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Cytidine Derivatives[1]

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The synthesis of Cl- and Br-cytidine by a photocatalytic process and the preparation of aminocytidine from the bromo derivative is described. Evidence that the site of substitution is the 5-position is presented. The 5-substituted cytidine compounds are more active than the corresponding uridine derivatives for inhibiting the growth of *Neurospora* 1298.

Derivatives of uridine with substituent replacing the hydrogen atom on the number 3 nitrogen,[2] the number 5 carbon,[3,4] or both atoms,[2] inhibit the growth of certain microörganisms,[5] molds,[2,3,6] and retard propagation of Theiler's GD VII virus *in vitro*.[7] Since cytidine is incorporated into PNA and DNA pyrimidines much more efficiently than is uridine,[8,9] it seemed reasonable to predict that cytidine derivatives might be better antimetabolites than the corresponding uridine derivatives. To investigate this possibility, 5-chlorocytidine, 5-bromocytidine and 5-aminocytidine sulfate were prepared. Dimethylcytidine[10] and 1-ribofuranosyl-5-methylcytosine[4] are derivatives of cytidine reported previously.

Synthesis of cytidine derivatives by condensation of bromoacetoribofuranose and the appropriate pyrimidine was not attempted because the yield of products is very low,[4] and there is evidence that pyrimidine nucleosides prepared in this manner may not be identical to enzymatically-prepared compounds.[11] 1-Ribofuranosyl-5-methylcytosine and 1-ribofuranosyl-5-methyluracil, prepared by total synthesis, are biologically inactive in the systems studied[4] and are not cleaved by resting cell suspensions of *E. coli*, whereas several uridine and cytidine derivatives prepared from naturally-occurring nucleosides are readily oxidized by *E. coli*.[5] For these reasons cytidine,[11a] isolated from RNA, was used as the starting material.

Although iodocytosine[12] and bromocytosine[13] have been prepared, the halogen derivatives of cytidine have not been reported. A derivative, presumed to be 5,5-dibromo-6-hydroxy-5,6-dihydrocytidine,[14] formed when cytidine was treated with bromine water in the same manner as described for the preparation of a corresponding uridine derivative.[15] Attempts to prepare bromocytidine from the dibromocytidine compound were unsuccessful. Halogenation of cytidine in anhydrous media,[16] therefore, was attempted as an alternative method.

After repeated attempts to chlorinate cytidine in anhydrous acetic acid and sufficient anhydrous pyridine to prevent precipitation of a cytidine salt, a small quantity of nucleoside was isolated which, after hydrolysis in ammoniacal methanol, was found to contain a halogen. Ionophoresis[17] of the product separated two components, one of which was cytidine. The other smaller fraction was assumed to be chlorocytidine. Separation of the products was not feasible because of the low yield of chlorinated product.

In an attempt to increase the yield of chlorocytidine, the same procedure of chlorination was repeated except that the reaction mixture was exposed to ultraviolet light. A triacetate of chlorocytidine crystallized from the photocatalyzed reaction mixture, and 5-chlorocytidine was obtained upon hydrolysis of the triacetate in ammoniacal methanol. The amino group of cytidine triacetate was acetylated as shown by the fact that it yielded no picrate or phosphotungstate, whereas the deacetylated product readily formed insoluble complexes similar to those produced with cytidine.[18] Bromination of cytidine in pyridine and acetic acid also required photocatalysis. A Pyrex (No. 7740) glass vessel[19] was used for the halogenations. The yield was not increased by adding the halogen in increment quantities, by increasing the amount of halogen, or by increasing the time of exposure to ultraviolet light. Light from a 300-watt incandescent lamp was ineffective.

It was observed that a solution of cytidine dissolved in anhydrous acetic acid emits blue-white fluorescence. The reaction mixture containing cytidine in pyridine and anhydrous acetic acid fluoresces yellow-green during irradiation with ultraviolet light, whereas in the absence of cytidine, the solvents do not fluoresce.[20a,b] Upon addition of chlorine or bromine, the fluorescence was completely quenched.

