2,4-Dimethyl-5-hydroxymethylpyrimidine, a Pyridoxol Antagonist[1]

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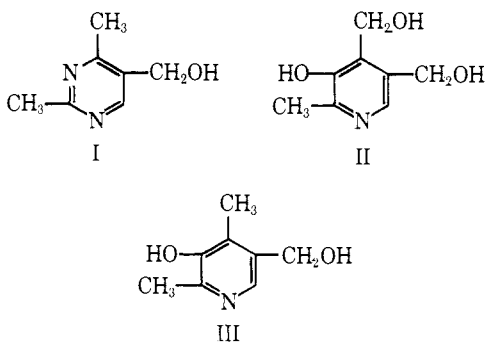
2,4-Dimethyl-5-hydroxymethylpyrimidine has been prepared from 2,4-dimethyl-5-carbethoxypyrimidine and has been evaluated as an antitumor agent and as a pyridoxol antagonist. Insignificant inhibition of several rodent tumors was found at maximum tolerated doses. No growth inhibition of Sarcoma 180 ascites tumor was observed in Swiss female mice on a B_6-deficient diet. Intraperitoneal injection of 3 mg./kg. or more produced lethal convulsions in mice. These seizures were reversed by pyridoxol in equivalent doses and by 4-deoxypyridoxol in a slightly larger dose.

As part of a program directed toward the preparation of "3-azapyridoxol," the synthesis and biological activity of 2,4-dimethyl-5-hydroxymethylpyrimidine (I) were investigated. The structural similarity of I to pyridoxol (II) and to the known antagonist, 4-deoxypyridoxol (III), suggested its synthesis. Furthermore,

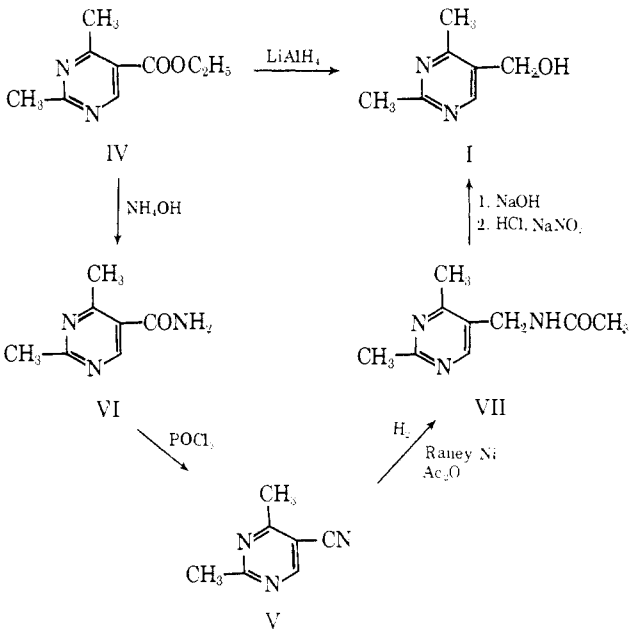


the observations of Pressman and Siegel[2] have indicated the hydration of annular nitrogens *in vivo*. Hydration of the 3-nitrogen of I in this manner would render its similarity to II and III even more pronounced.

Compound I has been synthesized from 2,4-dimethyl-5-carbethoxypyrimidine (IV) by two routes (Scheme I). Condensation of acetamidine with ethyl ethoxymethyleneacetoacetate, by the procedure of Urban and Schnider,[3] gave IV. Attempts to effect the conversion of IV to I by reduction with lithium aluminum hydride at room temperature led only to a product tentatively identified as a tetrahydro derivative of I. The desired conversion was effected by the $LiAlH_4$ reduction of IV at $-70°$.

Due to the early difficulty encountered, the preparation of 2,4-dimethyl-5-pyrimidinecarbonitrile (V) and its subsequent conversion to the carbinol I was investigated. Urban and Schnider[3] reported the preparation of 2,4-dimethyl-5-pyrimidinecarboxamide (VI) by treating IV with alcoholic ammonia at 100°. We have found that this conversion occurs in concentrated aqueous ammonia at room temperature.

