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Addition of Bisulfite to Cytosine Derivatives[1]

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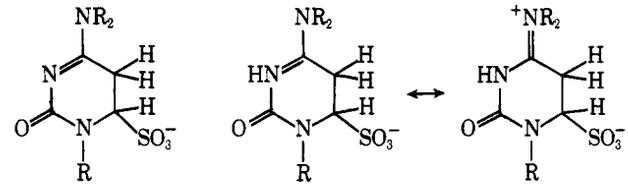
Studies of nucleophilic addition at the 5,6 double bond of pyrimidines with water,[2-4] alcohols,[5] bromine water,[6,7] hydroxylamines,[8,9] bisulfite,[10,11] etc.,[12] are essential in understanding chemical modifications of biologically active nucleic acids that may cause mutation and thus constitute a genetic hazard. The molecular structures of Ura addition products (such as photohydrates) were well established in 1956,[13] but actual isolation of Cyt addition products remains to be achieved.[14,15] Because of our interest in Cyt photohydrates[16] and because the Cyd bisulfite addition product has been isolated,[10,11] we prepared several Cyt bisulfite addition products. The UV absorption spectra for these compounds were measured, and IR and NMR spectra were obtained. This information should be valuable in bisulfite addition reactions, photohydration, and similar nucleophilic additions of Cyt derivatives.

Results and Discussion

In connection with our study of the chemistry of photodimerization of Cyt derivatives,[17] a series of methyl derivatives was prepared. It is advantageous to study these derivatives because $N(1)$-Me derivatives are analogues of biologically active compounds and $N(4)$-Me derivatives may provide information relating to the nature of amino–imino tautomerization of C(4)-NH_2 moiety in hCyt compounds.

Using a general procedure,[10,11] hCyt 6-sulfonate derivatives were obtained in yields varying from 20 to 95% (Table IA). Because adequate amounts could be obtained for our studies, no attempts were made to improve the product yields.

Table IB shows UV absorption spectral data of these compounds in aqueous solutions. The monoanions, as shown, probably exist in solutions with pH >9 (also see later discussion). The N^1-Me group causes an auxochromic shift of 3–4 nm in λ_{max} with a slightly higher ϵ_{max} that is consistent with that observed in hCyt [λ_{max} (pH 8) 239 nm] and Me^1hCyt [λ_{max} (pH 8) 243 nm].[18] N^4-Methylation produces more pronounced bathochromic shifts of >10 nm as seen in Me^1, $Me_2^{1,4}$, and $Me_3^{1,4,4}$ derivatives. A similar shift was reported in 1-cyclohexyl-hCyt [λ_{max} (pH 8) 245 nm] and 1-cyclohexyl-$Me_2^{4,4}$hCyt [λ_{max} (pH 8) 258 nm].[18,19]



monoanion zwitterion

In water (pH ~5), these addition products either exhibit λ_{max} <220 nm or end absorption with shoulders in the 240–260-nm regions. Characteristics of the former are analogous to those of hUra derivatives.[20] The position of the latter depends on the extent of N(4)-methylation, but their intensities are much reduced as compared to those observed at pH >9. This hypochromic effect indicates that monoanions are no longer the only or preponderant form under this condition. The appearance of strong absorption in the 220-nm region suggests that molecules with an exocyclic C=N bond exist predominantly for these bisulfite addition products because the predominant form of hUra compounds has the exocyclic C=O bonds and exhibits end absorptions. This could result from the amidinium resonance by protonation at N(3) in a zwitterion as shown.