Although a quantitative study would be required to interpret the mechanism of this photochemical reaction, it seems likely that radiation with ultra-

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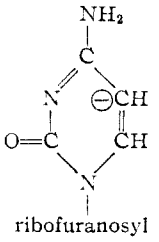
TABLE I

ULTRAVIOLET ABSORPTION OF SUBSTITUTED PYRIMIDINES

Compound	λ_{max}, mμ	$\Delta\lambda_{max}$ [a]	ϵ_{max}	λ_{min}, mμ	$\Delta\lambda_{min}$ [a]	pH
Uracil (2,4-dihydroxypyrimidine)[25,26]	259	..	8,200	228	..	7.2
2,4,6-Trihydroxypyrimidine[26]	255	4	24,500	224[d]	4	7
2,4,5-Trihydroxypyrimidine[26]	280	21	6,450[d]	243	15	7
2,4-Dihydroxy-5-aminopyrimidine[28]	290	31	8,600	258[d]	30	7
5-Chlorouracil[b]	274	15	7,463	238	10	7
5-Bromouracil[c]	276	17	7,194	241	13	7
2,4-Dichloropyrimidine[26,27]	259[d]	..	4,167[d]	233[d]	..	7
2,4,6-Trichloropyrimidine[27]	263[d]	4	4,556[d]	238[d]	5	7
2.4,5,6-Tetrachloropyrimidine[27]	277[d]	18	4,722[d]	255[d]	22	7
2-Amino-4-hydroxypyrimidine[28a]	267	..	6,500	245	..	7
2,6-Diamino-4-hydroxypyrimidine[28d]	267	..	12,070	..	..	6.5
2,6-Diamino-4-hydroxy-5-bromopyrimidine[28c]	276	9	14,100	251	6	6.8
Uridine[21]	262	..	9,820	230	..	7
5-Hydroxyuridine[4,30]	280	18	8,200	246	16	7
5-Aminouridine[4,30]	294	32	7,400	258	28	7
5-Chlorouridine[4]	276	14	10,000	239	9	7
5-Bromouridine[4,30]	279	17	9,600	242	12	7
Cytosine (2-hydroxy-4-aminopyrimidine)[25,28c]	267	..	6,200	249	..	7
6-Aminocytosine[28]	270	3	17,400	..	..	7.2
Cytidine[28b]	270	..	8,830	250	..	7
5-Aminocytidine	304	34	5,663	264	14	4.3
5-Chlorocytidine	287	17	7,250	261	11	7
5-Bromocytidine	289	19	7,131	264	14	7

[a] The extent of shift in ultraviolet absorption maxima and minima produced by 5- or 6-substitution. [b] Prepared according to the method of Barrett, Goodman and Dittmer, THIS JOURNAL, 70, 1753 (1948). [c] Prepared according to the method of Wheeler and Merriam, *Am. Chem. J.*, 29, 486 (1903). [d] Approximate value.

violet light serves to activate the pyrimidine ring, as well as halogen, thereby facilitating halogen attack on the pyrimidine nucleus. Position 5[21,22,23a] which is *meta to* the hetero nitrogen atoms, would be expected to be more susceptible than the 6-position to electrophilic attack,[23b] due to internal mesomerism.



The nucleophilic substituent at position 4 contributes to the ease of substitution at position 5 by mesomeric effects[22,23c] also. Uridine in anhydrous acetic acid is easily chlorinated at room temperature[3,16] without ultraviolet light activation. The difficulty encountered in the halogenation of cytidine using similar conditions may be due to salt formation between the 4-NH_2 group and acetic acid.[12] The resulting 4-NH_3^+ would be expected to lower the reactivity at position 5,[23c] thereby inhibiting halogenation at that position.