SCHEME I



The amide VI was converted to the nitrile V with phosphorus oxychloride in refluxing xylene. Hydrogenation of V using the method of Gould[4] gave 2,4-dimethyl-5-acetamidomethylpyrimidine (VII). Hydrolysis of VII to the amine with dilute alkali and subsequent deamination with nitrous acid gave carbinol I.

Noteworthy is the fact that the carbinol apparently was obtained in two crystalline habits, as shown by differences in their respective mineral oil infrared spectra in the 9–10-μ (C–OH) region. A lower melting material (55–57°) exhibited strong absorption at 9.66 μ; the higher melting substance (59.5–61.0°) was characterized by an intense band at 9.40 μ. The two samples, however, gave superimposible spectra in carbon tetrachloride and exhibited identical vapor phase chromatographic properties. Furthermore, transformation of the low-melting sample to the higher melting material occurred on prolonged standing at room temperature. Finally, recrystallization of the low-melting sample in one instance gave the higher melting material. Attempts to reverse this trans-

(1) This investigation was supported by Public Health Service Grant No. CA-02857-08 from the National Cancer Institute. Reported in preliminary form in a previous communication: T. J. Schwan, J. F. Holland, and H. Tieckelmann, *J. Heterocyclic Chem.*, **1**, 299 (1964).

(2) D. Pressman and M. Siegel, *J. Am. Chem. Soc.*, **79**, 994 (1957).

(3) R. Urban and O. Schnider, *Helv. Chim. Acta*, **41**, 1806 (1958).

(4) F. E. Gould, G. S. Johnson, and A. F. Ferris, *J. Org. Chem.*, **25**, 1658 (1960).

Table I
Effect of Vitamin B_6 Analogs on Growth of Cultures of L929 Mouse Fibroblasts[a]

Compd. III Concn., γ/ml.	Compd. I Concn., γ/ml. 0	10	100	500
0	9.0	9.4	9.8	9.1
10	8.8	7.5	9.0	8.8
100	7.9	7.2	5.0	6.7
1000	2.0	3.1	3.0	1.7

[a] Growth measured as protein accumulation expressed in multiples of initial tube content. Each value represents mean of three tubes. Medium contained 1.0 γ of II (pyridoxine)/ml. Tubes fed daily for 5 days.

formation have not been successful. The realization of two habits from the same solvent was due to variations in the crystallization procedures. These observations are similar to those of Okuda and Price[5] who have reported that 4-amino-5-hydroxymethyl-2-methylthiopyrimidine exhibits a similar polymorphism.

Materials and Results

Cultures of mouse fibroblasts strain L929 in Eagle's medium[6] and 10% horse serum showed no growth inhibition from I, and no potentiation of the growth inhibitory activity of III (Table I).

The growth of ascites tumors transplanted into the subcutaneous tissues was studied after intraperitoneal injections of I dissolved in water. Treatment was begun 24 hr. after tumor transplantation and continued daily for 6 of 7 days. Krebs 2 carcinoma, Ehrlich carcinoma clone 2, and Sarcoma 180 ascites tumors in 28-g. Swiss female mice, leukemia P815 in 25-g. BDF1 male mice, and leukemia L1210 in 25-g. DBA2 male mice were evaluated. Groups of five mice were treated with 0.1, 0.25, 0.5, 1.0, or 2.0 mg./kg./day of I and contrasted against 10 control mice receiving water for each tumor type. No reduction in excised tumor weight by as much as 40% of control was seen in any of the experiments, and in each tumor type some toxic deaths occurred at the highest dose.

Swiss female mice with subcutaneous Sarcoma 180 ascites tumor cells were studied while on a synthetic diet devoid of vitamin II, or the same diet containing 10 mg./kg. of II (Table II). Two to five animals in the control and treated groups had total leukocyte counts measured the day before tumor inoculation and one day before sacrifice. No growth inhibition was seen from I on either diet. In contrast, treatment with III on the deficient diet led to 74% inhibition of tumor growth in the presence of substantial weight loss. On the day before termination of the experiment, since there was no appreciable alteration in tumor size of animals receiving I at 0.1 mg./kg./day, a single dose of 2 mg./kg. of I was given. This led to convulsive death in 3 of 7 animals on the complete diet but all 7 of those on the deficient regimen. There were no significant alterations in total leukocyte counts.

