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The Stability of Carboquone in Alcohols. II.¹⁾ Kinetics and Mechanisms of Degradation of Carboquone in Methanol

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The kinetics and mechanisms of the degradation of carboquone (CQ) in methanol were investigated.

Eight peaks were observed as degradation products of CQ in high performance liquid chromatography, suggesting that the degradation process of CQ in methanol is rather complicated. However, conducting the reaction in proton-rich methanol and methylate-rich methanol made it clear that CQ is degraded in methanol through a combination of two mechanisms: methanolysis of the aziridine ring, which cleaved to a (2-methoxyethyl)amino group, and substitution of the aziridine ring by methylate. Further, some of the degradation products were obtained in pure form by this procedure.

Due to the asymmetric structure of CQ, two kinds of mono aziridinyl-mono methoxyethyl-amino compounds and two kinds of mono aziridinyl-mono methoxy compounds are produced. Kinetic studies of these four compounds and 2,5-bis(1-aziridinyl)-3,6-dimethyl-1,4-benzoquinone made it possible to assign their chemical structures. Their deduced structures were confirmed by mass spectroscopy and proton nuclear magnetic resonance spectroscopy.

Keywords—carboquone; antitumor agent; aziridinyl benzoquinone; kinetics; mechanism; ring cleavage; Michael reaction; HPLC; MS; ¹H-NMR

Carboquone [CQ; 2,5-bis(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-6-methyl-1,4-benzoquinone]^{2,3)} is one of the (1-aziridinyl)benzoquinone antitumor agents (Chart 1), which are classified as alkylating agents. CQ is fairly unstable in protic solvents such as water and alcohols. This may be mainly due to the lability of the aziridine moieties in the structure.

In aqueous solutions, CQ was found to be degraded with changes of the two aziridine rings: hydrolytic cleavage in acidic solutions, substitution by hydroxyl ion (radical) in basic solutions and a combination of these two mechanisms.⁴⁾ Further, difficulty in estimation of the structures of the two kinds of mono aziridinyl-mono hydroxyethylamino compounds and the two kinds of mono aziridinyl-mono hydroxy compounds (due to the asymmetrical structure of CQ) was overcome by comparing the labilities of these four compounds, CQ and 2,5-bis(1-aziridinyl)-3,6-dimethyl-1,4-benzoquinone (MEB, Chart 1).⁴⁾

On the other hand, in the previous study,¹⁾ 2,5-bis(1-aziridinyl)-1,4-benzoquinone (EB, Chart 1), which has the simplest structure among the CQ-related compounds, was chosen, and its stability in alcohols was investigated. In particular, the degradation mechanisms of EB in methanol were studied in detail: it is degraded by a combination of two mechanisms, methanolysis of the aziridine ring and substitution of the aziridine ring by methylate. Thus, the degradation mechanisms of EB in methanol were concluded to be essentially the same as those in aqueous solutions. Further, the use of proton-rich or methylate-rich methanol was proved to be an effective way to study the degradation mechanisms of EB in methanol, since it simplifies the degradation process by making one of the two mechanisms dominant.

In the present study, the degradation behavior of CQ in methanol was studied. The degradation behavior of MEB was also studied in order to assign the structures of some CQ

TABLE II. Molecular Peak in MS, Melting Point, Elemental Analysis and UV Spectral Data of Reference Compounds, CQ and CQ Degradation Products

Compound	Molecular formula	Molecular peak in MS	mp (°C)	Elemental analysis			UV spectral data ^{a)} λ_{\max} nm (log ϵ)
				Calcd	(Found)		
				C	H	N	
MEB	C ₁₂ H ₁₄ N ₂ O ₂	218	170	66.04 (65.92)	6.47 (6.44)	12.83 (12.71)	328 (4.20)
M-II	C ₁₃ H ₁₈ N ₂ O ₃	250	85	62.38 (62.39)	7.25 (7.30)	11.19 (11.18)	339 (4.33)
M-III	C ₁₄ H ₂₂ N ₂ O ₄	282	90	59.57 (59.55)	7.80 (7.98)	9.93 (9.92)	347 (4.41)
M-IV	C ₁₁ H ₁₃ NO ₃	207	115	63.76 (63.60)	6.32 (6.35)	6.76 (6.73)	303 (4.28)
M-V	C ₁₀ H ₁₂ O ₄	196	126	61.22 (61.02)	6.16 (6.14)	0.00 (0.00)	285 (4.15)
CQ	C ₁₅ H ₁₉ N ₃ O ₅	321	203	56.07 (56.17)	5.91 (6.05)	13.08 (13.19)	333 (4.21)
3	C ₁₆ H ₂₃ N ₃ O ₆	353					342
7	C ₁₆ H ₂₃ N ₃ O ₆	353	130	54.38 (54.33)	6.56 (6.65)	11.89 (12.01)	342 (4.34)
8	C ₁₇ H ₂₇ N ₃ O ₇	385	56	52.98 (53.07)	7.06 (6.97)	10.90 (10.84)	349 (4.43)
2	C ₁₄ H ₁₈ N ₂ O ₆	310					304
4	C ₁₄ H ₁₈ N ₂ O ₆	310					304
6	C ₁₃ H ₁₇ NO ₇	299					285

a) In ethanol.

