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REACTION OF 2-AMINO-2-DEOXY-D-GLUCOSE AND LYSINE: ISOLATION AND CHARACTERIZATION OF 2,5-BIS(TETRAHYDROXYBUTYL)PYRAZINE*

GIOVANNI CANDIANO†, GIAN MARCO GHIGGERI, ROSANNA GUSMANO,
Department of Nephrology, G. Gaslini Institute, Via 5 Maggio 39, 16148 Genova (Italy)

LUCIA ZETTA,
Istituto di Chimica delle Macromolecole del CNR, Via E. Bassini 15, 20133 Milano (Italy)

EMILIO BENFENATI, AND GIORGIO ICARDI
Istituto di Richerche Farmacologiche M. Negri, Via Gavazzeni 11, 24100 Bergamo, Via Eritrea 62, 20157, Milano (Italy)

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ABSTRACT

The reaction of 2-amino-2-deoxy-D-glucose with lysine in water under simulated physiological conditions gave several browning products, with characteristic optical (λ_{max} 340 nm) and fluorescent properties (emission at 430 nm for excitation at 362 nm). The major product was isolated and characterized by mass spectrometry and n.m.r. spectroscopy as 2,5-bis(tetrahydroxybutyl)pyrazine derived by the condensation of two molecules of 2-amino-2-deoxy-D-glucose.

INTRODUCTION

The reaction between sugars and free amino groups of mammalian proteins involves the initial formation of Schiff bases, followed by an Amadori rearrangement[3] and numerous subsequent rearrangement and dehydration steps with the ultimate formation of browning compounds. Little information is available on the chemistry of the browning derivatives formed under physiological conditions[1,2,4], although they have been implicated in the pathogenesis of diabetes mellitus[5,6]. The extensive literature[7] on the chemistry of browning compounds obtained under non-physiological conditions (preparation and storage of foods) cannot be transferred readily to biological systems.

We now report the characterization of a product obtained by reacting lysine and 2-amino-2-deoxy-D-glucose under simulated physiological conditions (37°, pH 7.4).

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†Author for correspondence.

EXPERIMENTAL

*Reaction of lysine and 2-amino-2-deoxy-*D*-glucose.* — Lysine (100mM) was incubated with 2-amino-2-deoxy-D-glucose (100mM) in 0.02M phosphate buffer (pH 7.4) for 1 week at 37° in the dark. The pH was monitored twice daily and did not change. The solution was then concentrated and the brown residue was extracted with aqueous 50% 1-butanol. The aqueous phase, which contained the major product (*A*), was concentrated three-fold and the residue was extracted with aqueous 80% methanol, then methanol. T.l.c. showed the residue (~70% of the brown residue) to contain a single brown component with R_F 0.08. However, staining with ninhydrin revealed an additional band relative to unreacted lysine (molar ratio brown component–lysine 2:1, as obtained by n.m.r. spectroscopy).

To a solution of the foregoing product (~100 μg) in *N,O*-bis(trimethylsilyl)trifluoroacetamide (50 μL) and pyridine (50 μL) was added chlorotrimethylsilane (5 μL), the mixture was kept for 30 min at 50°, and aliquots (1 μL) were then used for g.l.c.–m.s. of the octakis(trimethylsilyl) derivative. A solution of the foregoing product (~100 μg) in pyridine (50 μL) and acetic anhydride (50 μL) was kept for 1 h at 60° and then concentrated under a stream of nitrogen. The residue was dissolved in chloroform (100 μL); aliquots (1 μL) of this solution were used for g.l.c.

Physical methods. — T.l.c. was performed on silica gel (Merck 60 F_{254}, 5 × 10 cm), using 1-butanol–2-propanol–H_2O (5:14:6) with detection using a fluorescent lamp, the Pauly reagent[8], and charring with H_2SO_4.

U.v./visible spectra were recorded with a Cary 219 spectrophotometer and fluorescent spectra with a Perkin–Elmer MP 44A instrument in standard 10-mm quartz cuvettes and are uncorrected.

N.m.r. spectra were recorded with a Bruker AM-270 spectrometer controlled by an Aspect 3000 computer. The 2-D INADEQUATE ^{13}C-n.m.r. experiment involved quadrature detection in both dimensions, using an extended Ernst-type phase cycle[9] in order to suppress single quantum peaks and a ~125° conversion pulse to reduce F_1 image peaks[10]. The spectrum was accumulated with a sweep width of 11,000 Hz, covering aromatic and aliphatic resonances, over 4 K data points. The experiment was optimized for detection of sp^3–sp^3 carbon ($^1J_{C,C}$ ~30 Hz) connectivities. In the t_1 domain, 1024 traces were accumulated (2048 scans each), zero-filled to 2048 before Fourier transformation; the relaxation delay was 2 s.