Toxicity of I was assessed in female Swiss mice weighing 25 g. Doses of 1, 1.5, and 2 mg./kg./day were tolerated for 6 days without evident toxicity. A dose of 3 mg./kg./day given to 6 animals killed all by the third day. Single injections of 6 and 9 mg./kg./day caused deaths within 2 hr. from seizures.

The acute lethality of I was preventable by equal doses of II. The reversal was time dependent. When given at −60, −15, 0, or +15 min. with respect to I, II protected against lethality. However, injections of II at −24 or +1 hr. before or after I administration did not prevent I deaths. In another group of five animals treated with 3 mg./kg. of I, rescue with vitamin II was attempted when three showed evidence of intoxication, but this was unsuccessful in two.

Acute deaths from the drug are due to central nervous system activity of the compound leading to seizures. There is a sharp threshold for convulsant activity: 3 mg./kg./day is lethal within 2 hr. in the majority of all animals studied, and 2 mg./kg./day for 6 days usually causes no seizures. Thus, 24 hr. between intraperitoneal injections is enough to avoid cumulative toxicity. No clear abbreviation of killing time is apparent from increasing the dose above 3 mg./kg. (Table III).

Protection by simultaneous administration of II is partial at doses of II less than those of I, although no protection is evident when the ratio of I to II is 9:1 or greater (Table IV).

Table II
Treatment of Subcutaneous Sarcoma 180 in Swiss Female Mice[a]

Compd.	Dose, mg./kg./day	Complete diet: Mean tumor wt., mg.	Complete diet: Mean body wt., g.	Complete diet: Deaths	B_6-deficient diet: Mean tumor wt., mg.	B_6-deficient diet: Mean body wt., g.	B_6-deficient diet: Deaths
H_2O		1666	26.5	2	1912	33	5
I	1.0	2555	28	1	2546	24	4
	0.3	1841	26.6	1	1877	26.6	1
	0.1	2554	27.5	3[b]	...		7[b]
III	30.0				509	20	1

[a] Ten animals inoculated with 10^6 S180 ascites tumor cells subcutaneously in each water control group, 7 in each I treatment group, and 10 in III. All treatments were intraperitoneal for 6 days weekly; sacrifice was on the 22nd day. Animal weight at onset of experiment was 25 ± 2 g. [b] See text.

Table III
Convulsions from Single Intraperitoneal Doses of 2,4-Dimethyl-5-hydroxymethylpyrimidine[a]

Dose, mg./kg.	Deaths of 5 mice	Mean convulsive death time, min.
2	0	...
3	4	106
6	5	70
9	5	82
27	5	92

[a] Five Swiss female mice (25 g. each) at each dose.

(5) T. Okuda and C. C. Price, *J. Org. Chem.*, **22**, 1719 (1957).
(6) H. Eagle, *Science*, **130**, 432 (1959).

Table IV
Toxicity of 2,4-Dimethyl-5-hydroxymethylpyrimidine[a]

Compd. II Dose, mg./kg.	Compd. I Dose, mg./kg. 0	3	6	9	27
0		0.8[b]	1	1	1
0.1		1			
0.3		0.8	1	1	
1		0.4	0.8	1	
3	0	0	0.8	0.8	
6			0	0.2	
9	0		0	0	
27	0				0
Compd. III Dose, mg./kg.					
3	0	1	1		
6	0	0	0.8		
50			0		

[a] Five normal female Swiss mice (25 g. each) were given single injections of indicated drugs within 1 min. of each other. [b] Mortality for groups of five is indicated by 0 to 1. Deaths occurred at 60–120 min. with seizures, or not at all.

At equivalent II and I doses over the 9-fold range from 3 to 27 mg./kg. of each, lethality is prevented. Similarly, the B_6 analog III can protect against acute lethal toxicity of I but not with equivalent potency. Twice the dose of III eliminated acute fatalities.

Protection from seizures by III allowed the test of ordinarily supralethal doses of I as a tumor inhibitor without the objections that would attend concomitant use of the vitamin II. At 4.5 times an LD_{100}, significant tumor inhibition was not seen (Table V).