dissolve 50 mg of MEB or CQ in 10 ml of CHCl₃
 ↓
 add 40 ml of MeOH, H⁺-rich MeOH or CH₃O⁻-rich MeOH
 ↓
 store at room temp. or 60 °C (see Table I)
 ↓
 add 20 ml CHCl₃
 ↓
 wash with H₂O (ca. 100 ml)
 ↓
 collect CHCl₃ layer
 ↓
 evaporate the CHCl₃ layer and dissolve the residue in EtOH
 ↓
 load onto Lobar prepacked column (see Table I)
 ↓ (LiChroprep RP-8, E. Merck, Darmstadt, Germany)
 collect fractions (see Table I)
 ↓
 dilute the collected fraction 3—5 times with H₂O
 ↓
 extract into CHCl₃ and evaporate the CHCl₃ soln.
 ↓
 dissolve the residue in EtOH and store the solution in
 ↓ a refrigerator for crystallization
 collect crystals

Fig. 1. General Procedures Used to Obtain the Reference Compounds and CQ Degradation Products

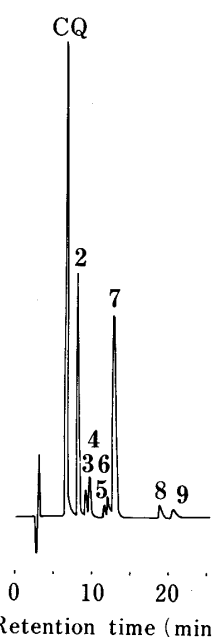


Fig. 2. Chromatogram Obtained by HPLC of CQ Stored in Methanol at 60 °C for 6 d

7, 8, 2, 4 and 6 in Fig. 2 were isolated by essentially the same procedure as for M-II, *etc.*, although the starting material was CQ. Storage and separation conditions are listed in Table I. Compounds 7 and 8 were obtained in crystalline form (Table II). On the other hand, 3, 2, 4 and 6 were obtained only as ethanol solutions, which were used as stock solutions for the kinetic study.

HPLC—Chromatography was performed on a Hitachi liquid chromatograph, model 655, with a variable-wavelength monitoring system (310 nm was used unless otherwise stated). ERC-ODS-1171⁷⁾ was used for the kinetic study of CQ and its degradation products. The mobile phase, the flow rate and the temperature were 25% acetonitrile in water, 1.7 ml/min and 40 °C, respectively. On the other hand, ERC-ODS-1262⁷⁾ was used for the kinetic study of MEB. In this case, 40% acetonitrile in water was used as the mobile phase. The flow rate and the temperature were the same as those for CQ.

Results and Discussion

Stability of CQ in Methanol

The degradation of CQ follows pseudo first-order kinetics in methanol. At 60 °C, the rate constant was $8.0 \times 10^{-3} \text{ h}^{-1}$, which is about 4 times greater than that for MEB under the same conditions, $2.1 \times 10^{-3} \text{ h}^{-1}$. This can probably be ascribed to the increase in the lability of the aziridine ring at the 2-position of CQ due to the presence of the carbamoyloxymethoxyethyl group at the 3-position of CQ.

A typical HPLC pattern of CQ stored in methanol is shown in Fig. 2. Eight peaks are observed as degradation products.

The position of λ_{max} of each peak component can be determined by comparing the peak heights in chromatograms obtained at various wavelengths by the same procedure as for the degradation products of EB.¹⁾ This position suggests how each component may be formed, as in the case of EB¹⁾: the ring opening of aziridine causes a red-shift in λ_{max} , and the substitution of aziridine causes a blue-shift in λ_{max} . However, further information concerning the chemical structures could not be obtained by this procedure.

Therefore, the degradation behavior of CQ in proton-rich methanol and methylate-rich methanol was examined, following the technique applied in the study of EB.¹⁾

Degradation Mechanism of CQ in Proton-Rich Methanol

When CQ was stored in proton-rich methanol at 60 °C (the apparent proton concentration was adjusted to 10^{-4} M), CQ was lost at a rate of 1.48 min^{-1} ,⁸⁾ and only 3, 7 and 8 were observed as degradation products up to at least five half-lives (Table III). The time courses suggest that CQ is degraded to 8 with 3 and 7 as intermediates (Fig. 3). Under the same conditions, 3 and 7 were degraded to 8 with rate constants of 0.21 and 1.27 min^{-1} , respectively. The ultraviolet (UV) spectra of 3 and 7 resembled that of M-II, and the UV spectrum of 8 resembled that of M-III (Table II, Fig. 4). The λ_{max} positions of 3, 7 and 8 are red-shifted compared to that of CQ.