The heteronuclear shift-correlated 2D spectrum was obtained with quadrature detection in both dimensions, using polarization transfer from ^{1}H to ^{13}C *via* $J_{H,C}$, without proton decoupling. In the t_2 domain, 11,000 Hz were sampled over 2 K data points, then zero-filled to 4 K. In the t_1 domain, 1200 Hz over 256 traces were accumulated (1024 scans per trace), then zero-filled to 512 before Fourier transformation; the relaxation delay was 2 s. The delay for polarization transfer was 3.125 ms, corresponding to a $J_{H,C}$ value of 160 Hz.

The 2D spectra were resolution-enhanced with a $\pi/3$-shifted squared sine-bell in both dimensions. The 2D-COSY spectrum was acquired with a sweep width of 2400 Hz over 2K data points.

F.a.b.-mass spectra were obtained with a VG 70-250 mass spectrometer equipped with a standard f.a.b. source operating at 8 kV and room temperature, with an accelerating voltage of 6 kV and xenon as the bombarding gas.

G.l.c.–m.s. was effected with a VG 70-250 mass spectrometer coupled to a Dani 6500 gas chromatograph with a fused-silica capillary column (25 m × 0.32 mm i.d.) of CPSil 5CB (Chrompack). A solid injector was used at 280° with a temperature programme of 140° for 1.3 min, then 140 → 280° at 18°/min. E.i.-mass spectra were obtained at 70 eV in the range *m/z* 50–1000 with an ionization source at 250° and a trap current of 100 mA.

RESULTS AND DISCUSSION

Incubation of lysine with 2-amino-2-deoxy-D-glucose under simulated physiological conditions (aqueous media, 37°, pH 7.4) yielded a heterogeneous product with the optical and fluorescent properties of browning compounds (strong absorbance in the range 250–340 nm, fluorescent emission at 430 nm when excited at 362 nm). Extraction with aqueous butanol of the material in the aqueous phase, followed by extraction with aqueous 80% methanol and then methanol, left a residue which appeared homogeneous in t.l.c.

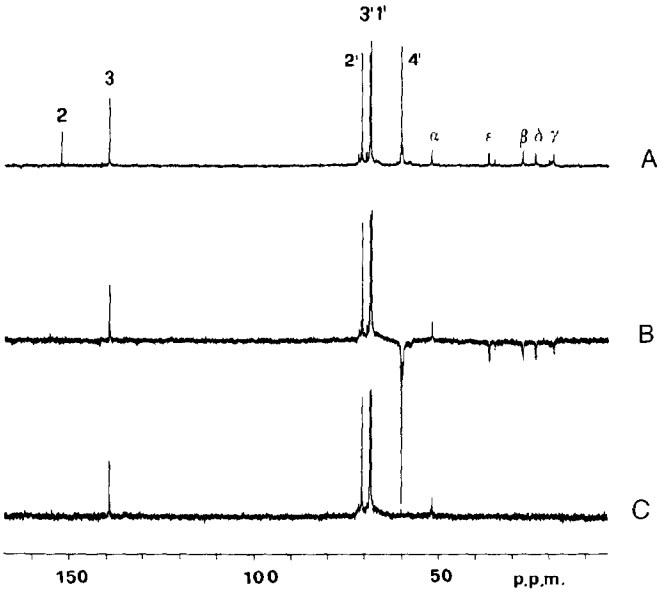


Fig. 1. 67.88-MHz ^{13}C-n.m.r. spectrum of a solution of *A* in D_2O: A, reference spectrum; B, DEPT experiment with refocussing of the even multiplets (d or q) positive and the odd multiplets (t) negative; and C, DEPT experiment with selective refocussing of the doublets.