Table V
Treatment of Subcutaneous Sarcoma 180 in Swiss Female Mice with Combinations of B_6 Analogs[a]

Compd.	Dose, mg./kg./day	Mean tumor wt., mg.	Δ mean body wt., g.	Deaths
Water		604	1.3	0
I	6	...	...	5
III	15	390	0.6	0
	30	562	−1.0	0
	60	580	0.2	0
I + III	6 + 15	454	1.6	1
	9 + 30	622	0.4	0
	27 + 60	380	−2.8	3

[a] Ten animals inoculated with 10^6 S180 ascites tumor cells subcutaneously in water control group, five in each treatment group; sacrifice was on the 7th day.

Discussion

The effects of I are manifest in the central nervous system where presumably it acts as a convulsant because of antagonism to vitamin B_6 in pyridoxal phosphate requiring enzymatic pathways. Since there is no abbreviation of convulsive death time by increase in the dose, the limiting phenomenon is probably saturation of an active transport mechanism into brain rather than passive diffusion. On dietary deprivation of B_6, the lethal toxicity of the drug is probably enhanced.

Catalytic doses of B_6 as obtained in complete diet, or minimum doses, do not protect, whereas pharmacologic doses of II have graded effects and can completely prevent seizures when, within specific time periods, it is given in doses equal to I. This suggests competition for a single pathway.

Finally, it is probable that III competes for the same transport mechanism or enzyme receptor site insofar as subconvulsive doses of this drug can reverse the lethal convulsive toxicity of I when given in 2–8 times the concentration of the convulsant. Thus, the relative affinities of the three compounds for the hypothetical transport receptor site would be II, I, and III, in that order. It is possible that activities on the central nervous system include a time lag for 5-phosphorylation to occur.

Compound I is ineffective in high dose against L929 cells in culture. It was inactive at the maximum tolerated dose in five transplanted animal neoplasms during complete dietary feeding and was ineffective against subcutaneous Sarcoma 180 transplants during dietary deprivation of B_6 under conditions when the known tumor inhibitor III[7,8] exercised a 74% growth inhibitory effect. Insignificant effect on tumor growth was found when convulsions expected with ordinarily supralethal doses were avoided by the simultaneous administration of III. Because of its extremely potent and rapid effects upon the central nervous system as a convulsant, the drug may prove a useful tool for elucidation of the role of vitamin B_6 functions in brain. Its similarity to "toxopyrimidine" (4-amino-2-methyl-5-hydroxymethylpyrimidine)[9] and other pyrimidines which cause convulsions in rodents,[10] however, provides alternative possible interpretations.

Experimental Section[11]

2,4-Dimethyl-5-hydroxymethylpyrimidine (I) from IV.—Lithium aluminum hydride (2.4 g., 0.064 mole) and 100 ml. of ether were stirred at room temperature for 30 min. After the slurry was cooled to −70° in a Dry Ice–methanol bath, a solution of 5.4 g. (0.03 mole) of IV[3] in 15 ml. of ether was added slowly. After stirring was continued at −70° for 30 min., 10 ml. of glacial acetic acid was added slowly, and the mixture was allowed to warm to room temperature. The reaction mixture was concentrated to dryness *in vacuo*, and the residue was then stirred with 120 ml. of 2.5 *N* HCl for 15 hr. at room temperature. After neutralization with dilute NaOH, the mixture was continuously extracted with ethyl acetate for 2 days. The solvent was removed *in vacuo*, and the residue was taken up in 100 ml. of water. The aqueous solution was extracted with two 50-ml. portions of benzene to remove unreacted ester and then concentrated to dryness *in vacuo* to give 2.2 g. (54%) of the crude carbinol which solidified upon cooling. An analytical sample was obtained by recrystallization from ligroin (b.p. 100–115°), m.p. 59.5–61.0°.

Anal. Calcd. for $C_8H_{10}N_2O$: C, 60.85; H, 7.30; N, 20.28. Found: C, 60.77, 60.88; H, 7.32, 7.47; N, 20.40, 20.42.

The infrared spectrum of a Nujol mull exhibited absorption at 3.17 (OH), 6.33 (C=N), and 9.40 μ (C–OH).

