Communications to the Editor

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Formation of Diastereomers of 5,6-Dihydrothymine-6-sulfonate by Deamination of 5-Methylcytosine with Bisulfite

The bisulflte-mediated deamination of 5-methylcytosine yielded a diastereomeric mixture of 5,6-dihydrothymine-6-sulfonate. One of the diastereomers was identical with the thymine-bisulfite adduct formed by treatment of thymine with bisulfite, and therefore was assigned the structure in which the hydrogen at position 5 and the sulfonate at position 6 are *trans*. The rates of degradation of this adduct into thymine in aqueous solutions were determined, and it was found that the adduct was considerably stable at pH 6.9, the half life being 13 hr at 37°, whereas at pH 8.9 it degrades with a half life of 70 min. The other diastereomer, in which the 5-H and 6-SO_3^- are *cis*, was stable in mildly alkaline conditions, as might be expected from the difficulty in undergoing elimination. This isomer rapidly generated thymine at pH 13.8.

Investigations on the reaction of bisulfite with cytosine nucleosides have shown that bisulfite brings about not only the deamination of cytosine nucleus, producing the 5,6-dihydrouracil-6-sulfonate derivatives,[1,2] but also the hydrogen-isotope exchange at position 5 of the cytosine.[3,4] It is believed that the exchange occurs *via* the formation of 5,6-dihydrocytosine-6-sulfonate by the reversible addition of bisulfite to the 5,6-double bond of the pyrimidine ring. From the proposed mechanism,[4] it may be expected that the deamination of 5-methylcytosine, a minor constituent of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA),[5] with bisulfite will yield diastereomers of the types I and II (Chart 1). Although a preliminary experiment on the 5-methylcytosine deamination was described in the early report,[2b] we have reinvestigated the reaction from this viewpoint and here report that the reaction does result in production of the diastereomers. Both I and II are convertible to thymine by treatment with alkali, but the conversion of II occurs only under strongly alkaline conditions. The latter fact distinguishes sharply the 5-methylcytosine deamination from the cytosine deamination in which the product, 5,6-dihydrouracil-6-sulfonate, readily generates uracil upon treatment with mild alkali.

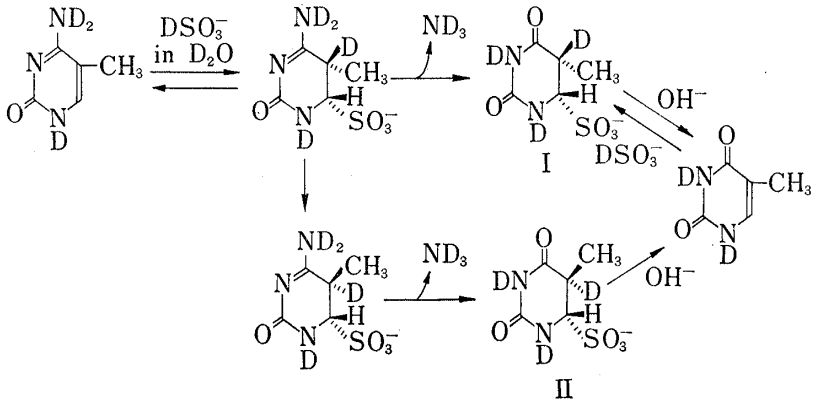


Chart 1

1) R. Shapiro, R.E. Servis, and M. Welcher, *J. Am. Chem. Soc.*, **92**, 422 (1970).
2) *a*) H. Hayatsu, Y. Wataya, and K. Kai, *J. Am. Chem. Soc.*, **92**, 724 (1970); *b*) H. Hayatsu, Y. Wataya, K. Kai, and S. Iida, *Biochemistry*, **9**, 2858 (1970).
3) K. Kai, Y. Wataya, and H. Hayatsu, *J. Am. Chem. Soc.*, **93**, 2089 (1971).
4) M. Sono, Y. Wataya, and H. Hayatsu, *J. Am. Chem. Soc.*, **95**, 4745 (1973).
5) "Handbook of Microbiology," Vol. II, ed. by A.I. Laskin and H.A. Lechevalier, CRC Press, Cleveland, Ohio, 1973, p. 428 and p. 789.

5-Methylcytosine HCl (Sigma, U.S.A.), 80 mg, was mixed with 5 ml D_2O containing 3M $NaDSO_3$, and the suspension, of which pD was 5.5, was incubated at 37° for 48 hr. Nuclear magnetic resonance (NMR) spectrum of the resulting solution showed four singlets corresponding to the methyl groups of four different compounds, *i.e.*, 5-methylcytosine, 5,6-dihydrothymine-6-sulfonates I and II, and thymine (Table I). From the intensities of the signals, the molar ratio of the compounds was estimated at 7% 5-methylcytosine, 31% I, 36% II, and 26% thymine. Complete disappearance of 5-methylcytosine required an incubation for 72 hr.

Table I. NMR Data of Compounds

Compound	Chemical shift (ppm)[a]		
	5-CH_3	6-H	5-H
I derived from 5-methylcytosine	1.36(s)	Nd	—
I from thymine[b]	1.36(s)	Nd	—
II	1.42(s)	4.48(s)	—
I (H)	1.36(d) J=7 Hz	4.51(d) J=6 Hz	3.41(m)
II (H)	1.42(d) J=7 Hz	4.26(s)	3.11(q) J=7 Hz
Thymine	1.84(s)	7.35(s)	—
5-Methylcytosine	1.95(s)	7.42(s)	—

a) NMR spectra in D_2O were recorded on JNM-PS-100 at 100 MHz, 3-(trimethylsilyl)propanesulfonic acid sodium salt being used as an internal standard. Abbreviations: s, singlet; d, doublet; q, quartet; m, multiplet; Nd, not detectable due to the overlapping signal of HOD.
b) Thymine, 64 mg, was suspended in 5 ml D_2O containing 3 M $NaDSO_3$, and the suspension (pD 5.5) was incubated at 37° for 72 hr. The mixture was filtered to remove undissolved thymine and subjected to the NMR measurement. As judged by the intensities of the CH_3- signals, the solution contained I and thymine in *ca.* 1:1 ratio.