In order to establish the site of halogen substitution, deamination of bromocytidine with acetic acid and sodium nitrite[24] was attempted. It was found, however, that bromocytidine, unlike cytidine, is very resistant to attack by nitrous acid. Ultraviolet absorption spectral analysis provides evidence that the halogeno-substitution occurs at position 5. It is apparent from Table I that pyrimidines substituted at the 5-position with a halogen, amino group, or hydroxyl group exhibit maximum absorption at longer wave lengths than the corresponding 6-substituted derivatives. Also, the shift in the absorption maxima and minima produced by the halogenated cytidines toward the longer wave lengths as compared to cytidine[25] is similar to that produced by the substitution of a halogen in the 5-position of uridine or uracil.

The aminocytidine (prepared from bromocytidine) gives a positive test with alkaline phosphomolybdate.[28c] This is additional evidence that the amino and halogeno substituents are at the 5-position since neither 6-aminopyrimidine derivatives[28c] nor cytidine give a positive test with this reagent. Thus, the prediction based on electromeric effects, the magnitude of shift in ultraviolet absorption maximum produced by the substitution, and the positive test with phosphomolybdate, collectively, provide good evidence that the site of cytidine substitution is carbon 5.

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Experimental

5-Chlorocytidine Triacetate.—To 5 g. (0.02 mole) of cytidine in a one-liter Pyrex florence flask was added 350 ml. of anhydrous acetic acid and 250 ml. of anhydrous pyridine. The flask was cooled and shaken until the cytidine dissolved. The solution was cooled further in the deep-freeze to below zero °. The cold solution of cytidine was removed from the deep-freeze and immediately exposed to ultraviolet light from a Westinghouse 100-watt projector lamp CH-4 at a distance of 9–10 cm. for about 5 to 7 minutes. Chlorine, 1.5 g. (5% in excess of theory) in about 10 ml. of anhydrous carbon tetrachloride, was added at once while the flask was shaken. Then the solution was removed from the light and allowed to stand at room temperature overnight.

The solvent was removed at reduced pressure at 35–40° until a thick sirup was obtained. This material was dissolved in a small amount of methanol and again reduced to a thick sirup. This process was repeated until crystalline material was obtained. The crude crystals of chlorocytidine triacetate were dissolved in a minimum of hot ethanol, filtered and allowed to crystallize at room temperature. Recrystallization from water yielded 3.5 g. (43%), m.p. 157.2–158°.[29]

Anal. Calcd. for $C_{15}H_{18}N_3O_8Cl$: C, 44.59; N, 10.46; H, 4.50. Found: C, 44.31; N, 10.20; H, 4.68.

5-Chlorocytidine.—Three grams (0.007 mole) of chlorocytidine acetate was dissolved in a minimum amount (approximately 50 ml.) of hot methanol and allowed to cool to room temperature. To the solution was added 200 ml. of absolute methanol containing 5 to 6% anhydrous ammonia. After standing for 3 days at room temperature the solvent was removed completely at reduced pressure. The residue was recrystallized from hot absolute ethanol. The yield was 1.86 g. (95%), m.p. 202–202.5°. The λ of maximum absorption is 287 mμ at *p*H 7. The melting point of chlorocytidine picrate is 173–174°.

Anal. Calcd. for $C_9H_{12}N_3O_5Cl$: C, 38.93; N, 15.13; H, 4.36. Found: C, 39.12; N, 15.10; H, 4.39.

5-Bromocytidine Triacetate.—Cytidine, 4 g. (0.017 mole), was added to 280 ml. of anhydrous acetic acid and 200 ml. of anhydrous pyridine and treated in the same manner as described for the chlorination. The theoretical amount of bromine, 2.6 g., in about 10 ml. of anhydrous carbon tetrachloride was added. Crude bromocytidine acetate, 5.8 g. (79%), was obtained. Recrystallization from hot water produced needles, m.p. 162–163°.

5-Bromocytidine.—Bromocytidine acetate was hydrolyzed in the same manner as described for hydrolysis of chlorocytidine acetate. Hydrolysis of 2 g. (0.004 mole) of bromoacetate yielded 1.13 g. (88%) of bromocytidine, m.p. 182–183°. The λ of maximum absorption is 289 mμ at *p*H 7. The melting point of bromocytidine picrate is 190.5–191.5°.

