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MECHANISM OF THE REACTION OF REDUCING CARBOHYDRATES WITH 2-CYANOACETAMIDE, USED FOR POSTCOLUMN LABELING IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR PHOTOMETRIC, FLUORIMETRIC AND ELECTROCHEMICAL DETECTION

SUSUMU HONDA*, KAZUAKI KAKEHI, KAZUMI FUJIKAWA, YUKIYE OKA, AND MASAYE TAKAHASHI
Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka (Japan)
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ABSTRACT

Two fluorescent compounds and one ultraviolet light-absorbing, electrochemically oxidizable compound were isolated from the reaction mixture of glucose and 2-cyanoacetamide by combination of column and paper chromatographies. On the basis of structural studies, the former compounds were considered to be 3-cyano-2-pyridone and 3-cyano-2-pyrrolidone derivatives, and the latter compound was identified as a conjugated diene-ol. These results suggest that the reaction was initiated by condensation of the aldehyde group of glucose with the active methylene group of 2-cyanoacetamide, followed by dehydration to give diene-ols, which were finally cyclized to afford pyridone and pyrrolidone derivatives.

INTRODUCTION

Previously, we proposed[1] for fluorimetric determination of reducing carbohydrates a convenient procedure using 2-cyanoacetamide. It was shown that this reagent reacts with reducing carbohydrates in weakly alkaline media to give highly intense fluorescence. Further investigation indicated that this reagent also produces substances that strongly absorb ultraviolet light under similar conditions, permitting sensitive, photometric determination at ~280 nm (where commercial mercury lamps emit most abundantly). In addition, the usefulness of this reagent for electrochemical detection was demonstrated[3]. Because of the versatility of the detection methods, and the high sensitivity and non-corrosiveness of the reagent, this type of reaction is quite suited for postcolumn labeling in high-performance liquid chromatography (h.p.l.c.). Thus, the 2-cyanoacetamide method has been widely used for automated analysis of various classes of carbohydrate[4–7].

However, the detailed mechanism of this type of reaction has not appeared, although structural requirements of carbohydrate for fluorescence generation[1], chromophore formation[2], and induction of electrochemical oxidizability[3] have been

*To whom correspondence should be addressed.

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briefly mentioned. We now discuss the mechanism of this type of reaction on the basis of structure elucidation of compounds isolated from a reaction mixture.

EXPERIMENTAL

Materials. — 2-Cyanoacetamide was obtained from Kanto Chemical Company (Nihonbashi, Chuo-ku, Tokyo). Avctivated charcoal (100–200 mesh) was purchased from Wako Pure Chemicals (Dosho-machi, Higashi-ku, Osaka). Other chemical compounds were of the highest grade commercially available. A column (25 mm i.d., 31 cm length) packed with silica RP-8, as well as thin-layer plates precoated with silica gel, silica RP-8, and cellulose, were obtained from Merck (Darmstadt, West Germany).

Instruments. — Fluorescence and ultraviolet absorption spectra were respectively recorded with a Hitachi 650-10 spectrofluorimeter and a Hitachi 557 spectrophotometer, by using a 1-cm quartz cell. Infrared (i.r.) absorption spectra were recorded with a Hitachi 260-38 spectrophotometer. ^{1}H-Nuclear magnetic resonance (n.m.r.) spectra were recorded with a JEOL JNM FX-200 spectrometer operated at 200 MHz in the pulse-Fourier transform mode at 25 ±2° with 90°-pulse at 6-s intervals. The homo-gated decoupling technique was applied to deuterium oxide solutions to eliminate the signal of water. Chemical shifts are expressed on the δ scale. The nuclear Overhauser enhancement (n.O.e.) was observed in deoxygenated chloroform-*d* with 30°-pulse at 30-s intervals. The enhancement of proton resonance was estimated from the mean value of duplicate measurements. Experiments for electrochemical detection were performed in the flow injection mode, by using an Irika E-502 amperometric detector equipped with a glassy carbon and a silver chloride electrode as the working and reference electrodes, respectively. An aqueous solution of D-glucose was injected, *via* a Rheodyne injector carrying a 20-μL loop, into a stream of water, supplied by a Hitachi 638 double-plunger pump at a flow rate of 0.50 mL/min, to which were consecutively added, *via* Y-shaped (PTFE) connectors, 0.20M borate buffer (pH 9.5) and an aqueous 1.5% solution of 2-cyanoacetamide at the same flow rae of 0.25 mL/min. The mixed effluent was allowed to flow through a 10-m PTFE coil immersed in a glycerol bath thermostated at 100 ±1°, and then introduced into the amperometric detector. The electrolytic current was recorded at an applied potential of 400 mV. For comparative studies, the isolated compounds were processed in the same manner, except that the 2-cyanoacetamide solution was replaced by water, and the reaction bath was maintained at 25 ±1°.

