Formation of Pyrazine Derivatives from D-Glucosamine and Their Deoxyribonucleic Acid (DNA) Strand Breakage Activity

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Two pyrazine derivatives [fructosazine (3) and deoxyfructosazine (6)] were simultaneously formed in a solution of D-glucosamine hydrochloride under various conditions. They showed deoxyribonucleic acid (DNA) strand breakage activity in plasmid pBR322 comparable to that of D-glucosamine. The DNA strand breakage by fructosazine (3) was stimulated by Cu^{2+}.

Keywords pyrazine; fructosazine; deoxyfructosazine; DNA strand breakage; plasmid pBR322; D-glucosamine; L-cysteine; aminosugar; copper ion

Previously, we found that some aminosugars caused single-strand breakage of pBR322 covalently closed circular (ccc)-deoxyribonucleic acid (DNA),[1)] and that the preheated sample of D-glucosamine (1) in solution had higher DNA breakage activity than that without heating (unpublished data). These findings led us to focus on a study of the chemical transformation of aminosugars in order to obtain more effective agents in terms of DNA breakage activity.

In this paper, we have reported the formation of pyrazine derivatives from D-glucosamine and their DNA strand breakage activity in plasmid pBR322.

Chemistry Under neutral (TE buffer) to basic (in aqueous ammonia) conditions, we isolated two pyrazine derivatives (3 and 6) as the sole products, the formation ratios (6/3) of which were dependent on the reaction conditions (Table I). We also detected formation of a small amount of the aldehyde, 5-(hydroxymethyl)-2-furaldehyde (7)[2–4)] by high performance liquid chromatography (HPLC) analysis of the reaction mixture in some runs (see Experimental). Under acidic condition, the formation of the aldehyde (7) was increased, however, the formation of pyrazines (3 and 6) were greatly decreased. Although these pyrazines were already reported independently in the literature and named for fructosazine and deoxyfructosazine (3 and 6, respectively),[5–10)] to the best of our knowledge, no report describing the simultaneous formation of these pyrazine derivatives has appeared, so far.

The formation process for the two pyrazine derivatives

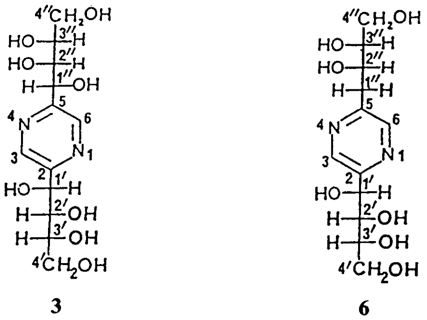

Fig. 1

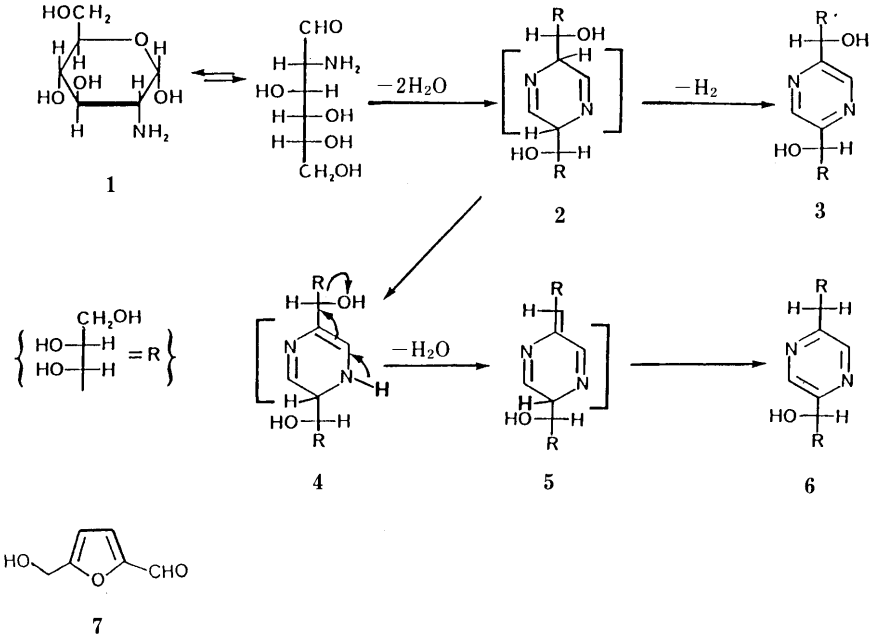

Chart 1

Table I. Ratios of the Products (**3**, **6**, and **7**) in the Reaction of **1** under Various Conditions[a]

Conditions[b]	Product ratio **3**	: **6**	: **7**
TE buffer 130 °C/4.5 h[c]	1	28	13
H_2O–pyridine 120 °C/16 h	1	1.74	0
H_2O–MeOH–pyridine–L-cysteine 120 °C/16 h	1	21	0
H_2O–IRA410 r.t./3 w	1	0.81	<0.2
H_2O–Tris (1 eq mol) r.t./10 min	1	0.55	Trace
H_2O–NaOH (1 eq mol) r.t./18 d	1	0.33	Trace
28% aq.NH_3 r.t./3 w	1	<0.01	0

a) Ratios were determined by HPLC monitering. *b*) Abbreviations: w; weeks, r.t.; room temperature, and Tris; tris(hydroxymethyl)aminoethane. *c*) Other unknown products were also detectable but the structures could not be established.