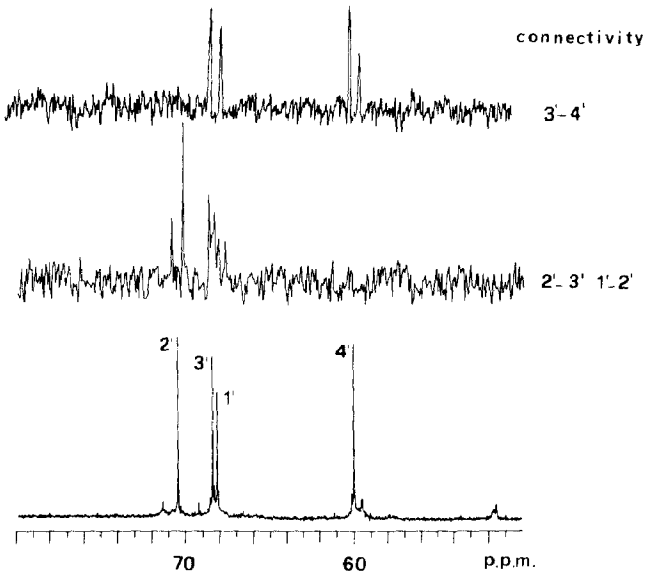


Fig. 2. ^{13}C 2D-INADEQUATE data matrix (67.88 MHz), corresponding to coupled aliphatic ^{13}C pairs of a solution of *A* in D_2O. The conventional ^{13}C-n.m.r. spectrum is shown at the bottom.

The ^1H-n.m.r. spectrum of this product contained broad multiplets corresponding to the lysyl protons and some of the 2-amino-2-deoxy-D-glucose protons. No resonances were observed for the sugar C*H*NH_2 group, whereas two signals were observed at ~5.0 and ~8.8 p.p.m., corresponding to a small amount of a new product (*A*).

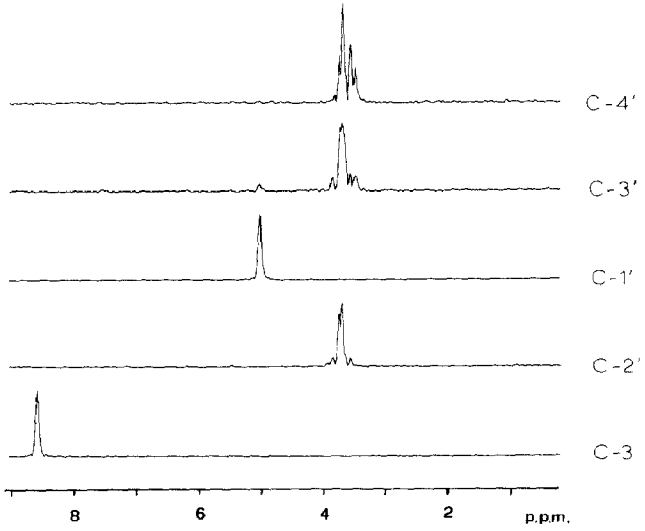


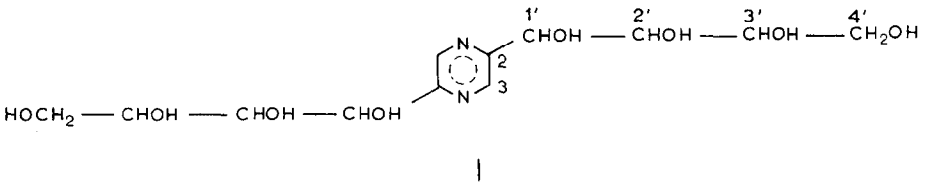
Fig. 3. 2D ^{13}C–^1H heteronuclear-correlated experiment (67.88 MHz) on a solution of *A* in D_2O: cross-sections through each ^{13}C chemical shift.

TABLE I

^{13}C AND ^{1}H CHEMICAL SHIFT DATA FOR 2,5-BIS(TETRAHYDROXYBUTYL)PYRAZINE

Signal	*Atom*	$^{13}C^{a}$	$^{1}H^{a}$
f	2	152.0	
e	3	139.0	8.8
d	2′	70.5	3.7
c	1′	68.5	5.1
b	3′	68.2	3.8
a	4′	60.0	3.6

[a]In p.p.m. from the signal for internal TSP [sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)propionate].



