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Syntheses and Biological Activities of N^1-(2-Formylethyl)-5-fluorouracil and Related Compounds

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Derivatives of 5-fluorouracil(5-FU) substituted at the *N*-1 position were synthesized in order to investigate their enzymatic conversion to N^1-(2-formylethyl)-5-FU, which is expected to give 5-FU with concomitant release of acrolein. Proton nuclear magnetic resonance spectroscopic studies of authentic N^1-(2-formylethyl)-5-FU suggested that the compound exists in an equilibrium mixture of free aldehyde and cyclic hemiacetal forms. Spontaneous liberation of 5-FU from N^1-(2-formylethyl)-5-FU at neutral pH was demonstrated by thin-layer chromatography using both the authentic compound and N^1-(3-aminopropyl)-5-FU after treatment with amine oxidase, but the release of 5-FU seemed to be limited. Cytotoxicity was observed with N^1-(2-formylethyl)-5-FU and N^1-(3-aminopropyl)-5-FU, but none of the derivatives of 5-FU presently prepared showed a positive effect on P388 leukemia inoculated into mice.

Keywords——5-fluorouracil; acrolein; cytotoxicity; drug design; amine oxidase

In the previous paper,[1] we reported that various cytotoxic 3-substituted propylamines [R-X-$(CH_2)_3NH_2$: R=alkyl, phenyl, benzyl; X=NH, O, S] were oxidatively deaminated *in vitro* by amine oxidase (AO) to give 3-substituted propanals, some of which, especially when R was phenyl, were then decomposed to yield R-XH and acrolein. It is conceivable that a combination of two mechanisms involving the propanals and acrolein is responsible for the cytotoxicity of 3-substituted propylamines. This mode of action suggests that compounds enzymatically convertible to 3-substituted propanals could be regarded as masked derivatives of the parent compound (R-XH) in the viewpoint of drug design. One example is cyclophosphamide, which is available as an anticancer agent or an immunosuppressor. Cyclophosphamide exerts its biological effects by spontaneous liberation of aldophosphamide, an alkylating agent, and acrolein after oxidation by mixed function oxidase (MFO).[2,3] Acrolein liberated from this drug was suggested to be responsible for its urotoxic side-effects.[4,5] However, in view of the significant role of acrolein in the teratogenicity of cyclophosphamide,[6,7] a possible co-operative effect of acrolein in the manifestation of the biological activity of this drug cannot yet be ruled out. Considering enzymatic reactions leading to the formation of 3-substituted propanals, we attempted to synthesize several derivatives substituted at the *N*-1 position of 5-fluorouracil (5-FU, **1**), in anticipation of the release of **1** and acrolein through the propanal formed by their enzymatic oxidation.

Chemistry

Syntheses

Derivatives of **1** that we planned to prepare in the present work were N^1-(3-aminopropyl)-5-FU (**6**), N^1-(3-hydroxypropyl)-5-FU (**5**), and 2,3-dihydro-7-fluoro-4*H*,8*H*-

