized from MeOH to give 550 mg of 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-hydroxymethyluracil, m.p. 182–183°C. Analysis calculated for $C_{13}H_{13}O_6N_2F$: C 43.48; H 4.74, N 10.14; found: C 43.61; H 4.77, N 10.08.

The UV, MS, NMR, and HPLC spectra for the isolated metabolite A were identical to those of the synthetic compound.

The retention times of metabolite C, in the mobile phase

described in fig. 2 with buffer, pH 3.0, 4.0, 5.0, 6.0, and 7.0, were 20.6, 17.2, 13.7, 11.8, and 5.8 min, respectively, which were consistent with the presence of an acidic moiety. The UV spectra of the metabolite in acid, water, and base was identical to that of FMAU. The possibility of metabolite C being FMAUMP was ruled out because it had retention times on HPLC different from synthetic FMAUMP and because it was resistant to alkaline phosphatase.

Mass spectral measurements did not show high molecular mass numbers. In conventional MS measurements, we obtained peaks at m/z 127 and 261 corresponding to thymine and FMAU. These spectra were similar to those obtained with FMAUMP. Silylated FMAUMP obtained by heating 10 μg of FMAUMP in 10 μl of tetrahydrofuran with 19 μl of *N,O*-bis(trimethylsilyl) acetamide and 1 μl of trimethylchlorosilane under a nitrogen atmosphere at 70°C for 0.5 hr showed an $(M + 1)^+$ at m/z 557 corresponding to FMAUwith 3 silyl groups. However, silylated metabolite C failed to give identifiable high molecular weight peaks. Although FMAUMP on negative Cf-252 fission fragment ionization gave a tiny peak at m/z 339 corresponding to $(M - 1)^-$, no high molecular weight peaks were obtained for metabolite C with either positive or negative ionization.

Fourier transform NMR of metabolite C showed the proton absorptions of metabolite A except that there was no peak at σ 4.1 corresponding to the 5-CH_2 protons but, rather, a singlet at δ 2.2 corresponding to a 5-CH_3 methyl group; there was an additional very broad complex multiplet at δ 3.6–4.6 whose number of protons was not determinable and another multiplet at δ 5.7 corresponding to one proton. Treating metabolite C and 6 N NaOH for 3 hr at 95°C caused some degradation but no conversion to FMAU. However, treating the compound with 6 N NCl at 95°C for 1 and 3 hr gave 50 and 100% conversions, respectively, to FMAU in addition to minor degradative products. β-Glucuronidase enzyme (2 mg) in acetate buffer, pH 4.5, completely hydrolyzed metabolite C (0.4 μg) to FMAU upon incubation at 37°C for 17 hr. This conversion was blocked by the presence of glucaro-1,4-lactone (0.5 μM), an inhibitor of β-glucuronidase (9).

The retention times for FMAU, metabolite C, FMAUMP, FMAUDP, and FMAUTP were 2.2. 6.5, 11.7, 19.3, and 30.2 min, respectively, on a Whatman SAX anion exchange column eluted with a mobile phase of 0.01 M phosphate, pH 4.1, for 5 min, followed by a gradient to 0.6 M phosphate, pH 4.1, over 25 min and then isocratic elution with the 0.6 M phosphate for 10 min at a flow rate of 1.5 ml/min at 40°C. In this system, metabolite C co-chromatographed with synthetic 5-fluorouridine glucuronide (10).

On the basis of the data, we postulate the structure of metabolite C to be *structure Ic* (fig. 1) with the position of glucuronide attachment to the furanose ring unknown.

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