Ionization constants of these compounds were estimated

Table I. Preparations and Ultraviolet Spectral Data of 5,6-Dihydrocytosine 6-Sulfonate Derivatives

	Methyl derivatives of cytosine					Nucleoside, Cyd
	1-	1,4-	4,4-	1,4,4-	4,5-	
			A. Preparation Conditions			
Amount, mmol	1.0	2.0	1.0	1.0	1.0	1.5
[in HOH, mL]	[1.5]	[1.0]	[0.5]	[No]	[2.0]	[No]
HSO_3^-, mmol	6.0	12.0	3.0	3.0	6.0	9.0
[in HOH, mL]	[1.0]	[2.0]	[0.7]	[0.7]	[1.0]	[1.8]
Time, ~day	1	2	1	2	4	1
Yield, ~%	60	95	20	70	80	37
Mp, °C	>280	176	207	149	202	205
			B. Ultraviolet Spectral Data			
Zwitterion (pH 5.1)						
λ_{max}, nm	217	217	221	222	218	218
$\epsilon_{max} \times 10^{-3}$	3.9	5.7	5.2	7.6	5.5	5.1
λ_{sh}, nm	242	238	255	260		241
$\epsilon_{sh} \times 10^{-3}$	2.6	3.9	2.1	3.0		3.6
Monoanion (pH 9.9)						
λ_{max}, nm	243	251	258	262	248	246
$\epsilon_{max} \times 10^{-3}$	8.3	9.5	8.1	9.9	8.0	8.5
λ_{min}, nm	229	233	234	238	229	230
$\epsilon_{min} \times 10^{-3}$	7.9	5.5	3.9	4.6	5.5	6.7
pK_a	~5.6	~5.7	~5.6	~5.7	~5.6	~5.4
λ_{aw}, nm	248	250	257	261	246	245

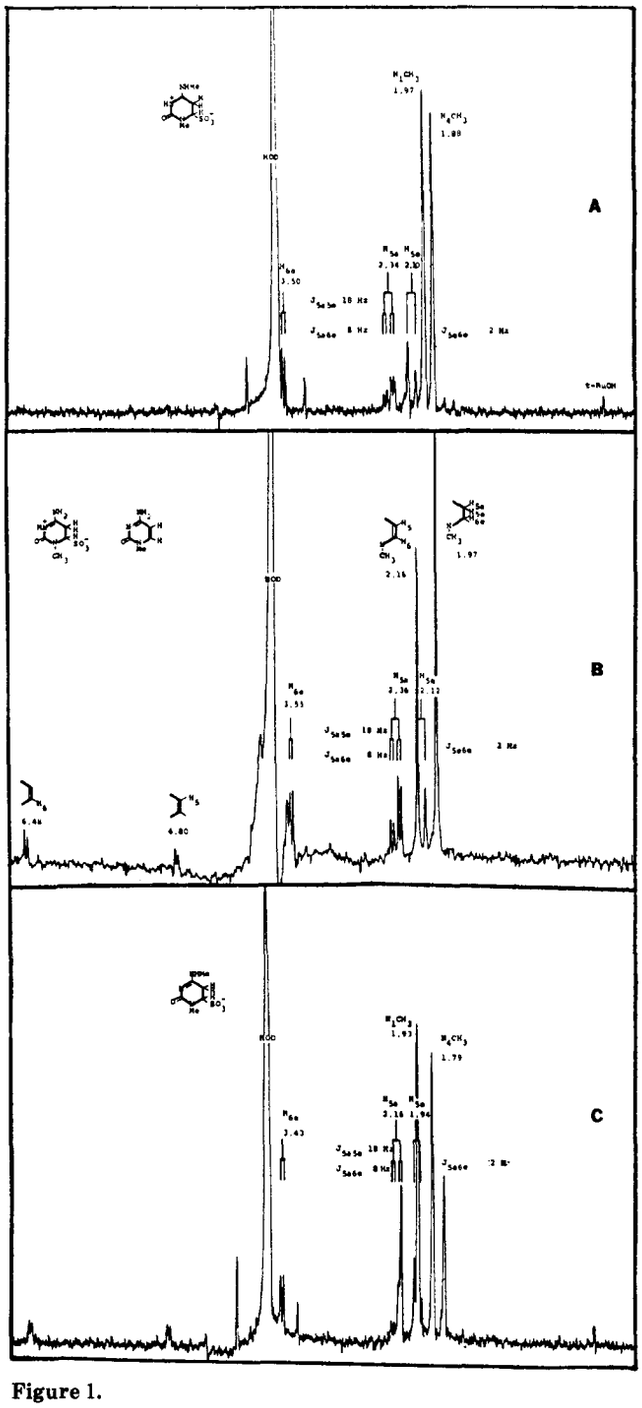


Figure 1.