2,4-Dimethyl-5-pyrimidinecarboxamide (VI).—A mixture of 40 g. (0.22 mole) of IV and 200 ml. of concentrated aqueous NH_3 was stirred at room temperature for 24 hr. The mixture was concentrated to dryness *in vacuo* to give 33.3 g. (99%) of the crude amide, m.p. 165–177°. An analytical sample was obtained by recrystallization from ethyl acetate; m.p. 189.5–191.0°, lit.[3] m.p. 191–192°.

(7) E. Mihich and C. A. Nichol, *Cancer Res.*, **25**, 153 (1965).

(8) H. E. Skipper, J. R. Thomson, and F. M. Schabel, Jr., *Cancer Chemotherapy Rept.*, **29**, 63 (1963).

(9) K. Makino, T. Konoshita, and T. Sasaki, *Nature*, **173**, 34 (1954).

(10) F. Rosen, J. F. Holland, and C. A. Nichol, *Proc. Am. Assoc. Cancer Res.*, **2**, 243 (1957).

(11) Melting points were taken on a Mel-Temp apparatus and are corrected. Infrared spectra were determined using a Beckman IR-5A spectrophotometer. Vapor phase chromatographic analyses were carried out on an F & M Model 500 gas chromatograph with a 0.25-in. o.d. 3-ft. stainless steel column packed with 20% silica gum rubber on glass beads. The chromatograms were run isothermally at 165° using helium as a carrier gas (60 cc./min.). Microanalyses were performed by Drs. G. Weiler and F. B. Strauss, Oxford, England.

Anal. Calcd. for $C_7H_9N_3O$: C, 55.62; H, 6.00; N, 27.80. Found: C, 55.82; H, 6.15; N, 27.74.

2,4-Dimethyl-5-pyrimidinecarbonitrile (V).—A mixture of 15.3 g. (0.101 mole) of VI, 50 ml. of $POCl_3$, and 200 ml. of xylene was stirred and refluxed for 8 hr. After excess $POCl_3$ and xylene were removed *in vacuo*, the residue was taken up in 400 g. of ice water, and the acidic solution was neutralized with dilute NaOH. Extraction with four 500-ml. portions of chloroform gave, after removal of the chloroform *in vacuo*, 6.5 g. (49%) of the crude nitrile. An analytical sample was obtained by vacuum sublimation at 35° (0.1 mm.), m.p. 50.5–53.0°.

Anal. Calcd. for $C_7H_7N_3$: C, 63.14; H, 5.30; N, 31.56. Found: C, 62.99; H, 5.33; N, 31.17, 31.31.

The infrared spectrum of a Nujol mull exhibited absorption at 4.49 (C≡N) and 6.34 μ (C=N).

2,4-Dimethyl-5-acetamidomethylpyrimidine (VII).—A mixture of 8.3 g. (0.067 mole) of V, 200 ml. of acetic anhydride, 3 g. of anhydrous sodium acetate, and 5 g. of Raney nickel was hydrogenated at 3–4 atm. for 24 hr. at room temperature on a Parr apparatus. The mixture was heated to boiling and filtered. After the catalyst was boiled with 100 ml. of additional acetic anhydride and the combined filtrates were concentrated to dryness *in vacuo*, the residue was extracted with two 400-ml. portions of boiling CCl_4. Removal of the solvent from the combined extracts gave 4.8 g. (40%) of the crude product. An analytical sample was obtained by recrystallization from heptanes; m.p. 88.5–90.0°.

Anal. Calcd. for $C_9H_{13}N_3O$: C, 60.32; H, 7.31; N, 23.45. Found: C, 59.98; H, 6.98; N, 23.60.

The infrared spectrum of a Nujol mull exhibited absorption at 3.05 (NH), 6.13 (amide C=O), and 6.31 μ (C=N).