Table II. Relative Amounts of Remaining ccc-DNA

Incubation time (h)	**3**	**6**	[**3**+Cu^{2+}][a]	**1**[b]
0	100.0	100.0	100.0	100.0
1	86.4	81.9	69.2	96.7
3	75.1	67.7	50.6	80.1
5	71.1	61.6	34.9	44.5

a) This experiment was carried out with 2 mM of **3** (1/50-fold amount compared to other runs) in the presence of 1 mM $CuCl_2$. *b*) Used as hydrochloride.

mentioned above would be rationalized as shown in Chart 1, in which an oxidative (dehydrogenation) process leading to fructosazine (**3**) and a dehydration stage, followed by isomerization, (**2**→**4**→**5**) for deoxyfructosazine (**6**) from a proposed intermediate dihydropyrazine (**2**) takes place competitively.[8,9] This hypothesis is well supported by the following experiment. Thus, the reaction of D-glucosamine carried out in the presence of a reducing agent (SH group) such as L-cysteine, afforded deoxyfructosazine in good yield, together with L-cystine having disulfide (-SS-) bond (see Experimental).

The fact that eight of the observed ^{13}C-resonances for the product (**3**) coincide, *i.e.*, the chemical shift of C1′, C2′, C3′, and C4′ overlap with those of C1″, C2″, C3″, and C4″ respectively, indicates that the molecule is symmetrical and that no epimerization at those chiral centers took place. Therefore, regarding the absolute configuration [C1′(1″), C2′(2″), and C3′(3″)] of two polyhydroxyalkyl substituents on the pyrazine ring, we are considering the possibility that products should have the same absolute configurations as those (C3, C4, and C5) in the starting aminosugar (**1**).

DNA Strand Breakage Activity The results with isolated pyrazines (**3** and **6**) of DNA strand breakage activity are shown in Table II, where relative activity was expressed as the ratio of ccc-DNA. The results obtained with D-glucosamine (**1**) used as a reference compound is also presented for comparison. As Table II shows, the pyrazine derivatives (**3** and **6**) possessed a DNA strand breakage activity, comparable to that of D-glucosamine. Compound **7** showed no activity. Since Cu^{2+} accelerated the ccc-DNA breakage activity of D-glucosamine, we examined the effect of Cu^{2+} on the compound **3**. As the same Table shows, Cu^{2+} was found to stimulate the ccc-DNA breakage. Cu^{2+} is known to catalyze the autoxidation of some reducing compounds to generate active oxygens and certain radicals in aqueous solutions containing oxygens.[11] Thus, these radicals might be involved in the pyrazines-induced DNA strand breakage. The elucidation of the mechanism of DNA strand breakage by pyrazines and the synthesis of more active compounds by chemical modification of these pyrazines are currently being investigated.

Experimental

Melting points were determined with a Yanaco micro melting point apparatus or a Yamato melting point apparatus, and uncorrected. Infrared (IR) spectra were obtained on a Hitachi 250 spectrometer. ^1H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded on a JEOL GX-400 spectrometer, with 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TSP) as an internal standard. High-resolution mass spectra were obtained with a JEOL JMX-DX300 instrument. Assignment for ^{13}C-resonances of the products were supported by 2D homo- or heteronuclear shift correlation spectra with a JEOL GX-400 spectrometer.

HPLC analyses were carried out on a Shimadzu LC-8A system consisting of a refractive index detector Shimadzu RID-6A with a data module Shimadzu C-R4A chromatopac. The columns used are mentioned below.

Fructosazine (3)[5—9] The following procedure is conventional for the preparation of fructosazine (**3**): D-Glucosamine hydrochloride (1.0 g, 0.0046 mol) was dissolved in 28% aqueous ammonia (10 ml) (pH 11), and the resulting solution was stirred at room temperature for 3 weeks.

After neutralization with 1 N-HCl, evaporation of the solvent gave solid material. Purification of the products with HPLC [Hibar Lichrosorb RP-18 (7 μm) 250 × 25/water as solvent] afforded fructosazine (**3**) in 47% yield, in addition to small amounts of deoxyfructosazine (**6**).