Paper and thin-layer chromatography. — Paper chromatography was performed in the descending mode on Whatman No. 1 and 3MM filter papers for analytical and preparative purposes, respectively. Papers were developed overnight with 6:1:2 (v/v) 1-butanol–acetic acid–water. Normal-phase thin-layer chromatography (t.l.c.) was performed on precoated silica gel and cellulose plates, with the same solvent as that used for paper chromatography. Reverse-phase t.l.c. was

carried out on silica gel RP-8 plates with 4:1 (v/v) acetonitrile–water as solvent. Spots were directly monitored under ultraviolet light at 254 and 365 nm. Spots on silica gel plates were additionally detected by charring with sulfuric acid.

Isolation of the fluorescent products (compounds II and III) and the ultraviolet light-absorbing, electrochemically oxidizable product (compound I). — (i) Reaction of D-*glucose with 2-cyanoacetamide.* D-Glucose (1.00 g, 5.5 mmol) and 2-cyanoacetamide (4.67 g, 55.6 mmol) were dissolved in 0.3M borate–phosphate buffer (pH 8.0, 350 mL) and heated on a boiling-water bath, and the solution was kept for 30 min at 100°; this procedure was repeated five times, and the reaction mixtures were combined. The whole was concentrated to 100 mL, the concentrate was kept overnight in a refrigerator, the resultant solid was filtered off, and the filtrate was evaporated to dryness, to yield 14.5 g of a dark syrup.

(ii) Fractionation on a column of charcoal. The residue was dissolved in a small volume of water, and the solution was applied to a column (5 cm i.d., 56 cm length) packed with activated charcoal (300 g). The column was eluted stepwise by using aqueous methanol with increasing methanol concentration, the fluorescence at 331 (excitation)/383 (emission) nm being recorded. From the 10% (fraction 3) and 50% (fraction 7) methanol fractions, dark syrups A (230 mg) and B (53 mg) were respectively obtained by evaporation.

(iii) Reverse-phase partition chromatography of syrup A. Syrup A (230 mg) was dissolved in 70% aqueous acetonitrile (2 mL), and the solution was applied to a column packed with silica RP-8. The column was eluted with 80% aqueous acetonitrile at a flow rate of 1.0 mL/min, with photometric and fluorimetric monitoring at 270 nm and 331 (excitation)/383 (emission) nm, respectively. The 70–100-mL fraction, which had strong absorption, was evaporated to dryness, to give crude compound I as a pale-yellow syrup; yield, 90 mg; λ_{max}^{water} 270 nm (ε_{mM} 18.30). A 10-ng sample of compound I gave an electrolytic current of 264 nA, whereas 1 nmol of D-glucose generated an electrolytic current of 87 nA when treated under the conditions described. Compound I (50 mg) was acetylated with a mixture of acetic anhydride (2 mL) and pyridine (2 mL) overnight at room temperature. The mixture was extracted with ethyl acetate, and the extract was washed with dilute hydrochloric acid, evaporated to dryness, and the residue purified on a small column packed with silica gel, with 3:1 (v/v) chloroform–acetone as the solvent. The purified acetate of compound I (55 mg) was obtained as a syrup after evaporation of the eluate; λ_{max}^{EtOH} 270 nm (ε_{mM} 19.20); $\nu_{max}^{CHCl_3}$ 2155, 1750, 1700, 1640, and 1590 cm^{-1}; ^{1}H-n.m.r. data ($CDCl_3$): δ 7.81 (s, 1 H), 6.51 (bd, 2 H, exchangeable with D_2O), 6.13 (d, 1 H, *J* 9.3 Hz), 5.87 (dd, 1 H, *J* 2.0, 9.8 Hz), 5.19 (dt, 1 H, *J* 5.2, 5.5 Hz), 4.23 (d, 2 H, *J* 5.0 Hz), 2.41 (s, 3 H), 2.09 (s, 3 H), 2.07 (s, 3 H), and 2.06 (s, 3 H). From the 120–150-mL fraction was obtained compound II, which had an absorption maximum at 327 nm, together with a shoulder at 236 nm; yield, 5 mg; recovery of fluorescence, 65%; ν_{max}^{KCl} 3350, 2140, and 1650 cm^{-1}; fluorescence (nm): 320 (excitation), 383 (emission); ^{1}H-n.m.r. data (D_2O): δ 7.77 (d, 1 H, *J* 8.0 Hz), 6.72 (d, 1 H, *J* 8.0 Hz), 3.4–3.8 (m, 3 H), 2.45 (dd, 1 H, *J* 2.0,

15.0 Hz), and 2.15 (dd, 1 H, *J* 10.5, 15.0 Hz). The electrolytic current generated by the oxidation of compound II (~10 ng) was 0.2 nA.