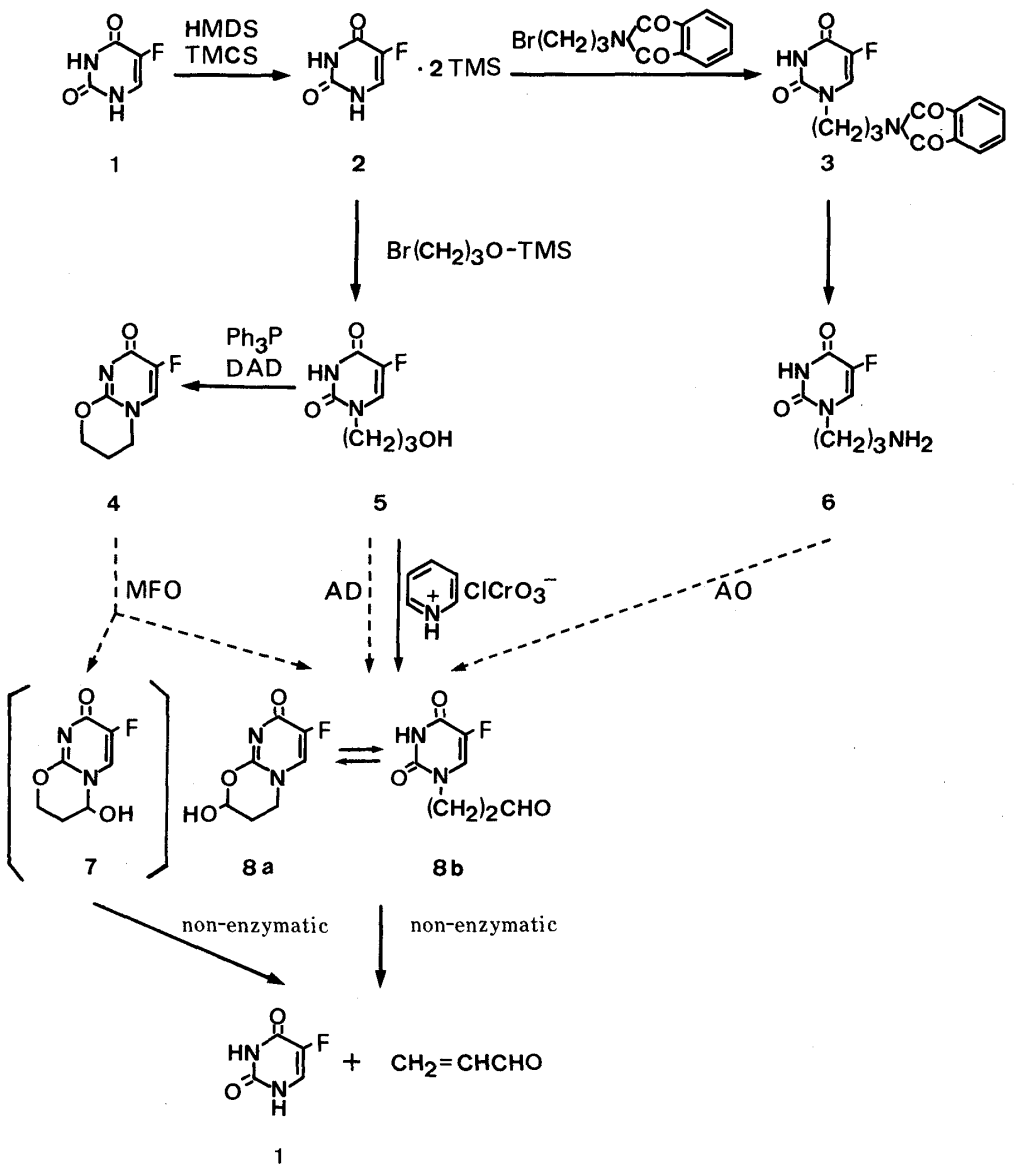

Chart 1

pyrimido[2,1-*b*][1,3]oxazine-8-one (cyclo 5-FU, **4**), which were expected to be converted to N^1-(2-formylethyl)-5-FU (**8b**) by AO, alcohol dehydrogenase (AD) and MFO, respectively, and **8b** as such. The procedures used for the syntheses of these compounds are summarized in Chart 1 together with the expected enzymatic oxidation of each product. Compound **6** (as the hydrochloride) and **5** were prepared by alkylation of trimethylsilylated 5-FU (TMS-5-FU, **2**) with *N*-3-bromopropylphthalimide and trimethylsilylated 3-bromopropanol, respectively, followed by deprotection. The yields were about 50% from **1**. Compound **4** was synthesized by dehydrocyclocondensation of **5** with triphenylphosphine (Ph_3P) and diethyl azodicarboxylate (DAD) according to the method used previously for the synthesis of 2,5′-anhydropyrimidine nucleosides.[8] The key compound **8b** was prepared by the oxidation of **5** with pyridinium chlorochromate.[9] An attempt to prepare the propanal (**8b**) by an alternative method based on direct alkylation of **2** with 3-halopropionaldehyde diethylacetal was unsuccessful.