These results and the result for EB¹⁾ indicate that 3 and 7 have a mono aziridinyloxy-

TABLE III. Products from CQ, 3, 7, 2 and 4 Stored in Methanol, Proton-Rich Methanol and Methylate-Rich Methanol

Compound	Products		
	MeOH	H ⁺ -rich MeOH	CH ₃ O ⁻ -rich MeOH
CQ	2-9	3, 7, 8	2, 4, 6
3	5, 8	8	5
7	8, 9	8	9
2	5, 6	6	5
4	6, 9	9	6

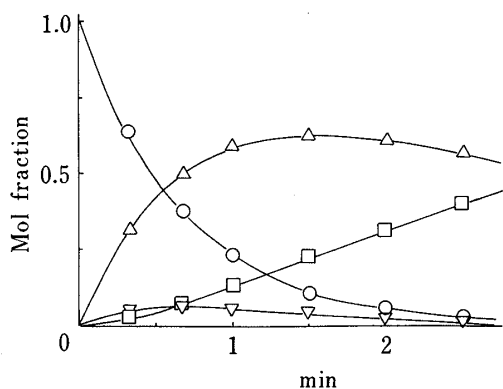


Fig. 3. Time Courses of CQ (○), 3 (▽), 7 (△) and 8 (□) During the Degradation of CQ in Proton-Rich Methanol at 60°C

The apparent proton concentration was adjusted to 10^{-4} M. The lines are calculated values based on the scheme in Chart 2, and obtained by trial-and-error fitting.

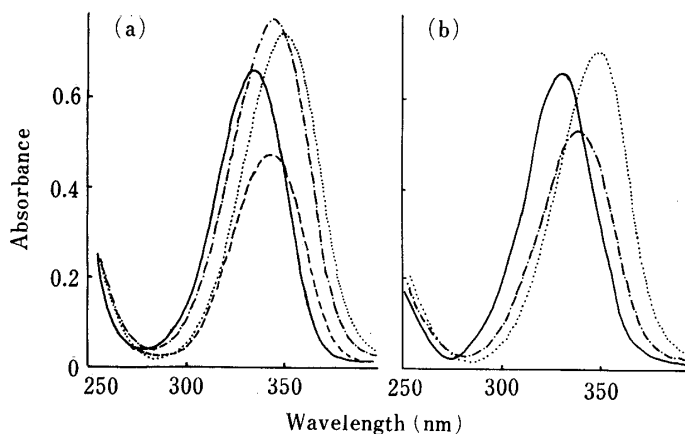


Fig. 4. UV Spectra of CQ, 3, 7, 8, MEB, M-II and M-III in Ethanol

(a) —, CQ (13.2 $\mu\text{g/ml}$); ---, 3; ···, 7 (12.7 $\mu\text{g/ml}$); - · - ·, 8 (11.0 $\mu\text{g/ml}$).
(b) —, MEB (9.13 $\mu\text{g/ml}$); ---, M-II (6.25 $\mu\text{g/ml}$); ···, M-III (7.68 $\mu\text{g/ml}$).

methoxyethylamino structure, although which aziridine (at the 2 or 5 position of CQ) is cleaved could not be identified, and that 8 has a dimethoxyethylamino structure. This speculation was supported by the molecular peaks of 3, 7 and 8 in the mass spectrum (MS), and the results of elemental analysis of 7 and 8 (Table II). These results suggest that the degradation mechanism of CQ in proton-rich methanol may be as shown in Chart 2.

