NEW METHOD OF SELECTIVE AND RAPID MODIFICATION OF THE CYTIDINE RESIDUES

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1. Introduction

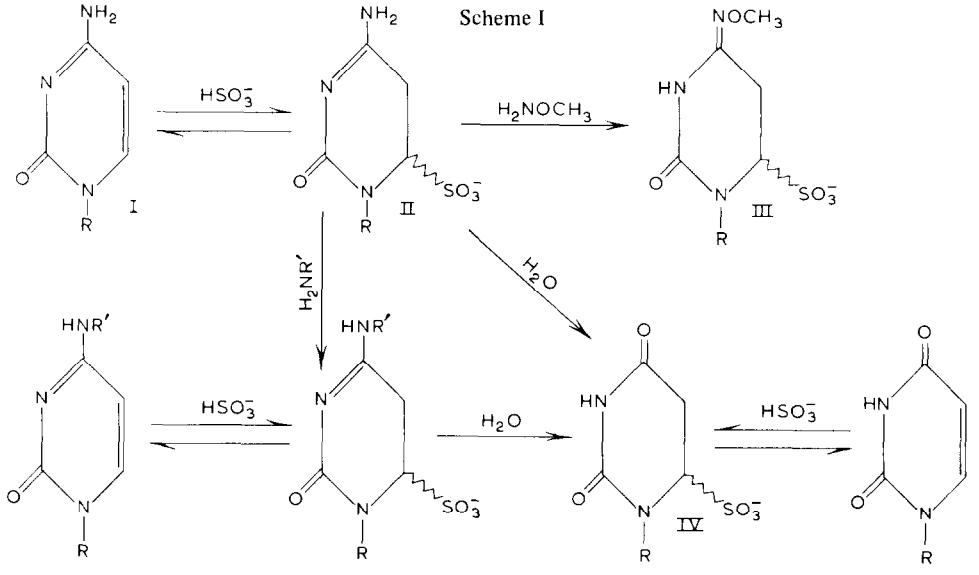
It has been demonstrated recently that the reaction of the cytosine ring system with bisulphite ions results in reversible and practically instantaneous addition of the bisulphite ion to the C^5–C^6 double bond. Saturation of the C^5–C^6 double bond strongly facilitates substitution of the exocyclic amino group. Even in the presence of weak nucleophiles – water or amines – slow hydrolysis or ammonolysis takes place giving uracil or N^4-alkylated cytosine adducts, respectively [1–4]. Obviously, much more speedy exocyclic amino group substitution of the cytosine–bisulphite adduct must be obtained with strong nucleophiles such as hydroxylamine, hydrazine and their derivatives.

Removal of bisulphite and alkalinization lead to conversion of saturated uracil or cytosine adducts into the respective aromatic pyrimidine derivatives [1–4].

It is known that some 6-substituted 4-hydroxyamino and 4-methoxyamino dihydropyrimidines are much more stable compared to the corresponding 4-oxo and 4 amino compounds [5–7]. For the reasons given, it was anticipated that addition of hydroxylamine or *O*-methylhydroxylamine to the bisulphite-containing reaction mixture would lead to the speedy formation of stable modified cytosine residues.

We have found that the action of a mixture of bisulphite and *O*-methylhydroxylamine on cytidine-5′-phosphate actually results in the rapid formation of

Scheme I



two diastereoisomers of 4-methoxyamino-2-oxo-5,6-dihydro-cytidine-6-sulphonate-5′-phosphate which are much more stable compared with the dihydrosulphonates of cytidylic and uridylic acids. Below are presented proofs of the structures of the compounds obtained, descriptions of some of their properties and a discussion of the possibility of the application of the new reaction to studies of nucleic acids and nucleoproteins.

2. Materials and methods

Sodium metabisulphite $Na_2S_2O_5$ (p.f.a., Chemapol) has been used as source of bisulphite ions. The reagent was purified by precipitation with ethanol from saturated aqueous solution. *O*-Methylhydroxylamine was prepared according to [8]. Cytidine-5′-phosphate was a preparation of Reanal. [^{14}C]cytidine-5′-phosphate of specific radioactivity 428 Ci/mole was obtained from the Radiochemical Centre, Amersham. In all the reaction mixtures the concentration of $Na_2S_2O_5$ was 1 M, and that of $CH_3ONH_2 \cdot HCl$ also 1 M.