Physicochemical Properties of 8b

In the course of a study on the proton nuclear magnetic resonance (^{1}H-NMR) spectra of

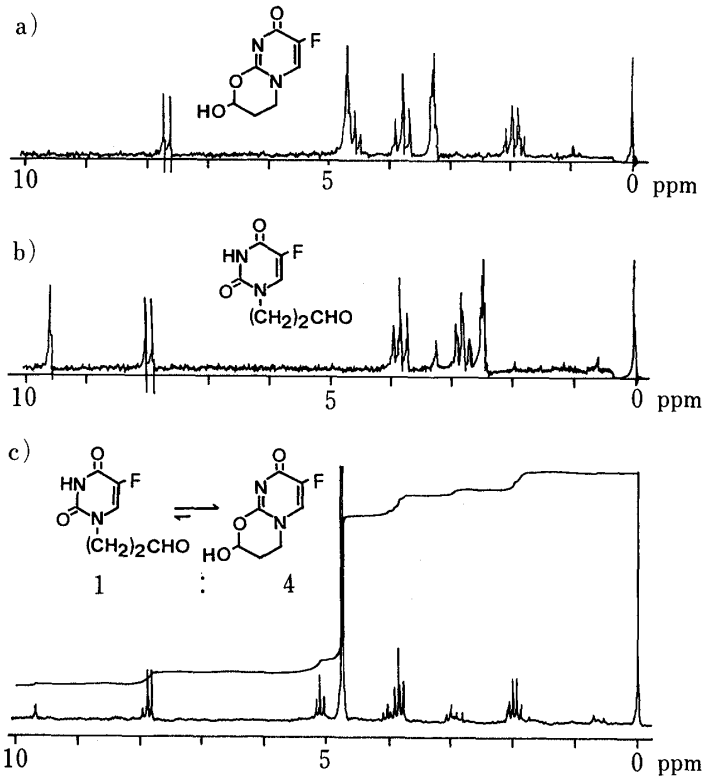

Fig. 1. NMR Spectra of N^1-(2-Formylethyl)-5-FU in Various Solvents

a) Recorded in CD_3OD (60 MHz). b) Recorded in DMSO-d_6 (60 MHz) after evaporation of CD_3OD. c) Recorded in D_2O (100 MHz, FT-^{1}H-NMR).

8b, it was found that the aldehyde group of this compound interacted with the carbonyl group at position 2 of the 5-FU moiety to form a cyclic hemiacetal (**8a**), depending on the solvent used. As shown in Fig. 1a, the spectrum obtained in deuteromethanol-d_4 (CD_3OD) clearly indicated the cyclic structure (Chart 1) for the compound, showing triplet and quartet peaks at 4.57 and 1.92 ppm assignable to a proton on the hemiacetal carbon and protons adjoining the hemiacetal, respectively, while no signal due to the aldehyde proton was observed. On the other hand, the sample redissolved in deuterodimethylsulfoxide-d_6 (DMSO-d_6) after removal of CD_3OD gave a spectrum (Fig. 1b) that showed a signal due to the aldehyde proton at 9.6 ppm and triplet peaks at 2.82 ppm assignable to the protons adjoining the aldehyde group, with the disappearance of the triplet at 4.57 ppm. These spectral data indicate that N^1-(2-formylethyl)-5-FU may exist in solution as an equilibrium mixture of the two forms, acyclic (**8b**) and cyclic (**8a**), depending on the solvent used. Indeed, the Fourier-transform (FT) ^{1}H-NMR spectrum recorded in deuterium oxide (D_2O) demonstrated the presence of the two forms in a ratio of approximately 4 to 1 for **8a** to **8b** based on the peak heights (Fig. 1c). Repeated ^{1}H-NMR spectral measurement of the same sample of N^1-(2-formylethyl)-5-FU in D_2O after standing for 20 h at 25 °C, showed no observable change in the spectrum, suggesting that the compound (equilibrium mixture) is fairly stable.

