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Aerial Oxidation of Some 2-Pyridyl Ketone Hydrazones Catalyzed by Cu^{2+}. Physical Properties of Reaction Products

Hisakazu MORI,* Kaori SAKAMOTO, Sachie MASHITO, Yohko MATSUOKA,
Mika MATSUBAYASHI, and Kazue SAKAI

Kyoritsu College of Pharmacy, Shibakoen, Minato-ku, Tokyo 105, Japan. Received May 7, 1993

Some 2-pyridyl ketone hydrazones were subjected to aerial oxidation catalyzed by Cu^{2+}. The oxidation product of di-2-pyridyl ketone hydrazone had the structure of a derivative of [1,2,3]-triazolo[1,5-*a*]pyridine, and this structure is different from that previously proposed by other investigators. The fluorescence spectra of the oxidation products were measured in solutions of a wide pH range. In contrast to the oxidation products of other 2-pyridyl ketone hydrazones, that of di-2-pyridyl ketone hydrazone showed very strong fluorescence in acidic media. The characteristic nature of this compound was also apparent in the ultraviolet spectrum. The generation of hydroxyl radical was demonstrated in the aerial oxidation of di-2-pyridyl ketone hydrazone catalyzed by Cu^{2+}, suggesting the formation of hydrogen peroxide as another oxidation product.

Keywords oxidation product; fluorescence spectrum; ^{1}H-NMR spectrum; aerial oxidation; hydrazone

We have studied the determination of amino acids[1] and proteins[2] in a flow system by the use of the aerial oxidation of phenyl 2-pyridyl ketone hydrazone (**1**)[1] or di-2-pyridyl ketone hydrazone (**2**)[2] catalyzed by Cu^{2+}. Since fluorescent compounds were formed in these reactions, amino acids or proteins were detected fluorometrically with high sensitivity. The sensitivity depends on the fluorescent character of the oxidation products and the rate of formation of the oxidation products in the flow systems. In order to find a substrate which affords high sensitivity for the determination of amino acids and proteins, we undertook the aerial oxidation of some 2-pyridyl ketone hydrazones catalyzed by Cu^{2+} and investigated several physical properties of the products. The compounds subjected to oxidation were **1**, **2**, methyl 2-pyridyl ketone hydrazone (**3**), and pyridine-2-aldehyde hydrazone (**4**). We also tried to detect hydrogen peroxide in the oxidation system as another reaction product.

Results and Discussion

Spectroscopic Characters of Oxidation Products of 2-Pyridyl Ketone Hydrazones The proton nuclear magnetic resonance (^{1}H-NMR) spectra of the oxidation product (**5**) of **3** and that (**6**) of **4** in $CDCl_3$ showed in common two triplet signals (including a double doublet signal with comparable coupling constants) and two doublet signals at δ 6.9—7.0, 7.1—7.3, 7.6—7.8 and 8.6—8.8, respectively (see Experimental). These signals can be ascribed to four protons of ring A in the structure I (Fig. 1). This structure was also supported by other data. The ^{1}H-NMR spectrum (Fig. 2) of the oxidation product (**7**) of **2** in $CDCl_3$ exhibited eight signals due to aromatic ring protons. Four of them were triplet signals and four of them were doublet signals.[3] Two triplet signals ($\delta = 7.06$, 7.38) and a doublet signal ($\delta = 8.77$) were observed at similar δ values to those of the signals in the spectra of **5** and **6**. These results favor the structure I in Fig. 1 for **7**. The doublet signal of H_a of **7** was observed at lower magnetic field ($\delta = 8.74$) than those of **5** and **6** ($\delta = 7.6$—7.8), due to the interaction of H_a with the nitrogen atom in the pyridyl group. An analogous effect had been reported[4] with respect to 1-vinyl-2-(2′-pyridyl)benzimidazole. The melting point (126—126.5 °C) of **7** was almost identical with that (126 °C)[5] of the compound synthesized by another method and assigned the structure I. Other spectral data were also consistent with the structure I. Thus, it can be concluded that compound **7** is 3-(2′-pyridyl)-[1,2,3]-triazolo[1,5-*a*]pyridine. On the other hand, Valcarcel *et al.*[6] proposed the structure II (Fig. 1) for the aerial oxidation product of **2** in the presence of Cu^{2+} as a catalyst, on the basis of the observation of the absorption due to H–C (aliphatic carbon) stretching in the infrared (IR) spectrum. However, no singlet proton signal was observed in the ^{1}H-NMR spectrum of **7**. Thus, the structure II in Fig. 1 can be excluded for **7**.[7] The oxidation of 2-pyridyl