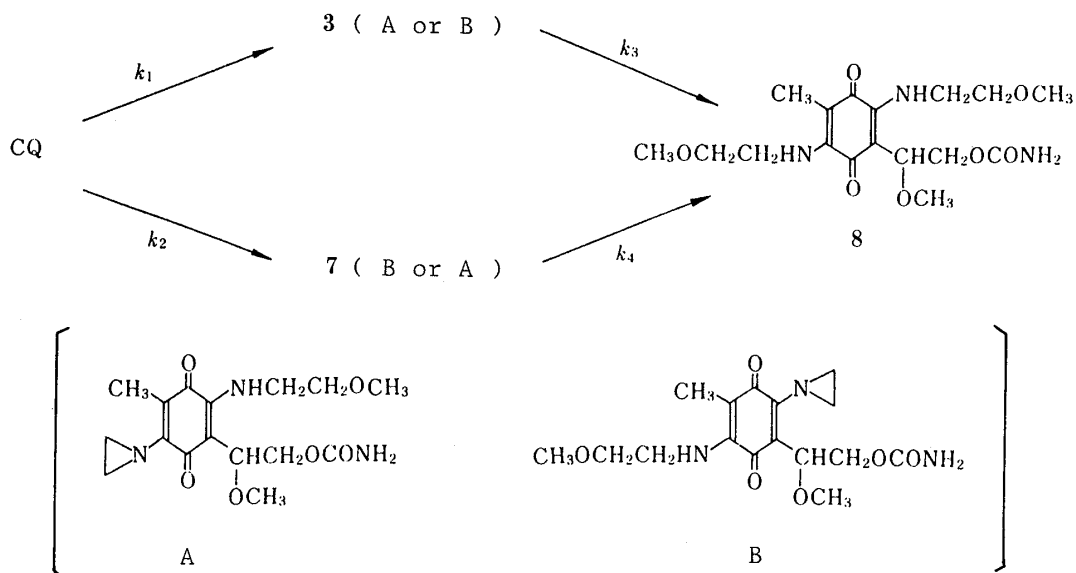


Chart 2

Experimentally, the mol fractions of CQ, 7 and 8 can be obtained from HPLC data using authentic compounds, and the mol fraction of 3 was obtained by subtracting the sum of the mol fractions of CQ, 7 and 8 from unity. As shown in Fig. 3, the calculated values agree well with the observed values assuming $k_1 = k_4 = 0.21 \text{ min}^{-1}$ and $k_2 = k_3 = 1.27 \text{ min}^{-1}$ (equations: see ref. 4). The value for k_1 here is very close to the degradation rate of MEB under the same conditions, 0.18 min^{-1} .

Therefore, the degradation of CQ in proton-rich methanol is concluded to occur as

shown in Chart 2: parallel sequential methanolysis of the two aziridine rings. In this degradation process, the aziridine ring is protonated to yield the iminium ion, then the carbon at the 2'-position of the aziridine ring is exposed to nucleophilic attack by methanol.¹⁾ The chemical structures of **3**, **7** and **8** are A [5-(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-2-(2-methoxyethyl)amino-6-methyl-1,4-benzoquinone] or B [2-(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-5-(2-methoxyethyl)amino-6-methyl-1,4-benzoquinone], B or A, and 3-(2-carbamoyloxy-1-methoxyethyl)-2,5-di(2-methoxyethyl)amino-6-methyl-1,4-benzoquinone, respectively.

Estimation of the Chemical Structures of **3** and **7**

As mentioned above, **7** is more stable than **3**. The formation rate of **7** is greater than that of **3**, which is comparable to the degradation rate of MEB. On the basis of these results, **7** is expected to be a methanolysis product of the more labile aziridine of CQ, *i.e.* the one at the 2-position. Also, **3** is expected to be a methanolysis product of the more stable aziridine of CQ, which has the same level of reactivity as MEB, *i.e.* the one at the 5-position. Consequently, the chemical structures of **3** and **7** are concluded to be B and A, respectively.

Degradation of CQ in Methylate-Rich Methanol

When CQ was stored in methylate-rich methanol at 60 °C (the apparent methylate concentration was adjusted to 10^{-3} M), CQ was lost at a rate of 0.099 h^{-1} ,⁹⁾ and only **2**, **4** and **6** were observed as degradation products until more than 60% of the original CQ was lost (Table III). The time courses suggest that CQ is degraded to **6** with **2** and **4** as intermediates (Fig. 5). Under the same conditions, **2** and **4** were degraded to **6** with rate constants of 0.08 and 0.11 h^{-1} , respectively. The UV spectra of **2** and **4** resembled that of M-IV, and the UV spectrum of **6** resembled that of M-V (Table II, Fig. 6). The λ_{max} positions of **2**, **4** and **6** are blue-shifted compared to that of CQ.

These results and the result for EB¹⁾ indicate that **2** and **4** have a mono aziridinyl-mono methoxy structure, although which aziridine (at the 2 or 5 position) is substituted could not be identified, and that **6** has a dimethoxy structure. This speculation was supported by the molecular peaks in the MS (Table II). These results suggest that the degradation mechanism of CQ in methylate-rich methanol may be as shown in Chart 3.

Experimentally, values of $k_5 + k_6$ ($=k_{\text{obs}}$) were readily obtained from the degradation

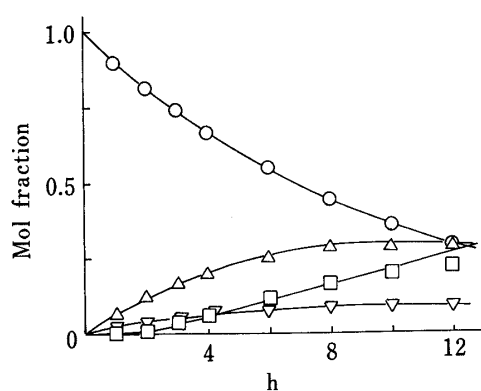


Fig. 5. Time Courses of CQ (○), **2** (△), **4** (▽) and **6** (□) During the Degradation of CQ in Methylate-Rich Methanol at 60 °C

The apparent methylate concentration was adjusted to 10^{-3} M. The lines are calculated values based on the scheme in Chart 3, and obtained by trial-and-error fitting.