Stability of N^1-(2-formylethyl)-5-FU at various pH values was examined by thin-layer chromatography (TLC). The compound was fairly stable at pH 3.0, no liberation of **1** being observed even after incubation for 24 h at 37 °C. At pH 7.0, liberation of a trace amount of **1** was observed after 3 h; the amount increased linearly in the course of incubation, and about 50% of the compound had decomposed to **1** after 9 h. In borate buffer (pH 9.0), the liberation of **1** was significant after 1 h, and only a trace amount of the compound remained as such after 24 h.

In order to examine the reactivity of N^1-(2-formylethyl)-5-FU under physiological conditions, the compound was incubated with amino acids such as L-lysine, L-serine, L-cysteine, and *N*-acetyl-L-cysteine in phosphate buffer (0.2 M, pH 7.0) at 37 °C, and the products were analyzed by TLC. It was found that of the amino acids used, L-cysteine alone reacted rapidly with N^1-(2-formylethyl)-5-FU. Thus, after 1 h incubation the compound had disappeared from the reaction solution with the concomitant appearance of a new spot which was visualized with ultraviolet (UV) light (254 nm) but not with 2,4-dinitrophenylhydrazine reagent and ninhydrin reagent. It seems reasonable to suppose that **8b** reacts with cysteine to form a new compound having a thiazolidine ring, in view of the general reaction of cysteine with aldehydes under physiological conditions.[10]

Enzymatic Oxidation

Compound **6** was incubated with commercial AO, and the products formed were examined by TLC (Fig. 2). As expected, the formation of N^1-(2-formylethyl)-5-FU was demonstrated with subsequent release of 5-FU in the course of incubation for 24 h. Although the amounts were not accurately determined, **6** seemed to be a much poorer substrate than benzylamine for the enzyme.

Compound **5** was also incubated with AD from a commercial source, and the products formed were examined by TLC. The reaction resulted in slight production of N^1-(2-formylethyl)-5-FU, but no release of **1** was observed even after incubation for 24 h (data not shown).

Possible enzymatic oxidation of **4** was examined by using crude MFO freshly prepared from rat liver according to the usual procedure.[11] There are two oxidation pathways through **7** and **8a**, both leading to the release of **1** and acrolein, as shown in Chart 1. However, neither N^1-(2-formylethyl)-5-FU nor **1** was detected by TLC after this oxidation.

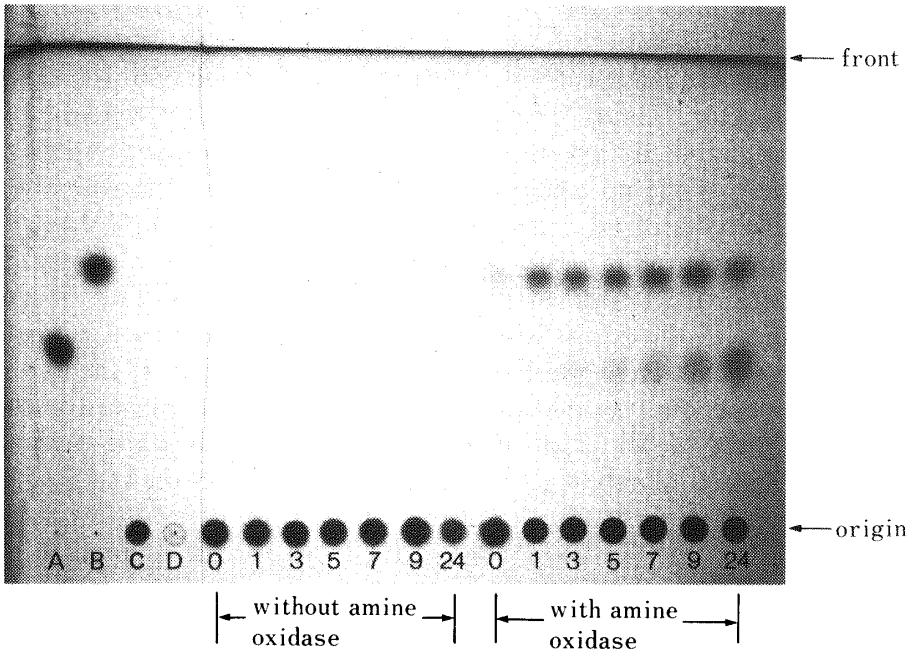

Fig. 2. Thin-Layer Chromatogram of an Incubation Mixture of **6** with Amine Oxidase