spectroscopically. First, preliminary experiments were carried out to determine the stability of these bisulfite addition products under the pH titration conditions. Second, the choice of an analytical wavelength (λ_{aw}), at which the two species differ most in optical density from one another, was made for each compound. They are listed in Table IB and were used for the determinations. The pK_a values were calculated according to the normal method[21] and are given also in Table IB. These values, ranging from 5.4 to 5.7, are comparable to that of Cyd hydrate[4] and are somewhat lower than those of hCyt derivatives.[18] These correspondences argue for the validity of these estimated pK_as despite the slight instability of these compounds at a higher pH which, of course, precludes assay of precise pK_a values. The values of p$K_a \sim 6$ imply that the UV spectra at pH ~10 are those of monoanionic and at pH ~5, a mixture of monoanionic and zwitterionic.

In further confirming the molecular structures of these bisulfite addition products, NMR studies of these compounds were made. Similar spectral patterns were seen for all compounds but the characteristic bands of parent compounds were also weakly displayed by some because of a slight reversion to original compounds. The spectra of two representative compounds, one methylated and the other unmethylated at N(4), are illustrated in Figure 1 showing the assignments, chemical shifts (δ), and coupling constants (J). Figure 1A shows the spectrum of a zwitterionic species of $Me_2^{1,4}$hCyt 6-sulfonate because the saturated solution was found to have a pH of 3.9. A pattern of ABX for $H_{5ax}H_{5eq}H_{6eq}$ is clearly discernible. While the signal of H_{5eq} appears as a doublet, the H_{5ax} exhibits a quartet. This variance occurs because the vicinal proton–proton coupling constant is very small for $H_{5eq}H_{6eq}$. The low solubility and instability of these compounds did not allow sufficient data acquisition time to show a splitting with $J \leq 2$ Hz using a 220-MHz spectrometer. (This was measured as 2 Hz with a 100-Mhz spectrometer.) This small coupling constant is best attributed to a 1,2-trans diequatorial coupling[22] and indicates that the sulfonate group occupies the 6-axial position.[10,11] Figure 1B shows the spectrum of Me^1hCyt 6-sulfonate and a similar ABX pattern is discernible. In addition, weak signals of Me^1Cyt are also displayed. Figure 1C shows the NMR spectrum of the monoanion of $Me_2^{1,4}$hCyt 6-sulfonate. While the pattern and the couplings remain the same as in the zwitterion, the expected upfield shifts of 0.12, 0.20, and 0.18 ppm for H_{6eq}, H_{5ax}, and H_{5eq}, respectively, are evident. These larger magnitudes of $\Delta\nu$ for the C(5) protons probably indicate that protonation occurs at N(3) for the corresponding zwitterion.

These findings should add to the knowledge of the addition of bisulfite to pyrimidines, which has considerable importance in nucleic acid chemistry and biology.[23–26]

Experimental Section

Addition of Bisulfite to Cytosine Derivatives. The method of preparing these addition products is essentially the same.[10,11] The pulverized Cyt derivative was added directly or was dissolved first in a minimal volume of water before it was added to a cold solution of sodium bisulfite. Total volume was also kept minimal to aid crystallization from the reaction mixture that was stirred at ~5 °C and then allowed to stand in the refrigerator. The deposited crystals were collected by filtration, washed twice with cold water (0.5 mL) and thrice with methanol (1 mL), and dried under vacuum. Reaction conditions are shown in Table IA.