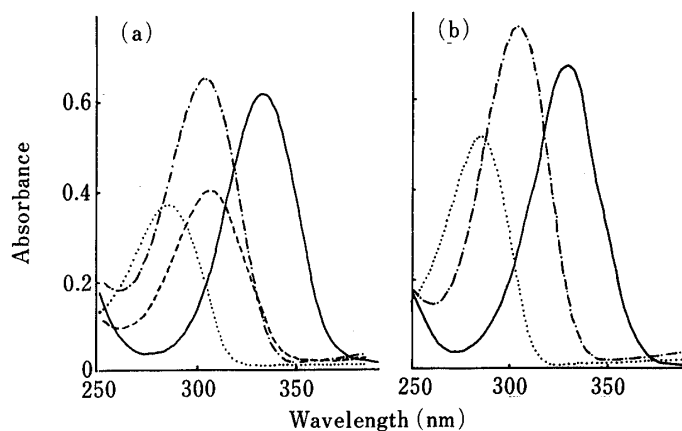
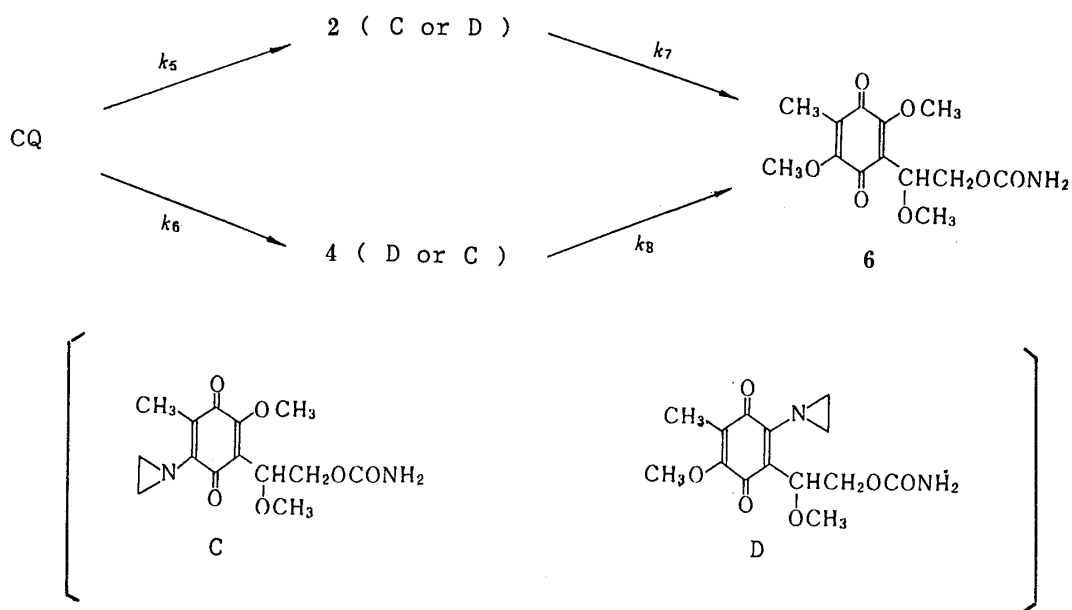


Fig. 6. UV Spectra of CQ, **2**, **4**, **6**, MEB, M-IV and M-V in Ethanol

(a) —, CQ (12.2 $\mu\text{g/ml}$); ---, **2**; ----, **4**; - · - ·, **6**.
(b) —, MEB (9.13 $\mu\text{g/ml}$); ---, M-IV (8.23 $\mu\text{g/ml}$); ----, M-V (7.17 $\mu\text{g/ml}$).



rate of CQ. Further, k_7 and k_8 were obtained from the degradation rates of **2** and **4**. Then, k_5 and k_6 can be obtained by proportional allotment of k_{obs} on the assumption that the k_5/k_6 ratio is equal to the ratio of the peak area of **2** to that of **4** during the initial part of the reaction (until about 70% of the original CQ is left), which is reasonable on the basis of the similarities of chemical structures. The time courses of peak heights corresponding to CQ, **2**, **4** and **6** showed patterns closely similar to the calculated ones. The ratio of peak height at an appropriate time (3 h when the apparent methylate concentration is 10^{-3} M at 60°C) to the calculated value at that time was used as the basis for fitting the curves, just as in the case of CQ in acidic and basic aqueous solutions.⁴⁾ As shown in Fig. 5, the calculated values agreed well with the observed values, assuming, $k_5 = 0.074 \text{ h}^{-1}$, $k_6 = 0.025 \text{ h}^{-1}$, $k_7 = 0.08 \text{ h}^{-1}$ and $k_8 = 0.11 \text{ h}^{-1}$. The value for k_6 here is very close to the degradation rate of MEB under the same conditions, 0.022 h^{-1} .