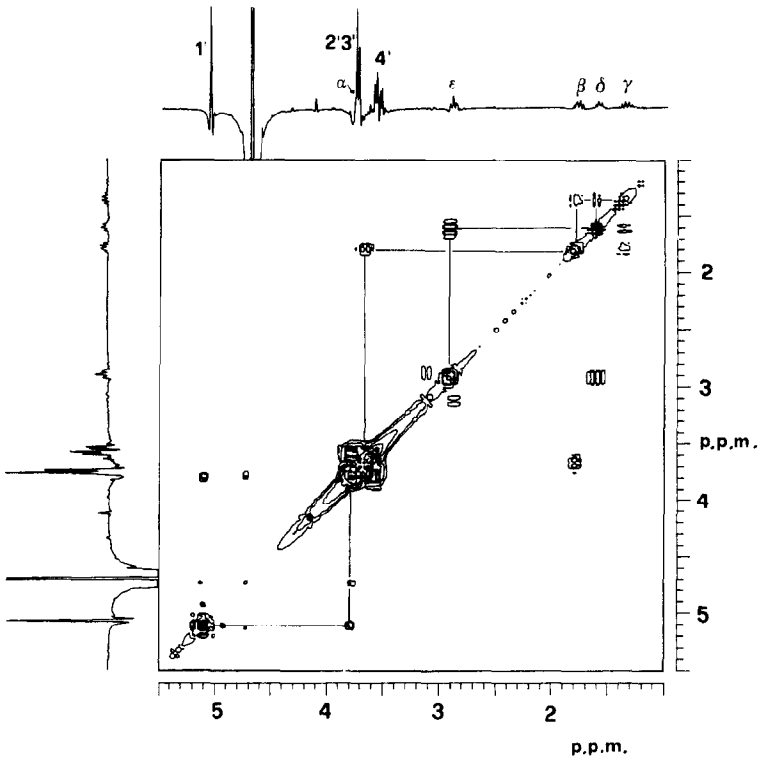


Fig. 4. ^{1}H-N.m.r. (270 MHz) 2D-COSY spectrum of a solution of *A* in D_2O.

The ^{13}C-n.m.r. spectrum of *A* is shown in Fig. 1A together with the DEPT spectra in which all the multiplicities are refocussed (d positive and t negative; B), and only the doublets are refocussed (C). In addition to the weak signals deriving from the four doublets and a triplet of the lysyl moiety, one triplet and three doublets were observed at 60.0, 68.2, 68.5, and 70.5 p.p.m., corresponding to CH and CH_2 groups bonded to oxygen. The resonances at 152 (s, absent from the DEPT spectra) and 139 p.pm. (d) are characteristic of aromatic carbons.

The rows of the 2D-INADEQUATE experiment are shown in Fig. 2, containing the connectivities between the aliphatic protons. Under the experimental conditions used, no connectivities were detected between the lysyl and the aromatic carbon atoms. However, the signals at 60.0 (t) and 68.5 p.p.m. (t) are correlated; that at 70.5 p.p.m. (d) is correlated to those at 68.5 (d) and 68.2 p.p.m. (d), indicating that the fragment CH_2OH–$(CHOH)_3$ is present in *A*.

The ^{13}C assignments in the INADEQUATE experiment made it possible to assign the ^{1}H resonances, *via* the heteronuclear correlations shown in Fig. 3. The ^{13}C and ^{1}H resonances are assigned in Table I.

The COSY spectrum (Fig. 4) shows the connectivities between the protons of the sugar fragment and between the lysyl protons. No connectivities were detected between the sugar and the lysyl moieties.

The molecular weight of *A* was established by positive-ion f.a.b.-mass spectroscopy (thioglycerol matrix), which gave an ion at *m/z* 321 (Fig. 5A). An ion at *m/z* 147 was also present, indicating the presence of lysine. When triethanolamine was used as the matrix, the ion at *m/z* 321 was still present, but there

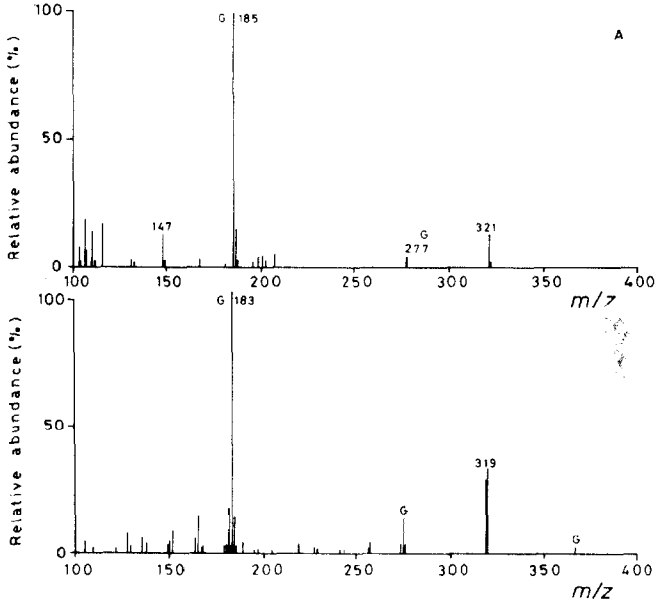


Fig. 5. F.a.b.-mass spectra of *A*: A, positive mode; B, negative mode.