(iv) Normal-phase, partition chromatography of syrup B. Syrup B (53 mg) was dissolved in a small volume of water, and the solution was applied to a column (10 mm i.d., 25 cm length) packed with silica gel (Kiesel gel 60, Merck; 15 g) pre-equilibrated with 6:1:2 (v/v) 1-butanol–acetic acid–water. The column was eluted with the same solvent at a flow rate of 0.25 mL/min. The 35–55-mL fraction was evaporated to dryness, to give an orange syrup which was purified by preparative paper chromatography with the same solvent. Zones having R_F 0.56 were extracted with water. The extracts were combined, and evaporated to dryness, to yield compound III as a yellowish-orange powder; yield, 3 mg; recovery of fluorescence, 11%; λ_{max}^{water} 440, 325, and 270 nm; ν_{max}^{KCl} 3350, 2140, and 1650 cm^{-1}; ^1H-n.m.r. data (D_2O): δ 7.80 (s, 1 H), 3.5–3.8 (m, 4 H), 2.56 (dd, 1 H, *J* 2.0, 14.0 Hz), and 2.25 (dd, 1 H, *J* 11.0, 14.0 Hz). The electrolytic current generated by the oxidation of compound III (~10 ng) was 0.1 nA.

Preparation of (2E,4E) (6R,7R)-4,6,7,8-tetraacetoxy-2-cyano-octadienamide. — To a solution of 2-cyanoacetamide (55 mg, 0.75 mmol) in pyridine (3 mL) was added 2,3,4,5,6-penta-*O*-acetyl-*aldehydo*-D-glucose[8] (195 mg, 0.50 mmol). The mixture was stirred overnight at room temperature in the presence of a few sticks of molecular sieve 4A, extracted with ethyl acetate (10 mL), and the extract washed with dilute hydrochloric acid, evaporated to dryness, and the residue purified as described for the acetate of compound I; yield, 96 mg (42.1%, syrup); λ_{max}^{EtOH} 270 nm (ε_{mM} 19.10); $\nu_{max}^{CHCl_3}$ 2155 (nitrile), 1750 (acetyl), 1700 (amide), 1640 (conjugated diene), and 1590 cm^{-1} (conjugated diene); ^1H-n.m.r. data ($CDCl_3$): δ 7.81 (s, 1 H, H-3), 6.53 (bd, 2 H, exchangeable with D_2O, NH_2), 6.14 (d, 1 H, *J* 9.4 Hz, H-5), 5.86 (dd, 1 H, *J* 2.0, 9.7 Hz, H-6), 5.19 (dt, 1 H, *J* 5.2, 5.5 Hz, H-7), 4.23 (d, 2 H, *J* 5.0 Hz, H-8,8′), 2.41 (s, 3 H, acetoxyl at C-4), 2.09 (s, 3 H, acetoxyl), 2.07 (s, 3 H, acetoxyl), 2.06 (s, 3 H, acetoxyl).

Fluorescence generation of compound I. — An aqueous solution (1 mL) containing crude compound I (10 μg) was dissolved in 0.3M borate–0.3M phosphate buffer (pH 8.0, 5 mL), and the solution was heated for 30 min on a boiling-water bath. After the solution had been cooled to room temperature, the fluorescence spectrum was recorded.

RESULTS AND DISCUSSION

Fluorescence and absorption spectra of the reaction mixture. — The fluorescence spectrum of the reaction mixture of D-glucose and 2-cyanoacetamide on a preparative scale (see Fig. 1a) showed exciation and emission maxima at the same wavelengths (331 and 383 nm, respectively) as those reported for fluorimetric analysis[1]. The absorption spectrum (see Fig. 1b) gave a maximum at 270 nm, also approximately the same as that for photometric analysis[2].