Therefore, the degradation of CQ in methylate-rich methanol is concluded to be as shown in Chart 2: parallel sequential substitution of the two aziridine rings by methylate. In this degradation process, methylate attacks the carbon at the 2 or 5 position of benzoquinone as a nucleophile (Michael reaction),¹⁰⁾ then the aziridine ring is eliminated from the adduct.¹⁾ The chemical structures of **2**, **4** and **6** are C [5-(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-2-methoxy-6-methyl-1,4-benzoquinone] or D [2-(1-aziridinyl)-3-(2-carbamoyloxy-1-methoxyethyl)-5-methoxy-6-methyl-1,4-benzoquinone], D or C, and 3-(2-carbamoyloxy-1-methoxyethyl)-2,5-dimethoxy-6-methyl-1,4-benzoquinone, respectively.

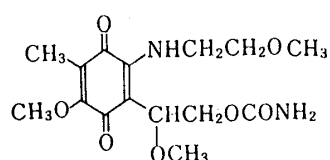
Estimation of the Chemical Structures of **2** and **4**

The formation rate of **4** is almost equal to the degradation rate of MEB, as mentioned above. Further, **2** is more stable than **4** in methylate-rich methanol. When stored in proton-rich methanol at 60°C (the apparent proton concentration was adjusted to 10^{-4} M), **2** and **4** were degraded at rates of 0.17 and 1.1 min^{-1} , respectively. Therefore, **2** is more stable than **4** in proton-rich methanol, too. On the basis of these results, **2** is considered to be a substitution product of the more labile aziridine of CQ by methylate, *i.e.* at the 2-position. Compound **4** is considered to be a substitution product of the more stable aziridine of CQ, which has the same level of reactivity as MEB, *i.e.* the one at the 5-position. Consequently, the chemical structures of **2** and **4** are concluded to be C and D, respectively.

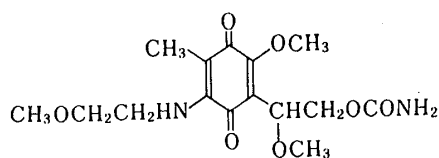
Degradation Process of CQ in Methanol

In methanol, CQ is expected to be degraded by a combination of the two mechanisms: methanolysis of the aziridine ring observed in proton-rich methanol and substitution of the aziridine ring by methylate observed in methylate-rich methanol. In addition to the six degradation products shown in Charts 2 and 3, therefore, two kinds of mono methoxy-mono methoxyethylamino compounds, E and F, are expected to be formed (Chart 4). In fact, two peaks, 5 and 9, were observed besides 2, 3, 4, 6, 7 and 8 on HPLC (Fig. 2). To estimate the structures of 5 and 9, compounds 3, 7, 2 and 4 were stored in methanol, proton-rich methanol and methylate-rich methanol. The products generated under these conditions are listed in Table III. On the basis of these results, 5 and 9 are considered to be F [3-(2-carbamoyloxy-1-methoxyethyl)-2-methoxy-5-(2-methoxyethyl)amino-6-methyl-1,4-benzoquinone] and E [3-(2-carbamoyloxy-1-methoxyethyl)-5-methoxy-2-(2-methoxyethyl)amino-6-methyl-1,4-benzoquinone], respectively.

Based on all these results, CQ is concluded to be degraded by a combination of the two mechanisms, as expected.



E



F

Chart 4

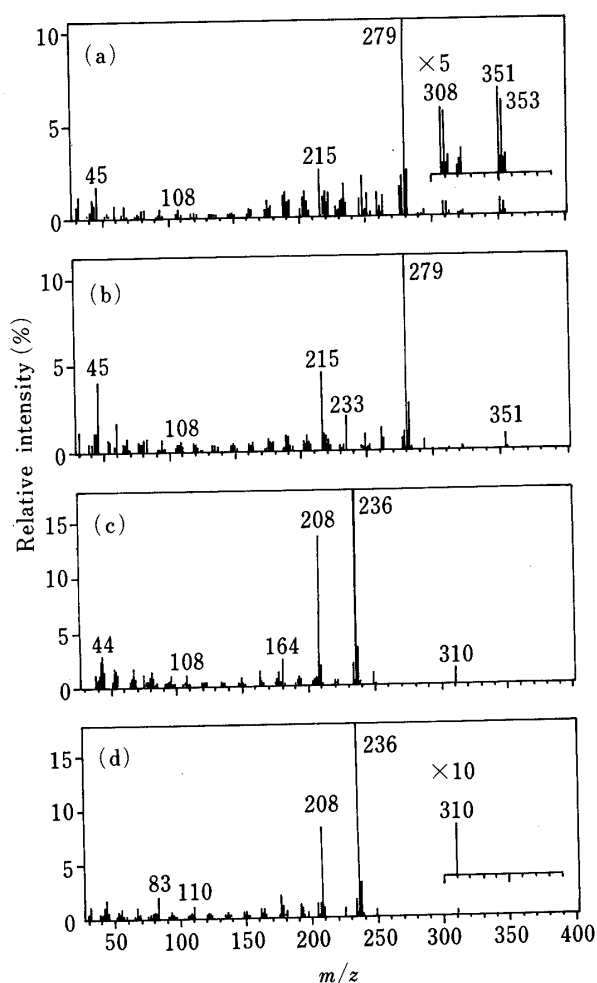


Fig. 7. MS of 3 (a), 7 (b), 2 (c) and 4 (d)
MS were obtained on a JMS D-300 instrument.