Determination of Ultraviolet Spectra of 5,6-Dihydrocytosine 6-Sulfonate Derivatives. A preliminary experiment was conducted for each bisulfite addition product. This was effected by dissolving ~1 mg of the compound in 50 mL of water cooled in an ice bath (~5 °C). The solution was titrated to pH ~10 by the addition of cold 0.01 N KOH solution. Then at 1-min intervals, UV spectra were recorded. The increase of the ϵ_{max} was found to be less than 3% in a 5-min interval, which is slower than that observed for the regeneration of Cyd from the Cyd–bisulfite addition product ($t_{1/2}$ 6 min) at a higher temperature (13 °C).[11] Also, the choice of the analytical wavelengths was made.

The determination of the pK_a value was made with 250 mL of a 1×10^{-4} M aqueous solution of the compound. The solution was kept at ~5 °C with stirring during titration. The optical densities at the λ_{aw} were recorded as soon as equilibrium was reached at pH ~10. Then a reading was taken when the solution was adjusted to pH ~7 with HCl. Adjustments between pH ~7 and ~4 were made in nine or ten steps, each resulting in a 0.3–0.5 pH change. The OD at λ_{aw} were recorded at each step and their corresponding pHs were accurately determined. A combination of 0.01, 0.1, and 1 N HCl was used to minimize the volume change of the solution. The OD at λ_{aw} of the solution at pH ~10 and pH ~3 were assigned for the monoanion (OD_M) and the zwitterion (OD_Z), respectively.

Nuclear Magnetic Resonance Spectra of $Me_2^{1,4}$-hCyt and Me^1-hCyt 6-Sulfonate. These spectra were obtained with a Varian HR 220-MHz spectrometer by Dr. G. McDonald of the Johnson Foundation at the University of Pennsylvania. They were taken at 5 °C in D_2O using *tert*-butyl alcohol as an internal standard.

Registry No.—Sodium bisulfite, 7631-90-5; 1-methylcytosine, 1122-47-0; 1,4-dimethylcytosine, 6220-49-1; 4,4-dimethylcytosine, 6220-48-0; 1,4,4-trimethylcytosine, 2228-27-5; 4,5-dimethylcytosine, 62006-34-2; 1-methyl-5,6-dihydrocytosine-6-sulfonic acid, 62006-35-3; 1,4-dimethyl-5,6-dihydrocytosine-6-sulfonic acid, 62006-36-4; 4,4-dimethyl-5,6-dihydrocytosine-6-sulfonic acid, 62006-37-5; 1,4,4-trimethyl-5,6-dihydrocytosine-6-sulfonic acid, 62006-05-7; 4,5-dimethyl-5,6-dihydrocytosine-6-sulfonic acid, 62006-06-8; sodium 1-methyl-5,6-dihydrocytosine-6-sulfonate, 62006-07-9; sodium 1,4-dimethyl-5,6-dihydrocytosine-6-sulfonate, 62006-08-0; sodium 4,4-dimethyl-5,6-dihydrocytosine-6-sulfonate, 62006-09-1; sodium 1,4,4-trimethyl-5,6-dihydrocytosine-6-sulfonate, 62006-10-4; sodium 4,5-dimethyl-5,6-dihydrocytosine-6-sulfonate 62029-61-2; 5,6-dihydrocytidine-6-sulfonic acid, 29725-37-9; sodium 5,6-dihydrocytidine-6-sulfonate, 62006-11-5.

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Coordinative Role of Alkali Cations in Organic Reactions. 1. Selective Methylation of the Alcoholic Group of Kojic Acid

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There is no method in the literature which describes methylation of an alcoholic group of an organic compound in the presence of its phenolic group. Kojic acid, 5-hydroxy-2-hydroxymethyl-γ-pyrone (HkH), can be methylated to 5-methoxy-2-hydroxymethyl-γ-pyrone (MkH) with diazomethane[1,2] or by treating a 1:1 mixture of HkH and KOH with a stoichiometric amount of dimethyl sulfate (DMS).[3] Both the hydroxyl groups of HkH can be methylated using an excess of KOH and DMS[2,4] to produce MkM.