2,4-Dimethyl-5-hydroxymethylpyrimidine (I) from VII.—A solution of 4.8 g. (0.026 mole) of VII and 50 ml. of 2 *N* NaOH was refluxed for 5 hr. The solution was neutralized with concentrated HCl and 2.5 ml. of the concentrated acid was added in excess. A solution of 5 g. (0.07 mole) of sodium nitrite was added. The mixture was stirred at 60° for 15 hr., neutralized with dilute NaOH, and continuously extracted with ethyl acetate for 19 hr. After removal of the ethyl acetate, the residue was extracted with two 400-ml. portions of boiling CCl_4. Removal of the solvent from the combined extracts *in vacuo* gave 1.4 g. (39%) of the crude carbinol. The product was recrystallized from ligroin to give a white crystalline solid, m.p. 55–57°.

The infrared spectra (mineral oil mulls) of various samples of I showed small but discernible differences in the 9–10-μ region and indicated that two crystalline habits were formed because of variations in the crystallization temperature. Solution spectra of the two samples in CCl_4 were identical. The infrared spectrum of the 55–57° melting material reported in this experiment exhibited absorption at 3.15 (OH), 6.31 (C=N), and 9.66 μ (C–OH).

Methanolic solutions of the two samples were analyzed by vapor phase chromatography using the conditions described above. Each of these solutions contained only one component, the retention time of which was identical in both cases. When the components from the two chromatograms were collected, they exhibited identical infrared spectra. Upon analysis of a mixture of the two solutions, only peak enhancement was observed.

The Effect of Some Sulfur-Containing Pyridine Derivatives on the Carbohydrate Metabolism of Ehrlich Ascites Tumor[1]

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The effect of 22 pyridine derivatives on the carbohydrate metabolism of Ehrlich ascites tumor was studied. Most of the compounds are pyridinethiols, sulfides, and disulfides; several of them are new compounds, of which the synthesis is described. 2,2′-Dithiodipyridine was found to inhibit respiration and glycolysis; pyridines containing the grouping $-(CH_2)_xS-$ (with $x = 1$ or 2) in the 4 position inhibited oxygen uptake and increased lactate accumulation; 5-nitro-2-pyridinethiol and the corresponding disulfide and thioether had the common property of stimulating oxygen uptake in the presence of added glucose.

In connection with a cancer chemotherapy research project, we have synthesized a series of pyridinethiols, sulfides, and disulfides, several of which are new. Although they were prepared primarily as model compounds, we have tested their effect on certain aspects of the metabolism of Ehrlich ascites tumor.

As is known, some key enzymes of the glycolytic pathway contain sulfhydryl groups in their active centers and are thus susceptible to interaction with other thiols or disulfides. Furthermore, nicotinamide adenine dinucleotide (NAD) acts as coenzyme for the dehydrogenases involved in glycolysis. It has been established[2] that certain carcinostatic alkylating agents exert their action by decreasing the availability of NAD to the cell, thus causing an inhibition of glycolysis. In addition, there are indications that NAD in ascites tumor cells may be synthesized by a route different from that of normal cells.[3] This may provide a basis for selective inhibition of the energy-yielding metabolism of tumor cells.

The properties studied are: oxygen uptake in the absence and presence of added glucose, and aerobic and anaerobic glycolysis. Among the 22 compounds reported here, we have found different types of activities, which can to some extent be correlated to structural features.

Materials and Methods

Manometric Experiments.—Swiss mice, bearing 5- to 10-day-old ascites tumors, were sacrificed by cervical fracture. The fluid was collected, heparinized, and used immediately. Manometric determinations were carried out in a conventional Warburg apparatus at 37°. Readings were taken every 5 min. for 1 hr. after introduction of the compound under study. Lactic acid was determined by the method of Barker and Summerson.[4]

When oxygen uptake was measured, the gas phase was air. The main compartment of the flask contained 2.5 ml. of heparin-

(1) This investigation was supported by Public Health Service Research Grant CA 07296, from the National Cancer Institute.

(2) (a) H. Holzer, P. Glogner, and G. Sedlmayr, *Biochem. Z.*, **330**, 59 (1958); (b) H. Holzer and H. J. Boltze, *Z. Krebsforsch.*, **64**, 113 (1961).

(3) (a) H. Holzer, G. Friedrich, and H. Grisebach, *Biochim. Biophys. Acta*, **51**, 600 (1961); (b) H. Holzer and H. Kroeger, *Biochem. Z.*, **330**, 579 (1958).

(4) S. B. Barker and W. H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).