The adduct I was identical with the one formed by the reversible addition of DSO_3^- to thymine.[1] The identification was based on the chemical shifts of the methyl groups in NMR (Table I), and the rates in the alkali-mediated conversion to thymine (Table II). In analogy to 5,6-dihydro-5-deuteriouracil-6-sulfonate (III), which is formed by treatment of uracil with DSO_3^-, the adduct I was therefore assigned the structure, 5,6-dihydro-5-deuterio-thymine-6-sulfonate, in which the deuterium and the sulfonate are situated *trans*.[1,2] 5,6-Dihydrothymine-6-sulfonate I(H), which possesses a hydrogen at position 5 instead of a deuterium (see below), showed an NMR spectrum consistent with the proposed structure (Table I).

Table II. The Pseudo-first-order Rate Constants for the Generation of Thymine from 5,6-Dihydro-5-deuterio-thymine-6-sulfonate I

Compound	k_{obsd} (min^{-1}) × 10^2, at pH[a]			
	6.9	8.0	8.5	8.9
I derived from 5-methylcytosine[b]	0.090	0.47	0.86	1.2
I from thymine[c]	0.090	0.46	0.74	1.1

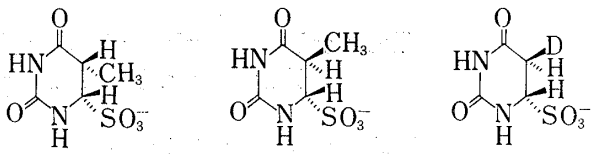
a) Buffers used: pH 6.9, 0.05 M Na-phosphate; pH 8.0, 8.5 and 8.9, 0.1 M tris-HCl.
b) The reaction mixture, in which 5-methylcytosine had been treated with 3 M $NaDSO_3$ at pD 5.5 and 37° for 72 hr, was diluted with a 500-fold volume of the buffer and the thymine formation at 37° was traced by measuring the increase of the absorbance at 264.5nm.
c) The 72 hr-incubated mixture of thymine and $NaDSO_3$, which was used for the NMR recording (Table I), was employed for this experiment. The rates were determined by the method given in *b*).

It should be noted that here the rates of the thymine-regeneration from I are for the first time described. Remarkably, I is not so labile at neutral pH as previously suspected.[2b]

The adducts I(H) and II(H) were prepared by treatment of 5-methylcytosine with $NaHSO_3$ in H_2O. By paper chromatographic fractionation of the reaction solution using the solvent system, *n*-butanol–acetic acid–water (2:1:1, v/v), a mixture of I(H) and II(H) (*Rf* 0.15—0.28)

was obtained free of thymine (*Rf* 0.85). A sample of II(H), free of I(H), was also prepared by rechromatography of this sample following a treatment with 0.001N NaOH at 22° for 24 hr which converts I(H) into thymine. The sample of II(H) showed an end absorption in ultraviolet region. On treatment with concentrated ammonia at 22° for 24 hr, II(H) gave thymine. The thymine was identified by ultraviolet spectra (at pH 7 and pH 14) and by comparison of the *Rf* values with an authentic specimen in paper chromatography using two solvents (*n*-butanol–acetic acid–water, 2: 1: 1; isopropanol–ammonia–water, 7: 1: 2, v/v). Tthymine was the only product in the alkaline degradation of II. This was demonstrated by the fact that, when the reaction mixture (5-methylcytosine in 3M $NaDSO_3$, pD 5.5, 37° 72 hr) was directly adjusted to pD 14 and NMR spectrum was measured, only the signals for thymine were detected. In contrast to I, the adduct II was stable in mild alkali. Half lives of II(H) in the degradation to thymine were 90 min at pH 12.8 and 4 min at pH 13.8.

Structures of compounds



I(H) II(H) III

Paper electrophoresis, at pH 7, of I(H) and II(H) showed that they are anionic, in agreement with the sulfonate structure.

In addition to these properties, the NMR spectra of both II and II(H) (Table I) showed that this adduct is indeed the diastereomer of I. The absence of coupling between 5-H and 6-H of II(H) is analogous to that previously observed for the uracil-DSO_3^- adduct (III),[1,2] and suggests a diaxial conformation[1] for the 5-CH_3 and the 6-SO_3^- groups. Whereas I can undergo a *trans* elimination to give thymine and DSO_3^-, the diastereomer II possesses the leaving group in *cis* positions. This provides an explanation for the stability of II in mild alkali.

On the basis of the previously reported hydrogen isotope exchange at position 5 of cytidine that is mediated by bisulfite,[3,4] it seems reasonable to postulate that the formation of the type II compound occurs through the formation of the *cis* isomer of 5,6-dihydro-5-methylcytosine-6-sulfonate (see Chart 1). It should be noted that the incubation of thymine under similar conditions gave only the *trans* adduct I (see Footnote to Table I), excluding the possibility that the formation of the *cis* isomer II might be a result of an isomerization of I.

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