Compound **3** had mp 234 °C (dec.) [lit.[8] mp 237 °C (dec.)]. $[\alpha]_D^{23.5}$ $-82.2°$ ($c=0.2$, H_2O) [lit.[8] $[\alpha]_D^{20}$ $-84.1°$ ($c=1.0$, H_2O)]. IR (KBr): 3300 (polymeric hydrogen bonded OH), 1035 (primary OH), 1090 (secondary OH) cm^{-1}. ^1H-NMR (D_2O) δ: 8.76 (2H, s, pyrazine ring protons), 5.19 [2H, d, $J=2.44$ Hz, C(1′ and 1″)-H], 3.92—3.84 [2H, m, C (2′ and 2″)-H], 3.92—3.84 [2H, m, C(3′ and 3″)-H], 3.92—3.67 [4H, m, C (4′ and 4″)-H]. ^{13}C-NMR (D_2O) δ: 65.84 (C4′ and C4″), 73.98 (C3′ and C3″), 74.32 (C1′ and C1″), 76.33 (C2′ and C2″), 144.82 (C3 and C6), 157.90 (C2 and C5). FAB-MS *m/z*: 321.1289 [$(M+H)^+$, $C_{12}H_{21}N_2O_8$]. The IR (Nujol) and Ultraviolet (UV) (in H_2O) data of the product were identical with the values reported previously.[8] *Anal.* Calcd for $C_{12}H_{20}N_2O_8$: C, 45.00; H, 6.29; N, 8.75. Found: C, 44.72; H, 6.38; N, 8.52.

Ratios of the two pyrazines (**3**/**6**) in the crude reaction mixture under various reaction conditions (monitered by HPLC analysis) are summarized in Table II. In some runs, a small amount of compound **7** which was identified with an authentic sample,[2] was isolated.

Deoxyfructosazine (6)[10] (Reaction of D-Glucosamine in the Presence of L-Cysteine) A solution of D-glucosamine hydrochloride (1.075 g, 0.005 mol), L-cysteine monohydrochloride monohydrate (0.878 g, 0.005 mol) and pyridine (39.5 g, 0.5 mol) in 45 ml of MeOH–H_2O (8 : 1) was refluxed for 32 h. After cooling, the precipitates were collected by filtration and washed with water to afford L-cystine (*ca.* 37%), which was identified with the commercially available sample. The filtrate was concentrated to a syrup and subjected to purification by HPLC [Prep PAK C18 cartrige/water as solvent] to give a light yellow powder, mp 130—141 °C. The product in the crude form was calculated at 76% by the internal standard method, however the isolated yield was 45%. Compound **6** had mp 156—158 °C (EtOH), [lit.[10] mp 161—162 °C]. $[\alpha]_D^{23.5}$ $-79.8°$ ($c=0.2$, H_2O) [lit.[10] $[\alpha]_D^{23}$ $-78°$ ($c=0.6$, H_2O)]. IR (KBr): 3290 (polymeric hydrogen bonded OH), 1040, 1055 (primary OH), 1075, 1100 (secondary OH) cm^{-1}. ^1H-NMR (D_2O) δ: 8.72 [1H, s, pyrazine ring C(3)-H], 8.55 [1H, s, pyrazine ring C(6)-H], 5.16 [1H, d, $J=2.44$ Hz, C(1′)-H], 3.25—2.96 [2H, m, C(1″)-methylene H], 4.08—4.03 [1H, m, C(2″)-H], 3.91—3.80 [1H, m, C(2′)-H], 3.91—3.80 [1H, m, C(3′)-H], 3.75—3.65 [1H, m, C(3″)-H), 3.91—3.50 [4H, m, C (4′ and 4″)-H]. ^{13}C-NMR (D_2O) δ: 40.38 (C1″), 65.37 (C4″), 65.82 (C4′), 74.01 (C3′), 74.20 (C2″), 74.23 (C1′), 76.36 (C2′), 77.25 (C3″), 145.04 and 146.95 (C3 and C6), 156.12 and 156.75 (C2 and C5). FAB-MS *m/z*: 305.1348 [$(M+H)^+$, $C_{12}H_{21}N_2O_7$]. *Anal.* Calcd for $C_{17}H_{20}N_2O_7$: C, 47.36; H, 6.63; N, 9.21. Found: C, 47.18; H, 6.72; N, 9.11.

Assay of DNA Strand Breakage Activity The reaction mixture (100 μl) containing 1 μg of plasmid pBR322 ccc-DNA, 0.1 M of sample compound, and 50 mM of Tris–HCl buffer (pH 7.4) was incubated at 37 °C. ccc-DNA of pBR322 was isolated from *Escherichia coli* W3350/pBR322 as described by Kupersztoch-Portony *et al.*[12] At intervals, 10 μl of the reaction mixture was mixed with 3 μl of 22.5 mM ethylenediaminetetraacetic acid (EDTA) (pH 8.2) containing 1.5% (w/v) sodium dodecyl sulfate (SDS), 25% (w/v) sucrose, and 0.22% (w/v) bromophenol blue, to stop the reaction. The resulting mixture was analyzed directly by agarose-gel electrophoresis as described in a previous paper.[1] After electrophoresis, the gels were stained with ethidium bromide (0.5 μg/ml) for 0.5 h, and washed with running water for 30 s. The stained DNA bands, ccc-DNA, nicked open circular (oc)-DNA and linear DNA, were made visible using an ultraviolet lamp (302 nm) and then photographed with Polaroid film. For quantitative analysis of DNA on the gel, the photographic negatives were scanned with a Shimadzu CS-920 TLC scanner for the OD measurement. The relative amounts of remaining ccc-DNA by this procedure are summarized in Table II.

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