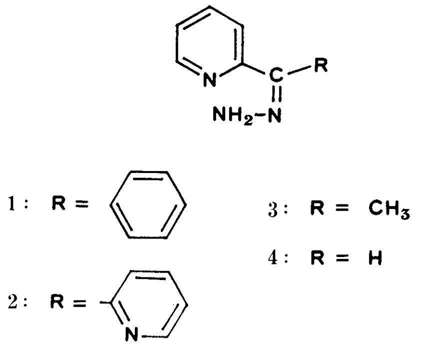

Chart 1

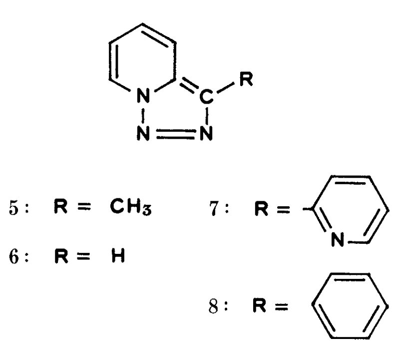

Chart 2

Fig. 1. Isomeric Structures of the Oxidation Product of 2-Pyridyl Ketone Hydrazone

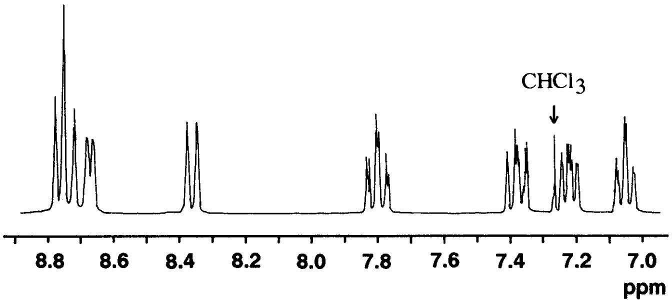

Fig. 2. ^{1}H-NMR Spectrum of **7** in $CDCl_3$

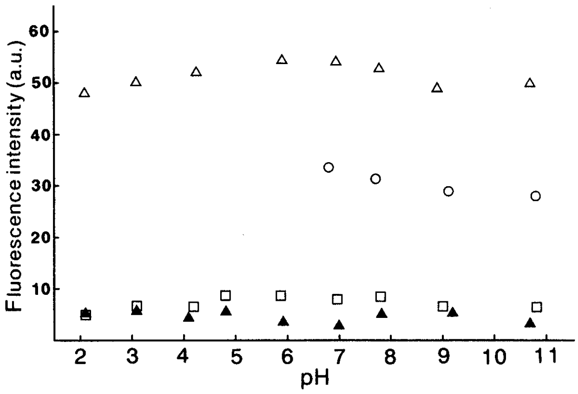

Fig. 3. The Dependence of the Fluorescence Intensities of the Aerial Oxidation Products of Some 2-Pyridyl Ketone Hydrazones on the pH of Their Solutions

□, **5**; ▲, **6**; ○, **7**; △, **8**. The concentrations of solutes were 5 μM. The solutions with pH in the range of 4.5—8 were prepared with citrate–phosphate buffers, those of pH less than 4.5, with a solution of HCl, and those of pH more than 8.9, with a solution of NaOH. The excitation and emission wavelengths which afforded maximum fluorescence intensities were automatically read with the instrument used. They differed somewhat for the samples of different pH. They were 281—287 and 391—399 nm for **5**, 236—243 and 391—394 nm for **6**, 305—307 and 403—407 nm for **7**, and 294—296 and 433—435 nm for **8**.

ketone hydrazones with nickel peroxide was investigated by Ogura *et al.*[8] We consider that all oxidation products of 2-pyridyl ketone hydrazones examined here had the structure I. However, the oxidation product of **2** with nickel peroxide seemed not to have the structure I, based on a comparison with the data for **7** obtained in the present study; the melting point[8] of the oxidation product of **2** with nickel peroxide was 165 °C, and the ^{1}H-NMR spectrum[8] of this compound seemed to be different from that of **7**.