Isolation of the fluorescent, ultraviolet light-absorbing, and electrochemically oxidizable entities. — The reaction mixtures was fractionated on a column of

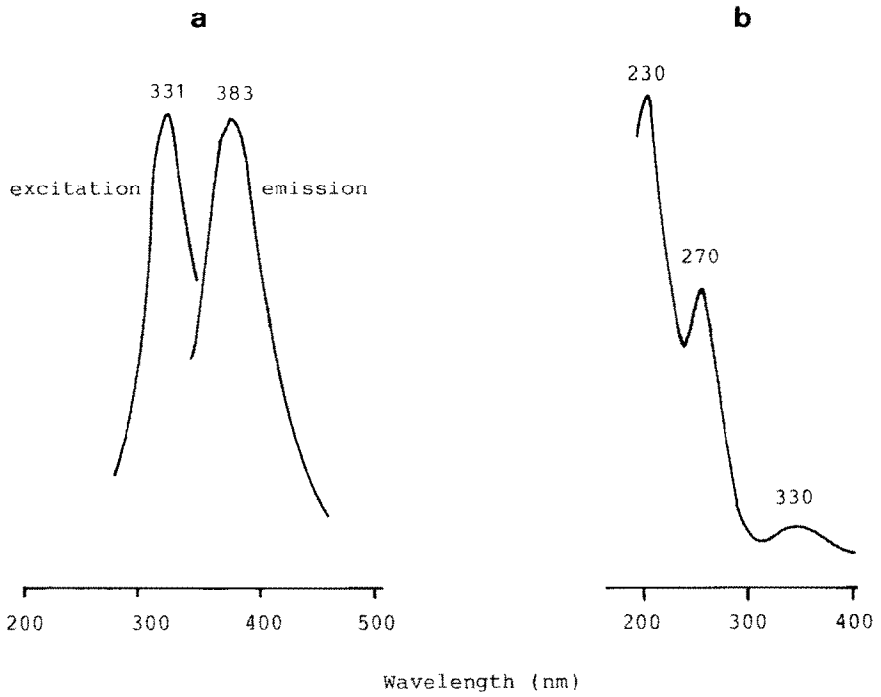


Fig. 1. Fluorescence (a) and absorption (b) spectra of a reaction mixture of D-glucose and 2-cyanoacetamide.

charcoal, to give two intensely fluorescent fractions, 3 and 7, with aqueous 10% and 50% methanol, respectively. These fractions were evaporated to dryness to give dark syrups, A and B, respectively.

Syrup A was then fractioned on a column of silica RP-8 in the reverse-phase partition mode, to give an ultraviolet light-absorbing, electrochemically oxidizable compound (I) and a fluorescent compound (II), in yields of 18 and 1 mg, respectively, per g of D-glucose. Another fluorescent compound (III) was obtained pure in yields of 0.6 mg per g of D-glucose from syrup B by fractionation on a column of silica gel, followed by paper chromatography. Although the yield of compound II was low, the recovery of fluorescence based on the original reaction mixture was as high as 65%, indicating that this compound was the major fluorescent product. With regard to compound III, the recovery of fluorescence was 11%. The yield of compound I was also low, but reasonable, considering its high millimolar absorptivity (18.30). The electrolytic current generated by the electrode oxidation of crude compound I was ~60 times that of the reaction mixture resulting from an equimolar amount of D-glucose. This value was in good agreement with the reciprocal of the yield of compound I, multiplied by 100, *i.e.*, 56.

Characterization and structure elucidation of the fluorescent products (compounds II and III). — Compound II was readily soluble in water, and fluoresced intensely, with excitation and emission maxima at 331 and 383 nm, respectively, as