The reactions were performed at 20°, pH 6.0. In the kinetic experiments, the concentration of cytidine-5′-phosphate in reaction mixtures was about 50 A_{260} units per ml, and their total volume was 2 ml. 0.1 ml aliquots were removed at time intervals, diluted with 10 ml of water and the UV-spectra measured over the 200–320 nm interval. The zero time control was removed immediately after mixing the reagents. Preparative isolation of the products was made after 3 hr incubation of the reaction mixture under the above conditions but at concentration of cytidine-5′-phosphate 62.5 mg/ml. The volume of reaction mixture was 8 ml. The products were separated by ion-exchange chromatography on anionite AG1 X 8 (Cl^-), 200–400 mesh, on 2 X 25 cm column. Before applying the products to the column, the reaction mixture was diluted to 1000 ml. The column was washed with 1000 ml of 0.005 M potassium acetate (pH 5). The elution was performed with linear gradient of NaCl (0–0.3 M) at flow rate 180 ml/hr; the total volume of the gradient was 3400 ml. The products were desalted by gel-filtration on Sephadex G-10. In some of the kinetic experiments the reaction mixtures contained 2.5 μCi [^{14}C]cytidine-5′-phosphate and 33 A_{260} units of non-labelled cytidine-5′-phosphate per ml. At time intervals, 0.2 ml aliquots were diluted to 20 ml with a solution containing 52 A_{260} units of cytidine-5′-phosphate and both the products of reaction (65 and 82 A_{230} units, respectively). The mixture was resolved on AG 1 X 8 column (1 X 10 cm) as described above but with proportionally reduced volumes of first buffer and gradient. The contents of components were found from the ratio of radioactivities of product peaks to total radioactivity of the effluent. Electrophoresis was done in 0.05 M sodium citrate buffer, pH 5, at a gradient of potential 7.6 V/cm on FN-12 paper (Filtrak, GDR). The UV-spectra were measured with Specord UV-Vis spectrophotometer (Carl Zeiss, Jena, GDR), IR-spectra with UR-20 IR-spectrophotometer of the same firm, the NMR-spectra with AN-100-D spectrometer (Varian) in D_2O at concentration of products 30 mg/0.3 ml. The radioactivity was measured with gas-flow counter after application of samples onto aluminium foil planchets and drying.

3. Results and discussion

The incubation of cytidine-5′-phosphate in 1 M sodium metabisulphite (pH 6, 20°) leads to decrease of the absorbancy of diluted aliquots of reaction mixture due to formation of the dihydrouridine sulphonate derivative [3] (IV, scheme I). The dihydrocytosine derivative (II) affords the starting compound under these conditions. The rate of the decrease of absorbancy becomes much higher in the presence of 1 M CH_3ONH_2 ($\tau_{1/2}$ 3.5 and 0.5 hr, respectively).

Absorption in the longer wavelengths region ($\lambda > 250$ nm) completely disappears under these conditions in 2–3 hr. Ion-exchange fractionation of the reaction mixture on anionite AG 1 X 8 at pH 5 after 3 hr of incubation reveals that it contains practically no cytidine-5′-phosphate and that two products are present, eluted at concentrations of sodium chloride 0.17 M (product IIIA) and 0.22 M (product IIIB) *. The products ratio remains constant in the course of the reaction and equal to 1 (table 1). The products exhibit similar UV-spectra suggesting

* The similar results were obtained for 2′-deoxycytidine-5′-phosphate.

Table 1
Distribution of radioactivity between components of the reaction mixture after modification of [^{14}C]cytidine-5′-phosphate with the mixture of bisulphite and *O*-methylhydroxylamine.

Time of reaction (min)	Per cent of radioactivity in fractions			A:B ratio
	CMP	Product IIIA	Product IIIB	
0	99	0.46	0.40	1.15
15	81	9.8	9.3	1.05
45	52	23.6	24.6	0.96
210	11.8	44.3	43.9	1.01

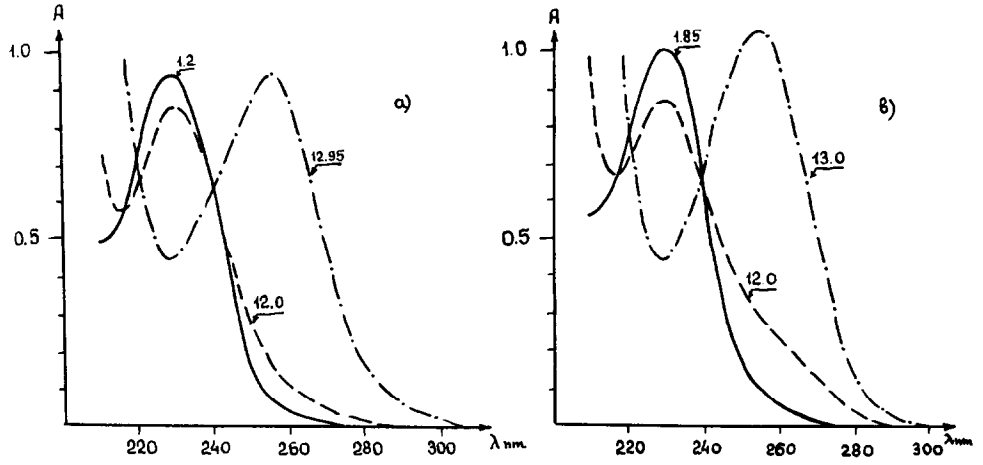


Fig. 1. UV-spectra of the products of reaction of cytidine-5′-phosphate with the mixture of bisulfite and *O*-methylhydroxylamine. a) Product IIIA; b) product IIIB. Figures on the curves are the pH values.

absence of conjugated structure (fig. 1). Their electrophoretic mobility at pH 5 is the same as that of cytidine diphosphate (1.3 relative to cytidine monophosphate). Thus, the products have an additional negative charge that must be due to the presence of the sulphonate grouping.