It was reported by Valcarcel *et al.*[6] that the fluorescence spectrum of **7** in solution changed markedly with change of the pH of the medium. Thus, the fluorescence spectra of the oxidation products of 2-pyridyl ketone hydrazones were measured in aqueous solutions over a wide pH range. Compounds **5** and **6**, and the oxidation product (**8**) of **1** showed no remarkable pH-dependence of the spectrum. The wavelengths of excitation and emission which afforded maximum fluorescence intensities were almost constant, and the fluorescence intensities did not vary much, as indicated in Fig. 3. In contrast, a great difference was observed between the fluorescence spectra of **7** in acidic and basic media. In the basic media, the wavelengths of excitation and emission giving maximum intensity were about 305 nm and about 405 nm, respectively, and the fluorescence intensity was comparable to that of **8** (Fig. 3). On the other hand, in the acidic media, the wavelengths of excitation and emission affording maximum intensity were about 348 nm and about 434 nm, respectively, and the fluorescence intensity increased with increase of the acidity of the medium, as shown in Fig. 4. The fluorescence spectra in the most acidic media gave enormous intensity values. The ultraviolet (UV) spectrum of **7** was also characteristic. As shown in Fig. 5, the absorption maximum shifted to longer wavelength in the acidic medium (347 nm at pH 1.4), compared to that in the basic medium (305 nm at pH 10.4). Such a characteristic was not found in the oxidation

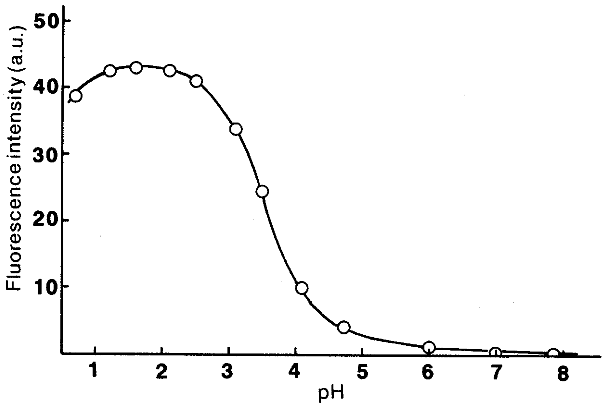

Fig. 4. The pH Dependence of Fluorescence Intensity of **7** in the Acidic Region

The concentration of **7** was 0.1 μM. The solutions of pH less than 4.1 were adjusted with a solution of HCl. The excitation and emission wavelengths giving maximum intensity were in the ranges of 347—348 and 433—435 nm for the samples of pH less than 4.7. For the samples of pH more than 6.0, the excitation wavelength was set at 348 nm, and the emission was measured at 435 nm. In such cases, the wavelengths applied did not afford maximum intensities.

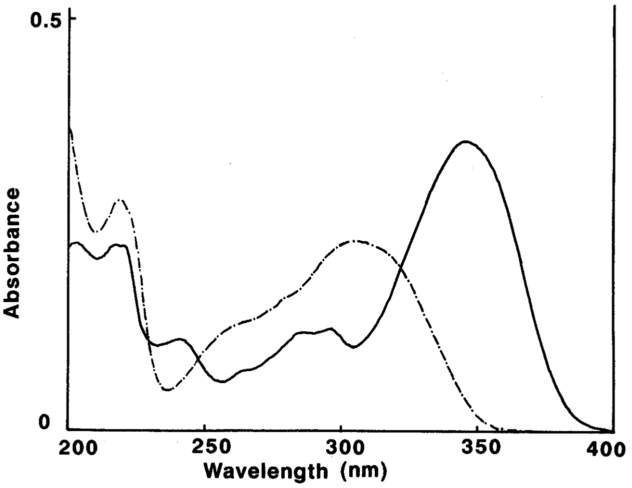

Fig. 5. UV Spectra of **7** in the Acidic and Basic Medium

——; pH 1.4, —·—; pH 10.4. The concentration of **7** was 20 μM.

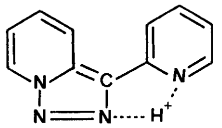

Fig. 6. The Postulated Structure of the Fluorescent Species

Table I. Fluorescence Intensities of Oxidation Products of Some 2-Pyridyl Ketone Hydrazones

Compound	pH	Excitation wavelength (nm)	Emission wavelength (nm)	Fluorescence intensity[a] (a.u.)
5	7.8	287	398	1.7
6	7.8	239	394	1.0
7	7.8	305	406	6.4
	1.6	348	434	431
8	7.8	296	434	10.5

[a] These intensities were corrected to those for solutions of the same concentration, 1 μM.