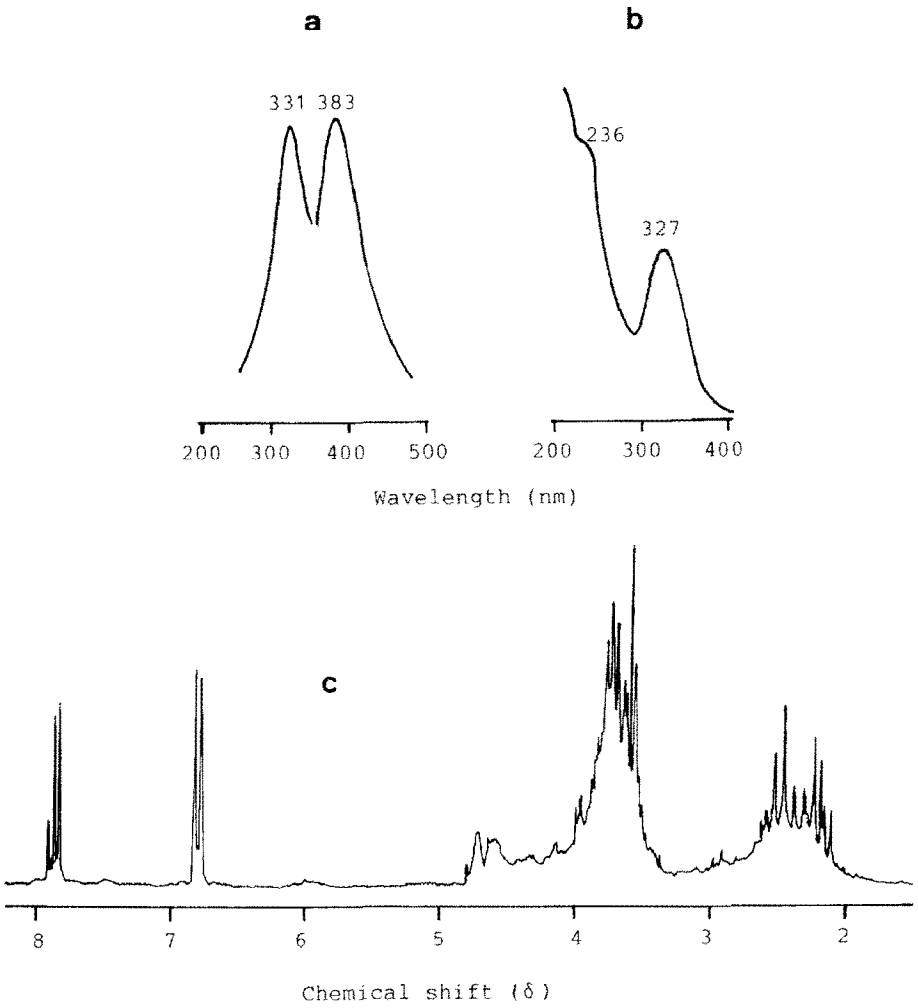


Fig. 2. (a) Fluorescence, (b) absorption, and (c) ^{1}H-n.m.r. spectra of compound II.

shown in Fig. 2a. However, its aqueous solution showed an absorption maximum at 327 nm, together with a shoulder at 236 nm, different from that on the reaction mixture (see Fig. 2b). The absorption spectrum was not changed by change in pH. In addition, compound II was not retained on a column of Amberlite CG-120 ion-exchange resin (H^+), as well as Amberlite CG-400 (acetate form), suggesting chemical neutrality. The ^{1}H-n.m.r. spectrum (see Fig. 2c) showed the presence of a pair of doublets, at 7.77 and 6.72 p.p.m., coupled with each other and giving a coupling constants of 6.3 Hz. Their chemical shifts and the large coupling constants indicated that these protons are oriented *ortho* in an aromatic or a heterocyclic ring. On the other hand, the absorption bands at 2140 and 1650 cm^{-1} in its i.r. spectrum were indicative of the presence of the cyano and amido groups. All these spectral characteristics were consistent with those of 3-cyano-6-methyl-2-pyridone,

synthesized according to the literature[9]. Thus, it was established that compound II had a 2-pyridone skeleton having the cyano group at C-3. Beside these signals, there were two sets of doublets of doublets in the range 2.1–2.6 p.p.m., which were assignable to methylene protons at the innermost carbon atom of the substituent dihydroxypropyl group at C-6 of the 2-pyridone ring. Three protons in the range 3.4–3.8 p.p.m. were ascribed to hydroxymethyl and hydroxymethylene protons. Therefore, the structure of 3-cyano-6-(1,2-dihydroxypropyl)-2-pyridone was proposed for compound II. The presence of minor doublets in the low field, and small signals in the methylene region (~2.3–2.6 p.p.m.) suggest the presence of an isomer of compound II.

Compound III, also soluble in water, had its excitation and emission maxima at the same wavelength as those of compound II (see Fig. 3a), but its absorption