Compound **6** (0.4 μmol) was incubated at 37 °C with amine oxidase (0.00564 units) in 0.2 M sodium phosphate buffer (pH 7.0, 40 μl). Aliquots (2 μl) of the incubation mixture were placed on plates at the time intervals indicated (0, 1, 3, 5, 7, 9, 24 h), and developed with solvent I.

A, **1** (20 nmol); B, **8a**, **b** (20 nmol); C, **6** (20 nmol); D, amine oxidase (0.000282 units).

There was a possibility that the enzymes involved in the conversion of the substrates to N^1-(2-formylethyl)-5-FU might have been inhibited by acrolein liberated after their own catalytic reactions, but this was ruled out, since an addition of acrolein had no significant effect on the enzyme activities, as tested with benzylamine or ethanol for AO or AD (data not shown). These *in vitro* studies, although carried out with only a few enzyme preparations, indicate that derivatives of **1** might not be efficiently metabolized to N^1-(2-formylethyl)-5-FU *in vivo*.

Biological Activities

Cytotoxicity

The cytotoxic effects of **1** and its derivatives on BALB/3T3, transformed BALB/3T3 (by 3-methylcholanthrene) and MDBK cells were examined by dye exclusion, and the results are shown in Table I. All the compounds except for N^1-(2-formylethyl)-5-FU reduced the cell viability by less than 30% at 0.4 mM (the highest concentration tested) in the presence of fetal bovine serum (FBS). On the other hand, **6** inhibited the viability of MDBK cells in the presence of calf serum (CS) with an IC_{50} value of 0.15 mM, and N^1-(2-formylethyl)-5-FU inhibited the viability of the three cell lines non-specifically, irrespective of the serum used, with a similar IC_{50} value. The results suggested the occurrence of oxidative deamination of **6** by AO in CS. The IC_{50} values observed with the three different cell lines again indicate the cytotoxicity of 3-substituted propanals, from which acrolein could be liberated.[1)]

Table I. Cytotoxicity of **1** and Its Derivatives[a)]

Compound No.	Cell line	IC_{50} (mM) BALB/3T3	Transformed BALB/3T3	MDBK	
	Serum	FBS	FBS	FBS	CS
1		—[b)]	—	—	—
6		—	—	—	0.15
5		—	—	—	—
4		—	—	—	—
8a, b		0.19	0.15	0.13	0.16

a) Details of the cytotoxicity test are described under Experimental. *b*) Less than 30% inhibition at 0.4 mM concentration.

Antitumor Effect

The effects of the newly synthesized compounds on P388 leukemia inoculated into BDF_1 mice were compared with that of **1**. At the same dose, *e.g.* 0.2 nmol/kg/d for 5 d, **1** showed a 93% increase in life span (ILS), whereas the synthetic compounds showed no ILS except for N^1-(2-formylethyl)-5-FU, which gave 9% ILS. These results might have been predicted from the *in vitro* enzymatic studies and the stability of N^1-(2-formylethyl)-5-FU, as described above.

The presently designed derivatives of 5-FU were unfortunately ineffective against P388 leukemia, but with reference to a report by Yamashita *et al.*,[12)] it may be useful to modify the carbonyl group in 5-FU (*e.g.*, O-substituted derivatives of 5-FU, which could be metabolized to 3-substituted propanals). It may be worthwhile to apply the principle described above to some other drugs.