Table II. The Absorbance of the Solution Obtained on Treatment with TBA of the Reaction Mixture of the Aerial Oxidation of **2** Catalyzed by Cu^{2+} Containing 2-Deoxy-D-ribose

Reaction time (h)	Without 2-deoxy-D-ribose	With 2-deoxy-D-ribose
2	0.049 (532)	0.513 (531)
20	0.201 (532)	1.980 (531)

The absorbance values shown above are those which were obtained by subtracting the absorbance at 600 nm from those of the maxima, taking into account the absorbance due to the turbidity of the sample solution. The maximum absorption wavelengths (nm) are indicated in parentheses.

products of other 2-pyridyl ketone hydrazones. These properties of **7** appear to be due to the pyridyl group. From Fig. 4, it can be seen that the fluorescent species is a monobasic acid having a pK of 3.6. On the other hand, neither pyridine nor 2,2′-bipyridine showed marked fluorescence in the acidic media,[9] indicating that pyridinium ion has no intrinsic fluorescence. Thus the structure shown in Fig. 6[10] is proposed for the fluorescent species, where one proton interacts with both a pyridine nitrogen atom and another nitrogen. Table I summarizes the fluorescence intensities of the oxidation products of 2-pyridyl ketone hydrazones examined. The fluorescence intensities of the compounds bearing an aromatic ring (**7** and **8**) were rather large, compared to those of **5** and **6**, and the fluorescence intensity of **7** in acidic media was strikingly large. Thus, the sensitive determination of amino acids and proteins using the aerial oxidation of **2** could be ascribed to the intense fluorescence of **7** in the acidic medium.

Attempt to Detect Hydrogen Peroxide in the Aerial Oxidation of 2 Catalyzed by Cu^{2+} A possible product other than **7** which might be involved in this oxidation reaction is hydrogen peroxide. Several methods were examined to detect hydrogen peroxide, including the reaction with Ti^{4+}[11] and that with peroxidase and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate).[12] However, no hydrogen peroxide was detected in the reaction mixture. It is conceivable[13] that in the presence of Cu^{2+}, hydrogen peroxide formed was decomposed to generate hydroxyl radical. Actually, in a model reaction, hydrogen peroxide was almost completely decomposed in 30 min. Thus, we attempted to detect hydroxyl radical formed in the aerial oxidation of **2** by the method using 2-deoxy-D-ribose and thiobarbituric acid (TBA).[13] In this method, hydroxyl radical reacts with 2-deoxy-D-ribose to produce malonaldehyde, which then reacts with TBA, forming a chromophore that absorbs at 532 nm. Table II lists absorbances at about 532 nm of the solutions obtained after TBA treatment of a portion of the reaction mixture of the aerial oxidation of **2**. The absorbances for the reaction mixture containing 2-deoxy-D-ribose were evidently larger than those for the reaction mixture without 2-deoxy-D-ribose. This tendency became marked as the reaction proceeded. These results would indicate that hydroxyl radical was formed in this reaction system, and perhaps originated from the decomposition of hydrogen peroxide. Thus the aerial oxidation of **2** could be expressed as shown below.

$$\mathbf{2} + O_2 \rightarrow \mathbf{7} + H_2O_2$$

Experimental

Melting points were measured with a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded with a JASCO FT/IR 5300 spectrometer. Mass spectra (MS) were measured with a JEOL JMS-DX 303 instrument. ^{1}H-NMR spectra were recorded on a JEOL JNM-FX200A spectrometer. Fluorometric and UV spectra were measured with a Hitachi F-3010 spectrofluorometer and a Hitachi U-3210 spectrometer, respectively.

Compounds **1**, **2**, **3**, and **4** were prepared by the reactions of the corresponding 2-pyridyl ketones and pyridine-2-aldehyde with hydrazine hydrate according to the literature.[6,8]