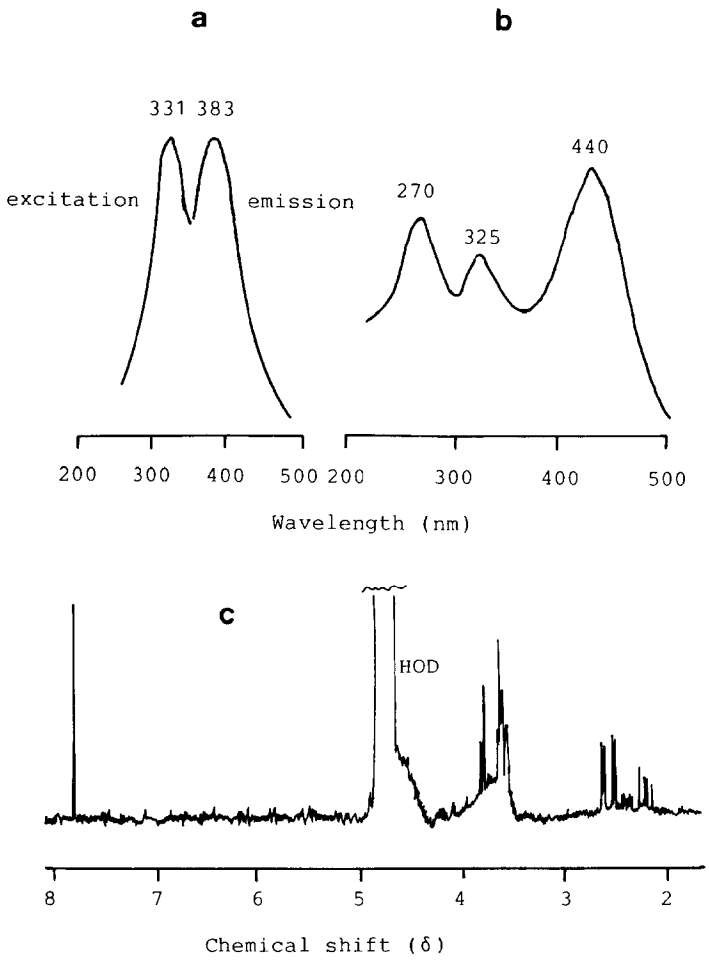


Fig. 3. (a) Fluorescence, (b) absorption, and (c) ^{1}H-n.m.r. spectra of compound III.

maxima were at 440, 325, and 270 nm (see Fig. 3b), quite different from those of compound II and of the reaction mixture. The ^{1}H-n.m.r. spectrum (see Fig. 3c) showed a singlet at 7.80 p.p.m. as the only signal in the aromatic region. Two sets of doublets of doublets in the range 2.0–2.6 p.p.m. were assigned to the methylene protons, and the signals for four protons in the range 3.5–3.8 p.p.m. were considered to be those of two hydroxymethyl and two hydroxymethylene protons. The absorption bands at 2140 and 1650 cm^{-1} in its i.r. spectrum indicated that compound III also contains the cyano and amide groups. On the basis of these spectral data, the structure of 3-cyano-5-(2′,3′,4′-trihydroxybutyl)-2-pyrrolidone was proposed for compound III. Neither of the fluorescent products (compounds II and III) was oxidized on a glassy carbon electrode. Based on the structures proposed, the yields of compounds II and III were 0.09 and 0.06%, respectively.

Characterization and structure elucidation of the ultraviolet light-absorbing, electrochemically oxidizable products (compound I). — Compound I showed an intense absorption at 270 nm, as shown in Fig. 4a. The ultraviolet and infrared absorption spectra, as well as the ^{1}H-n.m.r. spectrum of its acetate (see Fig. 4b) were identical with those of 4,6,7,8-tetraacetoxy-2-cyano-2,4-octadienamide. For stereochemical assignment of the 3- and 5-protons, the n.O.e. was examined.