There is no rationalized method of methylating HkH to obtain 5-hydroxy-2-methoxymethyl-γ-pyrone (HkM). However, the latter is reported[5] produced along with MkM when an aqueous solution of HkH (1 mol) and KOH (6 mol) is treated with DMS (3.7 mol). We feel that HkM is produced in this reaction because the amount of KOH overweighs that of DMS and the phenoxy site gets protected against the incipient CH_3^+ by K^+. This suggested to us the synthesis of HkM employing an excess of LiOH as alkali for a 1:1 reaction mixture of HkH and DMS. It was found that a threefold excess of LiOH could perfectly protect the phenoxy site (through the formation of partly covalent lithium kojate, $Li^+ {}^-kH$) in water against a stoichiometric amount of DMS at and below 40 °C.

Synthesis of HkM. Take 2.84 g (0.01 mol) of HkH and 2.60 g (0.03 mol) of $LiOH \cdot H_2O$ in 15 mL of water and maintain the reaction mixture at 35–40 °C. Add dropwise 1.7 mL (0.013 mol) of DMS in about 20 min while stirring constantly. Keep the reaction mixture for 30 min and add 2 N HCl to pH 6 and evaporate the solution to a semisolid employing a rotary evaporator. Extract HkM with six lots (10 mL) of benzene and crystallize it by expelling the latter at room temperature, yield ca. 55% (mp 72–74 °C, lit. mp 75–76 °C). The product can be recrystallized from ethyl acetate.[5]

Below 35 °C the reaction appears to be too slow whereas at 35–40 °C a fraction of DMS gets destroyed owing to alkali present in excess. Consequently, the yield of HkM is promoted by using a slight excess of DMS at 35–40 °C; DMS exceeding the recommended amount favors the formation of MkM. The product and an authentic sample of HkM both give a red color with ferric chloride. Infrared spectra of both show a broad band at 3300 cm^{-1} indicative of a free phenolic hydroxyl group ($-CH_2OH$ of HkH and MkH absorbs at about 3200 cm^{-1}). ^1H NMR spectra (80 MHz in D_2O) of both show an absorption at 2.7 ppm which is characteristic of the $-CH_2OCH_3$ methyl protons ($-OCH_3$ protons of MkH and MkM produce a singlet around 3 ppm).

Discussion

Methylation of $-CH_2OH$, obviously, is due to coordination of this group with Li^+, for this aids polarization of the alcoholic proton and its elimination with OH^-. The resulting oxide directly associates with the incipient CH_3^+ instead of Li^+ to produce $-CH_2OCH_3$. If ion pairing of the oxide with Li^+ should have taken place preferentially then conversion of $-CH_2OLi$ to $-CH_2OCH_3$ should have not been possible, for Li^+ cannot be replaced with CH_3^+ even from the more delocalized phenoxy site under these conditions as seen from the possibility of obtaining HkM. The idea of coordination of Li^+ derives its justification from the fact that even the low charge density K^+ and Cs^+ have been found to be coordinated (x-ray analysis) to $-CH_2OH$ in the compounds KI (phenacyl kojate)$_2$[6] and CsNCS(phenacyl kojate),[7] respectively.

Previous workers[5] failed to obtain the dienol by opening the γ-pyrone ring of HkH; we note that HkH and HkM do not undergo ring opening. This is probably because electron depletion (and hence bond weakening) of the ring through the carbonyl oxygen is overcompensated by the electron supply from the phenoxide created by the alkali. This should be true, in principle, for any γ-pyrone ring carrying an ionizable hydroxyl group.