Anal. Calcd. for $C_9H_{12}N_3O_5Br$: C, 33.66; N, 12.96; H, 4.04. Found: C, 33.88; N, 12.86; H, 4.10.

5-Aminocytidine Sulfate.—Two grams (0.006 mole) of bromocytidine was placed in a bomb tube and 20 ml. of liquid ammonia was added.[2,4] The tube was sealed and heated to 35–40° for 5 days. The orange-colored solution was transferred from the tube, and the solvent was completely removed. A colored residue was obtained which was dissolved in methanol, filtered and concentrated. The residue was dissolved in a minimum of water, acidified with 1 *N* sulfuric acid, and sufficient absolute ethanol was added to form a cloudy solution. Crystals of aminocytidine sulfate formed, 260 mg. (12%) upon standing in the cold. The compound begins to darken at 205° and melts at 211–212° (with decomposition). The product gives a positive diazo test,[4] forms an orange-brown oxidation product with potassium ferricyanide,[30] and gives a blue color with alkaline phosphomolybdate.[28c] The λ of maximum absorption is 304 mμ at *p*H 4.3. The picrate melts at 180–182.5°.

Anal. Calcd. for $C_9H_{14}N_4O_5 \cdot H_2SO_4$: C, 30.33; N, 15.73; H, 4.53. Found: C, 30.28; N, 15.38; H, 4.80.

Spectrophotometric Data.—The ultraviolet absorption maxima were measured in the Beckman spectrophotometer, model DU. Each compound was dissolved in distilled water (Table I).

Microbiological.—The 5-substituted cytidines were tested for growth-promoting or growth-inhibiting properties with a pyrimidine-requiring mutant of *Neurospora* 1298 using a modification[31] of the basal medium of Horowitz and Beadle.[32] The mutant was incubated at 25° for 3 days in 50-ml. erlenmeyer flasks, each containing a total volume of 10 ml. of liquid medium.

The mutant was grown in the presence of given amounts of uridine or cytidine as the pyrimidine requirement and varying amounts of the substituted nucleoside. The inhibition indices, the molar ratio of antimetabolite to metabolite which gives half-maximum growth, are summarized in Table II.

TABLE II

INHIBITION INDICES,[a] *Neurospora* 1298

Antimetabolite	Metabolite: Uridine	Metabolite: Cytidine
5-Chlorocytidine	0.98[b]	0.21[b]
5-Bromocytidine	1.04[b]	0.24[b]
5-Chlorouridine	3.04[b]	0.57[b]
5-Bromouridine	4.04[b]	...
5-Aminouridine[6]	14[b]	3.0[b]

[a] Inhibition index is defined as the molar ratio of antimetabolite to metabolite giving half-maximum growth. [b] Inhibition is competitive.

The inhibition index with aminocytidine was not determined due to insufficient material. However, a qualitative inhibition of growth was observed with the mutant and the wild-type *Neurospora*. The 5-chloro- and 5-bromocytidine had no effect on the growth of wild-type *Neurospora*.

Conclusions.—Substitution of an abnormal substituent at the 5-position of uridine or cytidine produces antimetabolites which are similar in their ability to inhibit growth of *Neurospora*, but differ in that the corresponding cytidine derivatives are inhibitory at lower concentrations. The cytidine derivatives, like the substituted uridines, inhibit *Neurospora* more effectively when cytidine provides the pyrimidine requirement. In both cases a chlorine substitution is more effective than substitution of the 5-hydrogen with a bromine atom. Furthermore, both aminocytidine and aminouridine inhibit growth of wild-type *Neurospora*, whereas the 5-halogeno derivatives of either nucleoside are inactive. From these data it is apparent that the kind of substituent in the 5-position of uridine or cytidine determines in large part the specificity of the antimetabolite produced by such substitutions. This suggests the possibility that the metabolic step or steps inhibited by similarly-substituted uridine and cytidine derivatives are the same.

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