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An Improved Synthesis of N^4-Aminocytidine

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N^4-Aminocytidine, a mutagenic nucleoside, was obtained as crystals on reaction of cytidine with a reagent mixture consisting of hydrazine, bisulfite and phosphate buffer at pH 7. The product can be precipitated directly from the reaction mixture, and therefore the procedure is suitable for preparing N^4-aminocytidine in large quantities.

Keywords——N^4-aminocytidine; large-scale synthesis; hydrazine; bisulfite; nucleoside analog; base-pair change; mutagen; transamination; cytidine

N^4-Aminocytidine (**1** in Fig. 1) and its N'-alkyl derivatives are potent mutagens in bacteria[1,2] and phages.[1-3] N^4-Aminocytidine is also mutagenic in cultured mammalian cells[4] and in *Drosophila*.[5] This nucleoside analog causes base-pair transitions, AT to GC and GC to AT, as shown by deoxyribonucleic acid (DNA) sequence determination of the mutant genes derived from phages ϕX174 *am3*[3] and M13 (unpublished work). When Chinese hamster V79 cells grow in the presence of N^4-aminocytidine in the culture medium, the cells incorporate this nucleobase analog into thier DNA.[4] N^4-Aminocytosine nucleotide can be incorporated into phage DNA *in vitro*, and the resulting DNA can induce the production of mutant phages in host bacteria.[6,7]

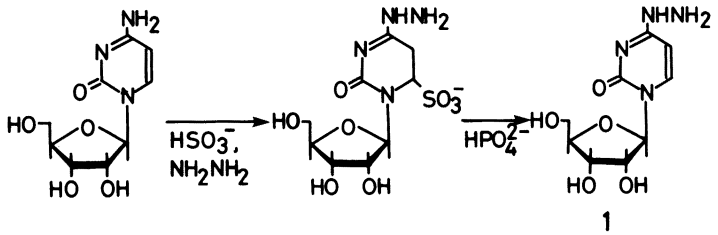

Fig. 1. Synthesis of N^4-Aminocytidine from Cytidine

In these studies, N^4-aminocytidine was prepared from cytidine by use of the bisulfite-mediated substitution of the N^4-amino group with hydrazine, which is a method originally used by Budowsky and coworkers for preparing N^4-aminodeoxycytidine 5′-phosphate from deoxycytidine 5′-phosphate.[8] N^4-Aminocytidine was obtained as a glassy material after fractionation of the reaction mixture by high-performance liquid chromatography (HPLC). Due to the necessity of the HPLC fractionation, the preparation has been limited to sub-gram scale. To extend the studies on the biological properties of N^4-aminocytidine, it is desirable to improve the method of isolation. Here we report the preparation of this compound in a crystalline form and describe a simple procedure to obtain the crystals in gram quantities.

Experimental Procedures

Synthesis and Purification of N^4-Aminocytidine——A reagent mixture was prepared by mixing hydrazine hydrate (4 ml, 80 mmol; Wako Pure Chemicals, Osaka), sodium bisulfite (0.2 g, 2 mmol; Wako), sodium dihydrogen phosphate dihydrate (0.3 g, 1.9 mmol; Wako) and water (10 ml). This mixture was neutralized to pH 7.0 by addition of concentrated HCl, and the volume was made up to 20 ml by adding water. Cytidine (0.3 g, 1.2 mmol) was dissolved in the reagent mixture (3 ml), and the resulting solution was allowed to stand at 60 °C for 4 h. The reaction mixture was then subjected to a preparative HPLC on a reversed phase column (ODS-20, i.d. 22 mm × 300 mm; Kusano Scientific Instruments, Tokyo) which had been connected to a precolumn (ODS-G3, i.d. 22 mm × 100 mm; Wako). After injection of the total reaction mixture into the column, elution was done with 50 mM formic acid, the pH of which had been adjusted to 4.5 by addition of ammonia. The flow rate was 3 ml/min. Ultraviolet (UV)-absorbing fractions ($A_{280} > 5$) were collected (t_R, 30—45 min) and evaporated to dryness under reduced pressure. The residue obtained was dissolved in water (2 ml) and rechromatographed on the same columns using water as the eluent. Fractions containing the product were combined and evaporated to dryness to give a glassy material (0.18 g). On storage in a freezer for several days, this material solidified. The solid was crystallized from water–ethanol to give colorless needles.

Gram-Scale Preparation of Crystalline N^4-Aminocytidine——Hydrazine hydrate (10 ml, 200 mmol), sodium bisulfite (0.5 g, 5 mmol), and sodium dihydrogen phosphate dihydrate (8 g, 32 mmol) were dissolved in water (30 ml). This solution was adjusted to pH 7.3 by addition of concentrated HCl (about 11 ml). Cytidine (15 g, 62 mmol) was added and the resulting solution was heated at 62 °C for 4.5 h. The solution was cooled in ice and a small amount of crystalline N^4-aminocytidine was added to it as seeds for the crystallization. Scratching with a spatula on the inside wall of the glass container facilitated the formation of crystals. After standing overnight at 4 °C, the crystals were collected by filtration, and washed with cold water (5 ml × 2), cold 50% methanol (30 ml × 2), methanol (50 ml × 2), and diethyl ether (50 ml × 3), successively. This crude sample (6 g) was recrystallized from water (16 ml) to give colorless crystals of N^4-aminocytidine hemihydrate (4.8 g; 29% of the theoretical yield). When this sample was analyzed by HPLC, it was 99.5% pure in terms of UV-absorbing (at 254 nm) material, the contaminating material being cytidine. For determination of the physical properties of the compound, the material was further recrystallized from water.