3-Methyl-[1,2,3]-triazolo[1,5-*a*]pyridine (5) Compound **3** (211 mg, 1.56 mmol) and cupric nitrate trihydrate (**9**) (3.2 mg, 0.013 mmol) were dissolved in 49 ml of water, and the pH of this mixture was adjusted to 11 with sodium hydroxide solution. It was stirred for 1 h in an open vessel at room temperature. Water (50 ml) was added to the mixture and extraction with 100 ml of benzene was repeated three times. The extract was dried over anhydrous sodium sulfate. The solid obtained after evaporation of the solvent was recrystallized from a mixture of benzene and petroleum ether to give colorless crystals. Yield 30%. mp 85.1—85.3 °C (lit.[8] mp 84.0—85.0 °C). MS m/z: 133 (M^+). IR (KBr) cm^{-1}: 3083, 3046, 1636, 1534, 1431, 1364. ^{1}H-NMR ($CDCl_3$) δ: 2.63 (3H, s, CH_3), 6.93 (1H, t, $J=7$ Hz, 6-H), 7.17 (1H, dd, $J=9$, 7 Hz, 5-H), 7.62 (1H, d, $J=9$ Hz, 4-H), 8.65 (1H, d, $J=7$ Hz, 7-H). *Anal.* Calcd for $C_7H_7N_3$: C, 63.14; H, 5.30; N, 31.56. Found: C, 62.77; H, 5.23; N, 31.72.

[1,2,3]-Triazolo[1,5-*a*]pyridine (6) Compound **4** (208 mg, 1.72 mmol) was dissolved in 50 ml of water and this solution was left at room temperature for 1 d to convert **4** completely to the E form. To this solution, **9** (3 mg, 0.012 mmol) was added and the mixture was made alkaline (pH 11) with sodium hydroxide solution. The reaction mixture was stirred for 1 h in an open vessel at room temperature, then extracted with 100 ml of ether three times. The extract was dried over anhydrous sodium sulfate, and the ether was evaporated off. The ^{1}H-NMR spectrum of the residue indicated the formation of **6** in 20% yield. For the isolation of **6**, the reaction was carried out on a large scale under more favorable conditions, that is, with a large amount of catalyst and a long reaction time. Colorless crystals were obtained by vacuum distillation of the liquid that remained after the evaporation of the solvent from the extract. mp 33 °C (lit.[14] mp 34—35 °C). MS m/z: 119 (M^+). IR (KBr) cm^{-1}: 3079, 1634, 1510, 1358. ^{1}H-NMR ($CDCl_3$) δ: 6.99 (1H, t, $J=7$ Hz, 6-H), 7.26 (5-H), 7.75 (1H, d, $J=8.5$ Hz, 4-H), 8.08 (1H, s, 3-H), 8.76 (1H, d, $J=7$ Hz, 7-H). The central part of the signal at $\delta=7.26$ overlapped with the signal of $CHCl_3$. ^{1}H-NMR (C_6D_6) δ: 5.96 (1H, t, $J=7$ Hz, 6-H), 6.35 (1H, dd, $J=9$, 7 Hz, 5-H), 6.86 (1H, d, $J=9$ Hz, 4-H), 7.73 (1H, s, 3-H), 8.04 (1H, d, $J=7$ Hz, 7-H). *Anal.* Calcd for $C_6H_5N_3$: C, 60.50; H, 4.23; N, 35.27. Found: C, 60.20; H, 4.15; N, 34.55.

3-(2′-Pyridyl)-[1,2,3]-triazolo[1,5-*a*]pyridine (7) Compound **2** (182 mg, 0.92 mmol) and **9** (10 mg, 0.041 mmol) were dissolved in 6 ml of water, and the mixture was made alkaline (pH 11) with a solution of sodium hydroxide. It was stirred for 5 h at room temperature in an open test tube, then poured into 300 ml of water and extracted with 100 ml of benzene four times. The extract was dried over anhydrous sodium sulfate. After the evaporation of the solvent, the solid obtained was recrystallized from a mixture of benzene and petroleum ether to provide colorless crystals. Yield 68%. mp 126—126.5 °C (lit.[5] mp 126 °C). MS *m/z*: 196 (M^+). IR (KBr) cm^{-1}: 3104, 3036, 3005, 1634, 1591, 1528, 1445, 1408. ^1H-NMR ($CDCl_3$) δ: 7.06 (1H, t, $J=7$ Hz, 6-H), 7.22 (1H, dd, $J=8$, 5 Hz, 5′-H), 7.38 (1H, dd, $J=9$, 7 Hz, 5-H), 7.80 (1H, t, $J=8$ Hz, 4′-H), 8.36 (1H, d, $J=8$ Hz, 3′-H), 8.67 (1H, d, $J=5$ Hz, 6′-H), 8.74 (1H, d, $J=9$ Hz, 4-H), 8.77 (1H, d, $J=7$ Hz, 7-H). ^1H-NMR (DMSO-d_6) δ: 7.25 (1H, t, $J=7$ Hz, 6-H), 7.34 (1H, dd, $J=7.5$, 5 Hz, 5′-H), 7.57 (1H, dd, $J=8.5$, 7 Hz, 5-H), 7.93 (1H, t, $J=7.5$ Hz, 4′-H), 8.25 (1H, d, $J=7.5$ Hz, 3′-H), 8.63 (1H, d, $J=8.5$ Hz, 4-H), 8.69 (1H, d, $J=5$ Hz, 6′-H), 9.14 (1H, d, $J=7$ Hz, 7-H). *Anal.* Calcd for $C_{11}H_{10}N_4$: C, 67.33; H, 4.11; N, 28.56. Found: C, 67.36; H, 4.05; N, 28.64.