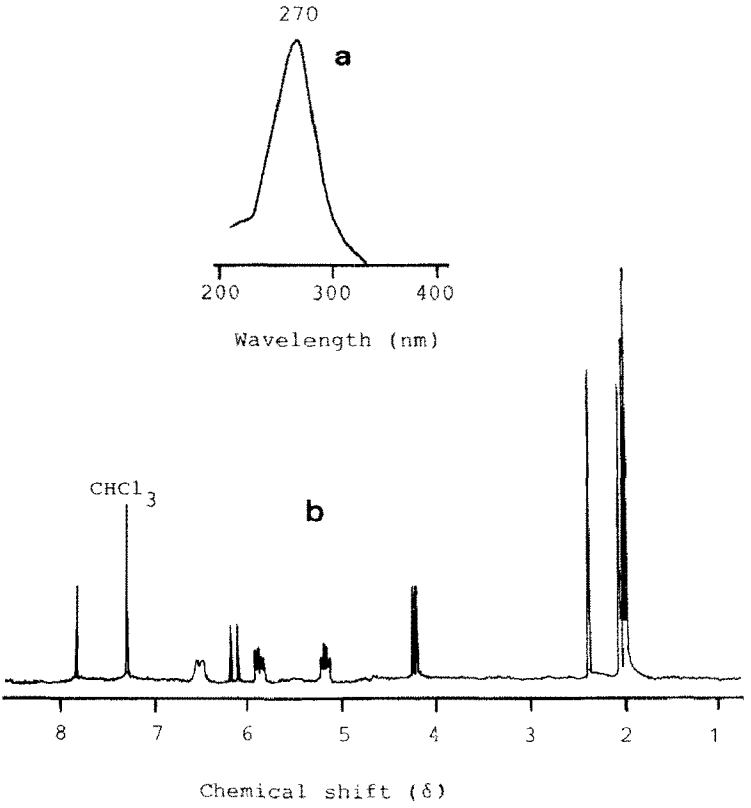
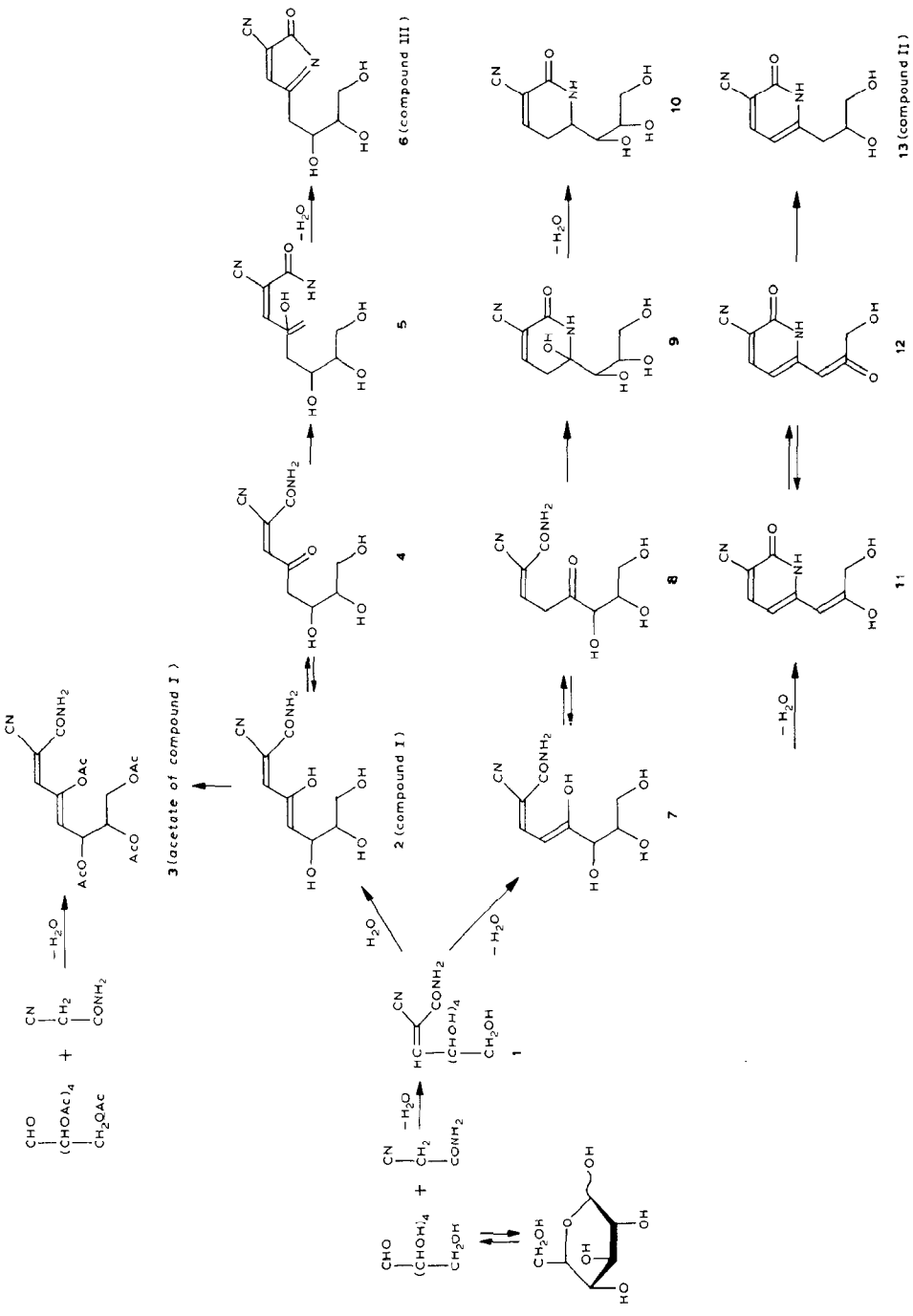


Fig. 4. (a) Absorption and (b) ^{1}H-n.m.r. spectra of the acetate of compound I.



Scheme 1. Proposed pathways of the reaction of D-glucose with 2-cyanoacetamide.