was a peak at *m/z* 470 also, corresponding to the protonated and solvated molecular ion. Such ions have been reported for carbohydrates[11] and support the hypothesis that 320 is the molecular weight of *A*,

In order to confirm this conclusion, some aggregated ions were studied. Addition of HCl, using 2:1 thioglycerol–glycerol as the matrix, gave peaks at *m/z* 357 and 359, with the typical isotopic pattern of chlorine, in addition to the ion at *m/z* 321. Addition of NaCl (thioglycerol matrix) produced a peak at *m/z* 343, confirming the inferred molecular weight. Further proof was obtained by f.a.b.-mass spectrometry in the negative ion mode (glycerol matrix). An $M - H^-$ ion was present at *m/z* 319 (Fig. 5B).

Thus, it is concluded that *A* is not covalently bonded to lysine and is formed from two molecules of 2-amino-2-deoxy-D-glucose to give 2,5-bis(tetrahydroxybutyl)pyrazine. The presence of a symmetrically substituted pyrazine accounts for the six-carbon-atom spectrum.

The product *A* was trimethylsilylated using *N,O*-bis(trimethylsilyl)trifluoroacetamide, and g.l.c.–m.s. of the derivative revealed a single component, the mass spectrum of which (Fig. 6) contains peaks at *m/z* 897 (M^+) and 882 ($M^+ - Me$), a typical fragmentation of these derivatives. There were also peaks at *m/z* 793 ($M^+ - CH_2OSiMe_3$) and 717 ($M^+ - 2\,Me_3SiOH$). The formation of an ion at *m/z* 662 can be explained by the mechanism shown in Fig. 7. The following ions are formed, *m/z* 590 [662 − $(CHOSiMe_3)_2CH_2OSiMe_3$] and 500 (590 − Me_3SiOH). The formation of a positive charge in the aromatic ring allows the expulsion of one of the chains, yielding an ion at *m/z* 409. Other ions are at *m/z* 355 [662 − $(CHOSiMe_3)_2CH_2OSiMe_3$], 307 [$(CHOSiMe_3)_2CH_2OSiMe_3^+$], 217 ($Me_3SiO^+$=CH–CH=$CHOSiMe_3$), 147 ($MeSi{=}O^+SiMe_3$), 103 ($CH_2{=}O^+SiMe_3$), and 73 ($Me_3Si^+$, base peak). These ions are common for trimethylsilylated polyols[12]. This mass spectrum confirms the proposed structure for *A*. Acetylation of *A* gave a single product (g.l.c.–m.s.), the mass spectrum of which is shown in

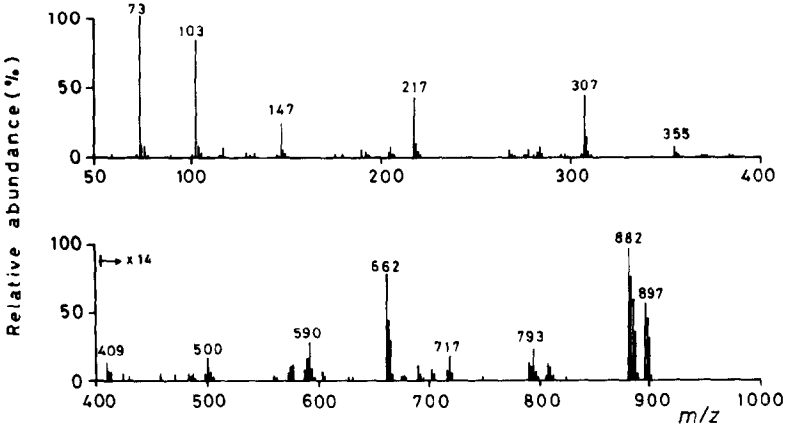


Fig. 6. E.i.-mass spectrum of trimethylsilylated *A*.