Experimental

Materials——Compound **1** was purchased from Asahi Chem. Inc. Co., Ltd. Hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), bis(trimethylsilyl)acetamide, bromopropanol, DAD and amino acids were obtained from Tokyo Kasei, Ltd. Ph_3P was purchased from Wako Pure Chem. Ind., Ltd. and *N*-(3-bromopropyl)-phthalimide from Aldrich Chem. Co., Inc. Amine oxidase (EC 1.4.3.4) from bovine plasma (28.2 units/g using benzylamine as a substrate) was purchased from Miles Laboratories (PTY) Ltd., while alcohol dehydrogenase (EC 1.1.1.1) from bakers' yeast (365 units/mg using ethanol as a substrate), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and glucose-6-phosphate were from Sigma Chemical Co., Ltd. Microsomes from the liver of Sprague-Dawley male rats (36 mg protein/ml) were prepared by the procedure described previously.[11] Protein concentration of the microsomes was determined by the method of Lowry *et al.*,[13] using bovine serum albumin as the reference standard. Nicotinamide adenine dinucleotide phosphate (NADP) was purchased from Oriental Yeast Co., Ltd., Tokyo. All organic solvents and reagents were of analytical reagent grade.

For column chromatography, a cation exchange resin (Dowex 50w-×8, 200—400 mesh, Dow Chemical Co.), and Sephadex LH-20 (Pharmacia Fine Chemicals) were used.

Cells——Three cell lines were used for the cytotoxicity testing. MDBK cells were kindly supplied by Dr. H. Shibuta, Institute of Medical Science, University of Tokyo. BALB/3T3 and transformed BALB/3T3 (by 3-methylcholanthrene) were gifts from Dr. T. Kuroki, Institute of Medical Science, University of Tokyo.

Animals——Male BDF_1 mice were used for the test on P388 leukemia and were obtained from Charles River Japan Inc., Kanagawa-ken.

Instrumental Analysis——UV absorption spectra were measured in 95% ethanol solution with a Hitachi 200-20 spectrometer. ^{1}H-NMR spectra were measured on a Hitachi R-24-B spectrometer at 60 MHz and a JNM FX-100 spectrometer at 100 MHz. Chemical shifts are expressed in δ with tetramethylsilane or 3-(trimethylsilyl)-propanesulfonic acid sodium salt as an internal standard; s=singlet, d=doublet, t=triplet, q=quartet, and m=multiplet.

TLC——TLC was performed on precoated silica gel plates (Silica gel 60 F-254, E. Merck AG, Darmstadt, G.F.R.). Preparative TLC was done on 20×20 cm glass plates (2 mm thickness). The following solvents were used: solvent I, $CHCl_3$–EtOH (9 : 1); solvent II, BuOH–AcOH–H_2O (3 : 2 : 2); solvent III, EtOAc–HCOOH (99 : 1); solvent IV, MeOH. Spots were visualized with UV light (254 nm), and N^1-(2-formylethyl)-5-FU was also detected with 2,4-dinitrophenylhydrazine reagent. Amino acids were detected with ninhydrin reagent.

***N*^{1}-(3-Hydroxypropyl)-5-FU (5)**——Compound **1** (1.3 g) was trimethylsilylated with HMDS (4.5 ml) and TMCS (0.45 ml) at 140 °C for 4 h, and removal of excess HMDS and TMCS by evaporation gave **2**. Trimethylsilylated bromopropanol, which was prepared by reacting bromopropanol (2.26 ml) with bis(trimethylsilyl)acetamide (7.42 ml) and TMCS (0.7 ml) at room temperature, was added to **2** in dried acetonitrile (20 ml) under argon gas. The mixture was allowed to stand at 125 °C for 3 d in a sealed glass tube. After addition of MeOH, the solvent was distilled off, and the residue was dissolved in H_2O and washed with $CHCl_3$. The aqueous phase was applied to a column of Dowex 50 (H^+) (60 ml) and eluted with H_2O. The fraction containing **5** was concentrated to a small volume, which was subjected to column chromatography on Sephadex LH-20 (190 ml). **5** (Rf=0.49, solvent III) was eluted with $CHCl_3$–MeOH–HCOOH (19 : 1 : 0.2). After evaporation of the solvent, the residue was recrystallized from 95% EtOH to give **5** in a yield of 44% (Table II).