Selective irradiation of the H-3 nuclei resulted in 20% enhancement of the resonance of H-5 and that of the H-5 nuclei caused an 18% increase of the H-3 signal. These results imply that these protons are located on the same side. On the other hand, no enhancements were observed between carboxamide protons and H-3 or H-5. Therefore, it is evident that compound I is a 2*E*,4*E* isomer. It was readily oxidized on a glassy carbon electrode, generating an electrolytic current of 264 nA from a 10-ng sample at an applied potential of 400 mV. The yield of compound I, based on the proposed structure, was 1.9%.

When an aqueous solution of compound I was heated under the same conditions as those used for fluorimetric determination, intense fluorescence having the same excitation and emission maxima as those for compounds II and III was generated. This provided evidence that compound I is an intermediate in the fluorescence reaction.

Reaction mechanism. — On the basis of the characterization and structure elucidation of the compounds isolated, the initial stage of the reaction is considered to be the Knoevenagel condensation between the aldehyde group in acyclic β-glucose and the active methylene group in 2-cyanoacetamide (see Scheme 1). The condensate **1** will be dehydrated *via* two routes, to give positional isomers of a conjugated octadienamide. The first isomer (**2**), carrying a hydroxyl group at C-4 will then be cyclized, *via* the keto form **4**, to the intermediate **5**, which will be dehydrated to give the 2-pyrrolidone derivative **6**, which is the same as compound III. In the processes, a methylene group will be introduced to the innermost carbon atom of the side chain. The second isomer (**7**), whose C-5 atom is hydroxylated, will be similarly cyclized, *via* the keto form **8**, to **9**, which will afford the 2-pyridone derivative **10** by dehydration. The formation of a methylene group in the side chain may be explained as resulting from a series of further reactions: dehydration between the HO-2 and HO-3 of the trihydroxypropyl group to give **11**, tautomerism to keto form **12**, and reduction to **13**. At the last stage, stereoisomers may be formed; this is in accord with the presence of minor signals at 7.80 and 6.72 p.p.m., for compound II. The reductant in the final process, namely, **12**→**13**, may be the reagent or its degradation products, although no evidence for this was educed in the present study. The high fluorescence recovery (65%) by **13** (compound II) indicated that this is the major entity of fluorescence. The production of **6** (compound III), which gave a fluorescence recovery of 11% with the same excitation and emission maxima as those of **13**, is considered to have played a subsidiary role in this fluorescence reaction. The reason why 5 mg of **13** gave about six times higher fluorescence recovery than 3 mg of **6** is presumably attributable to the difference of molar fluorescence intensity.

Of the two dienic key intermediates, **2** and **7**, only the former was isolated (as compound I), and purified as its acetate (**3**). No signals assignable to H-3 and H-4 of **7** were deteced in the spectrum of crude compound I. Transformation into the succeeding compounds was probably so rapid that **7** was not isolable as a stable intermediate. The intermediary formation of such dien-ols, at least **2**, was evidenced by fluorescence generation from compound I under the analytical conditions.

The foregoing mechanism indicates that fluorimetric and photometric analyses of reducing carbohydrates with 2-cyanoacetamide are based on the formation of different compounds, namely, the former on **13** and **6**, and the latter on **2**. Electrochemical detection is also considered to be based on the oxidation of **2**, because it is readily oxidized on a glassy carbon electrode, whereas **13** and **6** are not.

The mechanism proposed for the reaction of D-glucose may also be valid for other reducing carbohydrates, although the yields of fluorescent products, as well as the pyridone:pyrrolidone ratio may vary with the carbohydrate structure, especially that at C-2 and C-3; for example, the fluorescence intensities of 2-deoxy-D-*erythro*-pentose, 2-deoxy-D-*arabino*-hexose, and 3-*O*-methyl-D-glucose relative to that of D-glucose are as low as 0.01 (ref. 1), 0.01 (ref. 1), and 0.08 (ref. 10), respectively. These results indirectly support the aforementioned mechanism. In the cases of hexosamines and their *N*-acetates, which are as sensitive as aldoses, dienic compounds might be similarly formed as intermediates, but the amino or acetamido group must be removed before cyclization. The subsequent pathways may be the same as those in Scheme 1. Generation of fluorescence (having weak intensity) from ketoses may be explained as resulting from their partial isomerization to aldoses during reaction.

Although the yields of the isolated compounds are low, the capability of fluorescence generation, absorptivity, and electrochemical oxidizability of these compounds is so high that this type of reaction allows sensitive analysis of reducing carbohydrates by fluorimetric, photometric, or electrochemical methods.

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