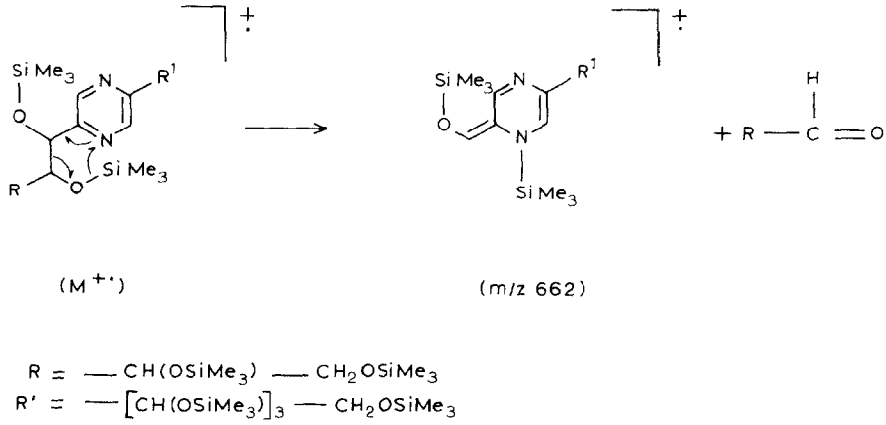


Fig. 7. Possible fragmentation pathway of trimethylsilylated *A* leading to the ion at *m/z* 662.

Fig. 8. As with similar compounds[13], there is the peak for M^+. Loss of Ac or AcO from M^+ yields ions at *m/z* 613 and 597, respectively, as expected[13]. The other ions are formed as follows: *m/z* 554 (597 − Ac), 537 (597 − AcOH), 495 (537 − ketene), 477 (537 − AcOH), 453 (495 − ketene), 440 [M^+ − $CH_2OAc(CHOAc)_2$], 435 (495 − AcOH), 398 (440 − ketene), 338 (398 − AcOH), 296 (338 − ketene), 236 (296 − AcOH), 175 [$(CHOAc)_2CH_2OAc^+$], and 115 (175 − AcOH). The loss of acetic acid and ketene species is typical of acetylated polyols[13].

It has long been known[14] that disubstituted pyrazines result from the condensation of sugars and amino compounds. Hough *et al.*[15] isolated and identified 2-methyl-5-(D-*arabino*-tetrahydroxylbutyl)pyrazine from a complex mixture of products formed by the reaction of D-glucose with aqueous ammonia. Reaction of

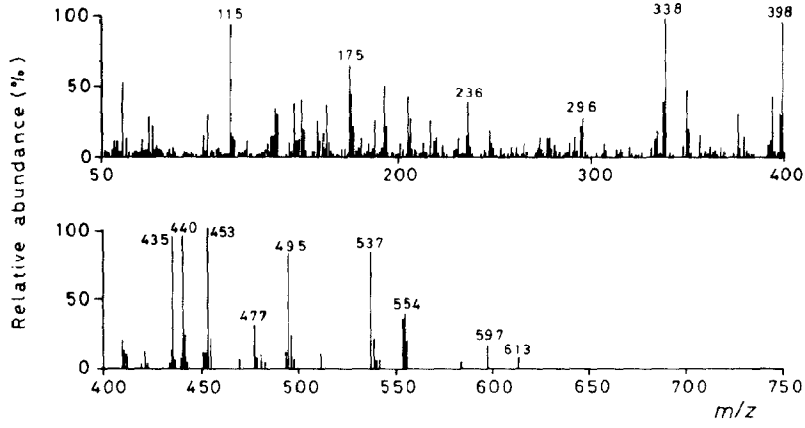


Fig. 8. E.i.-mass spectrum of acetylated *A*.

2-amino-2-deoxy-D-glucose with aqueous ammonia[16] or ethanolic ammonia[15] gave 2,5-bis(tetrahydroxybutyl)pyrazine. Methyl-substituted pyrazines have also been isolated from heated foods as the result of condensation between sugars and amino acids[17].

Symmetrically substituted pyrazines, obtained by spontaneous dimerization of α-aminoketones or aldehydes, are stable only as their salts. The dihydropyrazines are easily dehydrogenated to pyrazines[18]. None of these reactions occurs under physiological conditions, whereas browning compounds are produced at neutral pH under conditions resembling those of human serum. Thus, the characterization of the browning reaction of amino acids and proteins in systems resembling human serum is fundamental for studies of browning and aging of circulating and structural proteins in diabetes mellitus.

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