2,3-Dihydro-7-fluoro-4*H*,8*H*-pyrimido[2,1-*b*]-[1,3]oxazine-8-one (Cyclo 5-FU) (4)——Compound **5** (117.1 mg) and Ph_3P (445.9 mg) were dissolved in pyridine (5 ml) and the mixture was stirred at room temperature for 30 min. DAD (0.2 ml) was added dropwise to the above mixture under cooling, and the whole was allowed to stand for 10 min, then stirred at room temperature overnight. The precipitate produced was washed with a mixture of pyridine (0.1 ml) and ehter (0.2 ml), and recrystallized from MeOH to give **4** (Rf=0.38, solvent IV) in a yield of 22% (Table II).

***N*^{1}-(3-Aminopropyl)-5-FU (6)**——A solution of *N*-(3-bromopropyl)-phthalimide (2.68 g) in dried acetonitrile (20 ml) was added to **2** (corresponding to 1.3 g of **1**) under argon gas. The mixture was allowed to stand at 125 °C for 3 d in a sealed glass tube. After addition of MeOH, the solvent was removed by distillation, and the residue was suspended in $CHCl_3$ and washed with H_2O. A yellow solid residue obtained after removal of $CHCl_3$ from the organic layer was recrystallized from dioxane to give crystalline **3** (Rf=0.26, solvent I) in a yield of 77%. **3** (0.485 g) was treated with a mixture (10 ml) of acetic acid and conc. HCl (1 : 1) in a sealed glass tube at 110 °C for 4 d. The reaction mixture was allowed to cool, and precipitated phthalic acid was removed by filtration. The filtrate was concentrated to dryness, and the residue was dissolved in H_2O (250 ml). After removal of insoluble phthalic acid, the solution was applied to a column of Dowex 50 (H^+) (80 ml) using 2 N HCl as the eluent. The first eluate (40 ml) was discarded, and the following eluate (100 ml) was collected. After evaporation of hydrochloric acid, the residue was recrystallized from 95% EtOH to give **6** as the hydrochloride (Rf=0.43, solvent II) in a yield of 54% (overall yield from **1**) (Table II).

***N*^{1}-(2-Formylethyl)-5-FU (8a, b)**——Pyridinium chlorochromate (431.1 mg) was added to a solution of **5** (188.1 mg) in acetonitrile (20 ml). The mixture was allowed to react at 70 °C for 4 h, then cooled and passed through a Celite bed. The solvent was removed and the residue was subjected to preparative layer chromatography (developed

TABLE II. Physicochemical Properties of 5-FU Derivatives

Compound No.	mp (°C)	Analysis (%) Calcd (Found) C	H	N	^{1}H-NMR (δ)	UV λ_{max} nm (ε)
6	234—236	37.60 (37.70	4.96 4.95	18.79 18.70)	In DMSO-d_6 1.89 (m), 2.79 (t), 3.70 (t), 8.16 (d), 8.6—9.3 (br)	269—271 (5703)
5	148—149	44.68 (44.74	4.82 4.80	14.89 14.79)	In DMSO-d_6 1.82 (m), 3.53 (t), 3.78 (t), 4.3—4.4 (br), 7.66 (d)	272—274 (9011)
4	244—248	49.41 (49.39	4.15 4.25	16.46 16.27)	In CD_3OD 2.27 (m), 4.05 (t), 4.52 (t), 7.76 (d)	256 (7762) 231 (9110)
8a, b	140—141	45.15 (45.03	3.79 3.72	15.05 14.78)	In DMSO-d_6 2.82 (t), 3.86 (t), 7.98 (d), 9.6 (s) In CD_3OD 1.92 (q), 3.78 (t), 4.57 (t), 7.68 (d) In D_2O 1.99 (q), 3.02 (t), 3.86 (t), 4.04 (t), 5.12 (t), 7.85 (d), 7.91 (d), 9.69 (s)	273 (8989)

three times with III). The fraction positive to 2,4-dinitrophenylhydrazine reagent was extracted with EtOAc to give N^1-(2-formylethyl)-5-FU (Rf=0.40, solvent III; Rf=0.35 solvent I) in a yield of 48% (Table II).