The IR-spectra of products IIIA and IIIB are also similar. Characteristic bands are at 1640 cm^{-1} (amide I and $\nu_{C=N}$), 1225 cm^{-1} ($\nu_{p=0}$ and SO_3H), 515 cm^{-1} (δ_{SO} [2,3]) suggesting the presence of a sulfo grouping and a carbonyl-containing heterocycle. The NMR-spectra (fig. 2) demonstrate the presence of a single CH_3O-grouping in each of the compounds. The simple character of the signals of H'_1 protons suggests that the products are individual compounds. Absence of signals around 7–8 ppm, in accord with the UV-spectra, reveals the absence of protons at the double bond. The same products IIIA and IIIB are obtained by reaction of N^4-methoxycytidine-5′-phosphate with 1 M sodium metabisulphite at pH 6.

All the above evidence proves that the products IIIA and IIIB are N^4-methoxy-5,6-dihydrocytidine-6-sulphonate-5′-phosphates (III).

The differences of the chemical shifts and of the spin–spin coupling constants of interacting protons of the compounds are analogous to those characteristic of the diastereomers of 5,6-dihydro-6-hydroxyuridine [9, 10] and 5,6-dihydrouridine- and 5,6-dihydrocytidine-6-sulphonates [1–3]. We believe for this reason that the products IIIA and IIIB are diastereomers of III differing in configuration at C^6. In accord with this conclusion is the different character

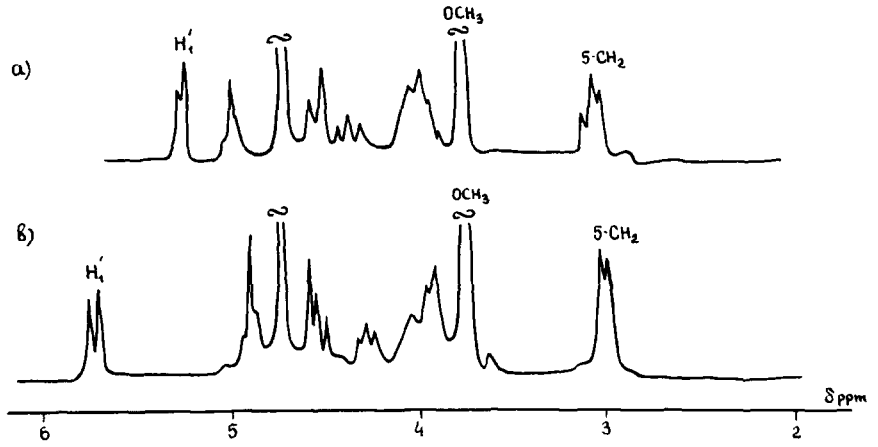


Fig. 2. NMR-spectra of the products of reaction of cytidine-5′-phosphate with the mixture of bisulfite and *O*-methylhydroxylamine. Solvent, D_2O; internal standard, tert.-butanol. a) Product IIIA; b) product IIIB.

of the CD-spectra and the opposite signs of rotation ($[\alpha]_D^{20} = +16.9$ and -24.8 for IIIA and IIIB, respectively).

Hence, the reaction of cytidine-5′-phosphate with mixture of HSO_3^- and CH_3ONH_2 proceeds according to the above scheme.

According to kinetic evidence, a minor amount of dihydrouridine sulphonate (IV) should be also formed, but we failed to detect the compound in ion-exchange chromatography.

As expected, both the diastereomers appeared to be rather stable compounds whose sulphonate grouping is not removed by 24 hr incubation at pH 0 (30°) or at pH 10 (35°). Thus, it is possible to work with the compounds over a wide range of conditions.

The presence of an additional negative charge in the modified residues of type III must be an aid in separation of isopleths differing in cytidine content. The modification may complicate the interaction of the modified nucleoside residue with enzymes used for polynucleotides structural analysis, particularly, with snake venom phosphodiesterase. Conversion of the cytosine nucleus into the acid-resistant dihydropyrimidine derivative of type III strongly decreased stability of the *N*-glycosidic bond in the corresponding nucleoside residue, opening up the possibility of removing the modified base in weakly acidic conditions.

Attack of nucleophilic reagents at the C^5-C^6 double bond of the pyrimidine nucleus occurs perpendicularly to its plane. Thus, the isomers ratio must depend on the shielding of the two sides of this plane by adjacent nucleoside residues or protein. This seems an interesting possibility for studies of the three-dimensional structure of polynucleotides and nucleoproteins.

Preliminary experiments confirm the suggestions outlined above (the results will be published elsewhere) and demonstrate the usefulness of the new method of selective modification of the cytosine nucleus for studies of the structure and function of polynucleotides and nucleoproteins.

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