Care must be taken to avoid direct contact with this compound, because it is a potent mutagen.

Spectra——UV spectra were recorded on a Hewlett Packard 8450A spectrophotometer, nuclear magnetic resonance (NMR) spectra on a Hitachi R-22FT NMR spectrometer at 90 MHz with 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt as an internal standard, and Raman spectra on a JASCO NR-1000 spectrophotometer, with Ar-laser excitation at 514.5 nm (NEC 3300).

Results and Discussion

By a simple one-step reaction, gram quantities of N^4-aminocytidine were prepared from cytidine. In this procedure, the reaction mixture contains a high concentraion of the nucleoside, and the product can be precipitated directly from the mixture as a crystalline solid. The reaction solution also contains a high concentration of sodium phosphate, which serves as a catalyst to remove bisulfite from the 5,6-dihydropyrimidine 6-sulfonate intermediate.[9] The results of elemental analysis of the material indicated that it was a hemihydrate of N^4-aminocytidine: $C_9H_{14}N_4O_5 \cdot 1/2H_2O$ requires C, 40.45%; H, 5.66%; N, 20.97%; the values found were C, 40.20%; H, 5.60%; N, 20.82%. The mass spectrum (MS) gave a signal at m/z: 258 (the molecular weight of N^4-aminocytidine is 258.24). The UV spectra of N^4-aminocytidine at several pH values are shown in Figure 2: at pH 1, λ_{max} 281 nm (ε, 13700); at pH 7, λ_{max} 236 nm (ε, 7700) and 273 nm (ε, 10900); at pH 13, λ_{max} 265 nm (ε, 12600). The ε value at the λ_{max} at pH 7 is identical as that reported earlier for N^4-aminodeoxycytidine 5′-phosphate, which was determined on the basis of phosphorus analysis.[1] The spectra at pH 7 and 1 are very similar to those of cytidine at these pH values. The spectrum at pH 13, however, is very different from that of cytidine. The spectrum of N^4-aminocytidine at pH 13 is not that of some degradation products, because the spectrum reverted to that at pH 7 on neutralization of the alkaline solution. Probably, the hydrogen at the N^4 position dissociates under alkaline conditions. Titration of an acidic solution of N^4-aminocytidine with sodium hydroxide showed that this compound has a pK_a value of 4.50. In this titration, consumption

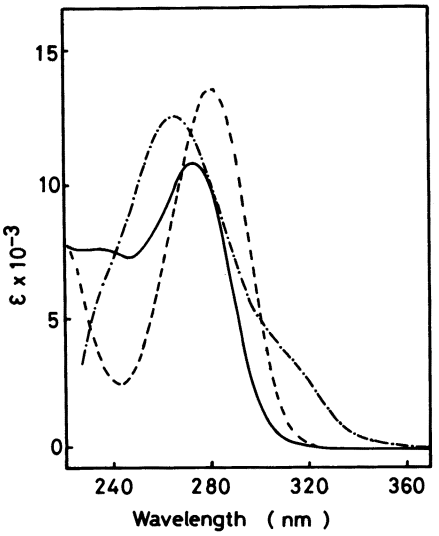

Fig. 2. UV Spectra of N^4-Aminocytidine

Spectra of N^4-aminocytidine solutions at various pH values.
pH 1, ----; pH 7, ——; pH 13, —·—.

of alkali was also observed at the pH region of 11—13, but the end point of the consumption was obscure. This poorly defined end point was apparently due to the dissociation of the sugar hydroxyl groups which occurs under strongly alkaline conditions. The dissociation of the N^4-hydrogen probably has its pK_a value at around 12.

In the Raman spectrum of solid N^4-aminocytidine, ν-NH_2 signals were observable at 3200 cm^{-1} and 3313 cm^{-1}. Under the same conditions, cytidine gave ν-NH_2 signals at 3300 cm^{-1} and 3452 cm^{-1}. The location of the signals for N^4-aminocytidine at smaller wave numbers than those for cytidine is consistent with the structure of the compound, in which the NH_2 is not directly linked to the pyrimidine ring.

Proton nuclear magnetic resonance (^{1}H-NMR) spectra recorded in CF_3COOD–D_2O (3 : 10, v/v) gave signals at δ 5.90 ppm (1H, d, J=3.5 Hz) for 1′-H, δ 6.25 ppm (1H, d, J= 7.5 Hz) for 5-H, δ 8.14 ppm (1H, d, J=7.5 Hz) for 6-H, and complex signals in the 4 ppm region due to protons in the sugar moiety.

The preparation of N^4-aminocytosine nucleoside derivatives has been described.[8,10-12] However, N^4-aminocytidine is not among those reported. The method of preparation described in the earlier literature[10-12] involved the substitution of 4-thio- or 4-alkylthio-uracil (or thymine) nucleosides with hydrazine. Obviously, the bisulfite-mediated amine exchange of cytosine nucleosides[8] is a simpler way of preparing N^4-aminocytosines. An advantage of this method is that the reaction takes place in neutral aqueous solution, so that it can be adapted to the preparation of N^4-aminocytosine nucleotides and polynucleotides.

The availability of large quantities of crystalline N^4-aminocytidine will facilitate its use in chemical and biological studies. For example, a study on metal complexes of N^4-aminocytidine is in progress, and a test for carcinogenic potential of N^4-aminocytidine in animals is also under way.

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