Confirmation of the Structures by Mass Spectroscopy and Proton Nuclear Magnetic Resonance ($^1\text{H-NMR}$) Spectroscopy

Confirmation of the chemical structures of **3**, **7**, **2** and **4** deduced from their kinetic properties was attempted by mass spectroscopy and $^1\text{H-NMR}$ spectroscopy. MS of **3** and **7** are shown in Fig. 6. Although the $M^+ - 2$ peak is observed only in the spectrum of **3**, this is not sufficient to determine which is A. The $^1\text{H-NMR}$ spectra of CQ, **7** and **8** are shown in Fig. 7. Accompanying the change of CQ to **7**, marked alterations of the chemical shifts of 7-H and 8-H in the side chain of CQ are seen. In addition, the chemical shift of 6-Me is quite different in **7** and **8** (Table IV). On the basis of these $^1\text{H-NMR}$ data, it is concluded that the degradation of CQ to **7** is caused by methanolysis of the aziridine at the 2-position, and that the degradation of **7** to **8** is caused by methanolysis of the aziridine at the 5-position. Therefore, **7** is concluded to be A, so that **3** is B. These results agree with the results estimated from the kinetic properties.

MS of **2** and **4** are also shown in Fig. 6. While the base peak (m/z 236) and the next largest peak (m/z 208) are common to **2** and **4**, some characteristic peaks are also observed: m/z 108 for **2**, and m/z 110 and 83 for **4**. This can be explained as shown in Chart 5, if **2** and **4** are supposed to be C and D, respectively. Therefore, the chemical structures of **2** and **4** deduced from their kinetic properties were also confirmed.

The present study has provided strong evidence for the proposed chemical structures of the two mono aziridinyl-mono hydroxyethylamino compounds and two mono aziridinyl-mono hydroxy compounds generated in an aqueous solution of CQ as degradation products, based on comparisons of the kinetic properties. Since isolation of the degradation products is very difficult due to the increase of their polarity as compared to CQ, this approach is a convenient one for determining the chemical structures.

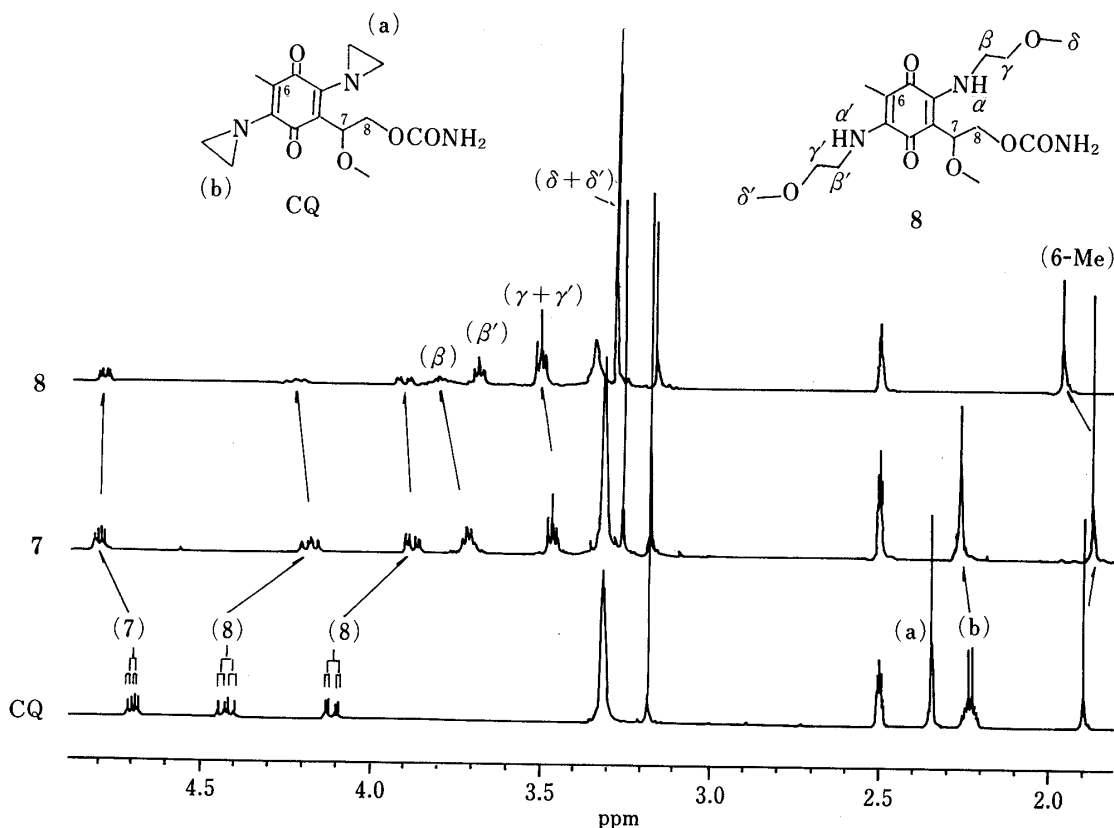


Fig. 8. $^1\text{H-NMR}$ Spectra of CQ, **7** and **8**

Each compound was dissolved in $\text{DMSO-}d_6$ (ca. 3 mg/0.55 ml) with tetramethylsilane as an internal standard. Spectra were obtained on a JNM GX-400 instrument.