3-Phenyl-[1,2,3]-triazolo[1,5-*a*]pyridine (8) Compound **1** (305 mg, 1.55 mmol) and **9** (15 mg, 0.062 mmol) were dissolved in 100 ml of water, and the pH was adjusted to 10.5 with sodium hydroxide solution. The mixture was stirred for 14 h at room temperature in an open vessel. Then 100 ml of water was added, and extraction with 100 ml of benzene was carried out three times. The extract was dried over anhydrous sodium sulfate, and the solvent was evaporated off to leave crystals, which were recrystallized from a mixture of benzene and petroleum ether. Colorless crystals. Yield 61%. mp 113.5—114 °C (lit.[8] mp 113—114 °C). MS *m/z*: 195 (M^+). IR (KBr) cm^{-1}: 3104, 3052, 1630, 1603, 1534, 1455. ^1H-NMR ($CDCl_3$) δ: 7.01 (1H, t, $J=7$ Hz, 6-H), 7.31 (1H, dd, $J=9$, 7 Hz, 5-H), 7.40 (1H, t, $J=7.5$ Hz, 4′-H), 7.52 (2H, t, $J=7.5$ Hz, 3′,5′-H), 7.97 (2H, d, $J=7.5$ Hz, 2′,6′-H), 8.01 (1H, d, $J=9$ Hz, 4-H), 8.76 (1H, d, $J=7$ Hz, 7-H). *Anal.* Calcd for $C_{12}H_9N_3$: C, 73.83; H, 4.65; N, 21.52. Found: C, 73.89; H, 4.50; N, 21.72.

Model Reaction for the Decomposition of Hydrogen Peroxide in the Presence of Cu^{2+} To 11 ml of aqueous sodium hydroxide solution (pH 10.5) were added 1 ml of 50 mM hydrogen peroxide and 1 ml of 21 mM Cu^{2+} solution with stirring. At appropriate times after the start of the reaction, 0.1 ml of the reaction mixture was added to 1 ml of water. A portion (0.1 ml) of this solution was mixed with 2.8 ml of a reagent solution which contained peroxidase (20 μg/ml) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (1 mg/ml) in 0.1 M phosphate buffer (pH 7.0). The absorbance of this solution was measured at 420 nm. The amount of residual hydrogen peroxide was 41% and 0.7% of the initial value at 10 and 26 min after the start of the reaction, respectively.

Trapping of Hydroxyl Radical Formed in the Aerial Oxidation of 2 Catalyzed by Cu^{2+} A hundred milligrams (0.51 mmol) of **2**, 6 ml of 0.1 M 2-deoxy-D-ribose, 5 mg (0.021 mmol) of **9** in 3 ml of water, and 3 ml of water were mixed, and the pH was adjusted to 10.5 with sodium hydroxide solution. At appropriate times after the start of the reaction, 2 ml of the reaction mixture was sampled and 2 ml of 5.6 mM EDTA solution was added to it. The mixture was centrifuged (2500 rpm, 10 min) to remove a precipitate. To 2 ml of supernatant were added 12 ml of TBA solution (1% (w/v) in 50 mM sodium hydroxide) and 1 ml of acetic acid, and the mixture was heated at 100°C for 15 min, then allowed to cool. The visible spectrum exhibited absorbance at about 530 nm.

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9) Fluorescence spectra of 10 μM aqueous solutions of pyridine and 2,2′-bipyridine were measured. Fluorescence intensity of pyridine solution (pH 3.5) was 2.0 (a.u., $\lambda_{ex}=243$ nm, $\lambda_{em}=396$ nm) and those of 2,2′-bipyridine solution of pH 1.9 and in 3 M HCl were 5.0 ($\lambda_{ex}=230$ nm, $\lambda_{em}=344$ nm) and 2.1 ($\lambda_{ex}=230$ nm, $\lambda_{em}=345$ nm), respectively.
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