Stability of N^1-(2-Formylethyl)-5-FU at Various pH——A buffer solution (1 ml) containing N^1-(2-formylethyl)-5-FU (186.7 μg) was incubated at 37 °C, and at 1, 3, 6. 9 and 24 h, and aliquot (1 μl) of the solution was examined by TLC using solvent I. The buffer solutions used were as follows: 0.2 M citrate buffer, pH 3.0; 0.2 M phosphate buffer, pH 7.0; 0.2 M borate buffer, pH 9.0.

Reaction of N^1-(2-Formylethyl)-5-FU with Amino Acids——A mixture of N^1-(2-formylethyl)-5-FU (0.01 M) and an amino acid (0.01 M) in phosphate buffer (0.2 M, pH 7.0) was incubated at 37 °C, and at 1, 3 and 24 h, an aliquot (1 μl) of the reaction mixture was examined by TLC using solvents I and II.

Enzymatic Oxidation——**6**: AO (0.00564 units) in phosphate buffer (0.2 M, pH 7.0) solution (20 μl) was added to **6** (0.02 M) in the same buffer solution (20 μl) and the mixture was incubated at 37 °C. After 1, 3, 5, 7, 9 and 24 h, an aliquot (2 μl) of the incubation mixture was examined by TLC using solvents I and II. Phosphate buffer was used instead of enzyme solution in the blank.

5: The method of Racker[14] was used, based on the absorption of NADPH at 340 nm. Thus nicotinamide adenine dinucleotide (NAD) (1.0 mg), **5** (1.38 μmol) and pyrophosphate buffer (0.06 M, pH 8.8) solution (3.0 ml) were placed in a quartz cell, and the absorption at 340 nm was read. The reaction was started by the addition of 0.04 ml of AD (14.6 units/ml). The mixture was allowed to stand at room temperature, and the absorbance was again read at 340 nm. Ethanol was used as a positive control.

4: The incubation mixture contained 360 μg of microsomal protein, Tris–HCl buffer (0.1 M, pH 7.4), NADPH-generating system (1.5 mM NADP, 15 mM glucose-6-phosphate, 15 mM $MgCl_2$, and 1.47 units of glucose 6-phosphate dehydrogenase) and 50 mM **4** in a final volume of 0.1 ml. The mixture was incubated at 37 °C, and at 1 and 24 h, an aliquot (1 μl) was examined by TLC using solvent IV.

Cytotoxicity——The dye exclusion test was carried out as follows: BALB/3T3 cells and transformed BALB/3T3 cells were grown in Eagle's MEM with 10% FBS (Lot No. 97951, M.A. Bioproducts, MD, U.S.A.). MDBK cells were grown in Eagle's MEM with 10% FBS or CS (Lot No. R265423, GIBCO, NY, U.S.A.). The cell suspension (5×10^5 cells/5 ml of medium) was seeded in a disposable bottle (NUNCLON, 25 cm^2, Nunc, Denmark). After incubation of the cells at 37 °C for 3 d, 0.1 ml of test compound dissolved in phosphate-buffered saline was added to the bottles through a Millipore filter. After further incubation for 16 h, cells were harvested by the usual 0.05% or 0.125% trypsin treatment and viable cells were estimated by the trypan blue exclusion test. Cell counting was done with a microscope.

Antitumor Activity——Male BDF_1 mice were inoculated intraperitoneally (*i.p.*) with 10^6 P388 leukemia cells and given test compound (*i.p.*) once a day for 5 consecutive days beginning 24 h after inoculation of the cells. The inhibitory effects of test compounds were expressed in terms of ILS.

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