TABLE IV. Chemical Shifts (δ) of 6-Me, 7-H and 8-H of CQ, 7 and 8

Proton	δ (ppm)			$\Delta\delta$	
	CQ	7	8	CQ \rightarrow 7	7 \rightarrow 8
6-Me	1.990	1.876	1.965	+0.024	-0.089
7-H	4.701	4.808	4.793	-0.107	+0.015
8-H	4.113	3.881	3.909	+0.232	-0.028
8-H	4.426	4.186	4.226	+0.240	-0.040

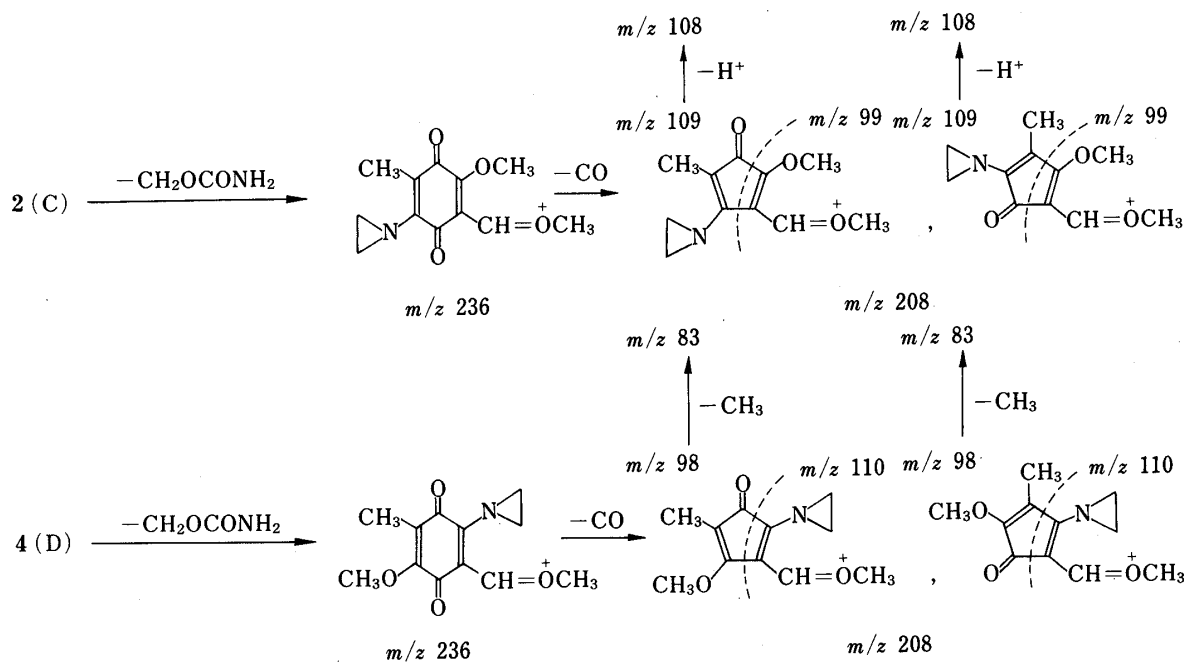


Chart 5

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References and Notes

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- 6) D. W. Cameron and R. G. F. Giles, *J. Chem. Soc.*, **1968**, 1461.
- 7) Erma Optical Works, Ltd., Tokyo, Japan.
- 8) This reaction depends on the concentrations of CQ and protons, *i.e.* it is a second-order reaction. However, the concentration of protons is constant throughout the reaction, because the proton consumed at the initial step (protonation of the aziridine ring to yield the iminium ion) is regenerated at the next step (methanolysis of the aziridine ring to yield the 2-methoxyethylamino group) (see Chart 3 in ref. 1). Therefore, the reaction follows pseudo first-order kinetics.
- 9) This reaction depends on the concentrations of CQ and methylate, *i.e.* it is a second-order reaction. However, the concentration of methylate is constant throughout the reaction, because the proton consumed at the initial step (nucleophilic attack of methylate on the carbon at the 2 or 5 position of benzoquinone) is regenerated at the next step (elimination of the aziridine ring from the adduct) (see Chart 5 in ref. 1). Therefore, this reaction also follows pseudo first-order kinetics.
- 10) C. K. Ingold, "Structure and Mechanism in Organic Chemistry," 2nd ed., Cornell University Press, Ithaca, 1969, pp. 1015-1023.