Mechanism of Decomposition of Cyclic Peroxides, 4-Alkoxy-1,4-dihydro-2,3-benzodioxin-1-ols, to Afford Hydroxyl Radical

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The decomposition of 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ols (1, Bd) in aqueous media was examined. Increasing the water content of the medium accelerated the decomposition of 1 and increased the formation of the corresponding 2-formyl benzoic acid ester (2) as the decomposition product. Electron spin resonance (ESR) studies using dimethylpyrroline *N*-oxide (DMPO) as a spin trapping reagent had revealed that hydroxyl radicals are formed during the decomposition of 1 (Matsugo *et al.*, *FEBS Lett.*, 184, 25 (1985)). Thus, water-mediated decomposition of 1 was suggested to occur, affording the ester 2 and hydroxyl radical. Direct involvement of water was confirmed by an ^{18}O isotopic tracer experiment which revealed that ^{18}O was incorporated exclusively into the formyl position of the ester 2. It is plausible that a hydrated hydroperoxide (5) is formed by the addition of water at the formyl position of the ring-opened structure of 1 at the initial stage of the decomposition of 1. Preliminary studies on the antibacterial activities of 1 showed moderate cell-killing activity, especially to *Pseudomonas* strains, and the activity was found to be related to the decomposition of 1.

Keywords hydroxyl radical; 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ol; antibacterial activity; cyclic peroxide; minimum inhibitory concentration; decomposition kinetics; ESR spectrum; dimethylpyrroline *N*-oxide

The toxicity of peroxides, typically lipid peroxides, has been widely recognized and is considered to be due to active oxygen species generated by their decomposition.[1] In the action mechanism of certain anti-tumor drugs such as adriamycin[2] or bleomycin,[3] hydroxyl radicals are considered to play a significant role in tumor cell killing. These facts suggest that if one could develop a chemical system to generate active oxygen radicals in a specified site, a strong cell toxicity would be produced at that site. As candidate compounds, we chose cyclic peroxides, such as 4-methoxy- and 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ols (**1a, b** Bd), and studied their reactions with cytochrome c.[4–6] These studies indicated that Bd generated active oxygen radicals including hydroxyl radical on its decomposition in aqueous media and indeed the generation of hydroxyl radical was confirmed by electron spin resonance (ESR) using dimethylpyrroline *N*-oxide (DMPO) as a spin trapping reagent.[7] We have studied the precise decomposition path of Bd in aqueous media using ^{18}O as a tracer and we propose here a water-mediated Bd decomposition which accompanied with hydroxyl radical generation. Preliminary studies on the antibacterial activity of Bd are also described and the antibacterial activities of cyclic peroxides are discussed in relation to the mechanism of hydroxyl radical generation.

Results and Discussion

Decomposition of Bd in an Aqueous Medium The decomposition of 4-methoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1a**) and 4-ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1b**) was quite slow in aprotic media such as acetonitrile, chloroform, and acetone at 30 °C, but, in aqueous media, **1a** and **1b** smoothly decomposed to afford 2-formyl-benzoic acid (**3**) and corresponding esters (**2a, b**). The product distribution studies revealed that as the water content in the medium was increased, the formation of esters (**2a, b**) increased, while the formation of the acid **3** was low in every case (Figs. 1 and 2).

The rate constant for the decomposition of **1a** and **1b** became larger with increasing water content in the medium.

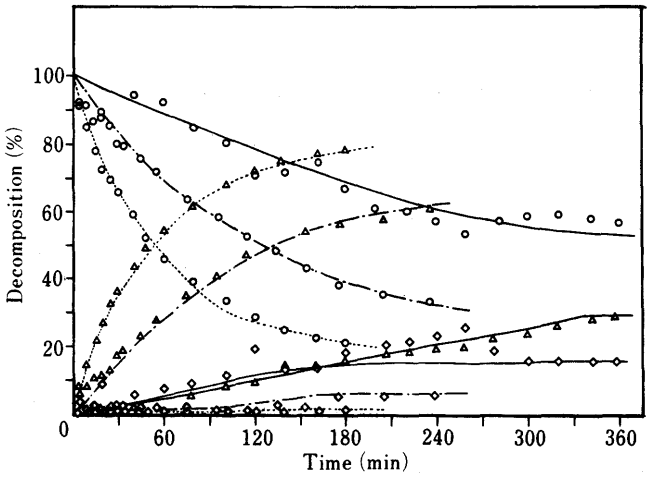

Fig. 1. Decomposition Profiles of 4-Methoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1a**) in Aqueous MeCN (90, 70, 50%) Solutions at 29.5±0.5 °C

○, peroxide, △, ester; ◇, acid. —, $CH_3CN:H_2O$ (9:1); ———, $CH_3CN:H_2O$ (7:3); – – –, $CH_3CN:H_2O$ (5:5).

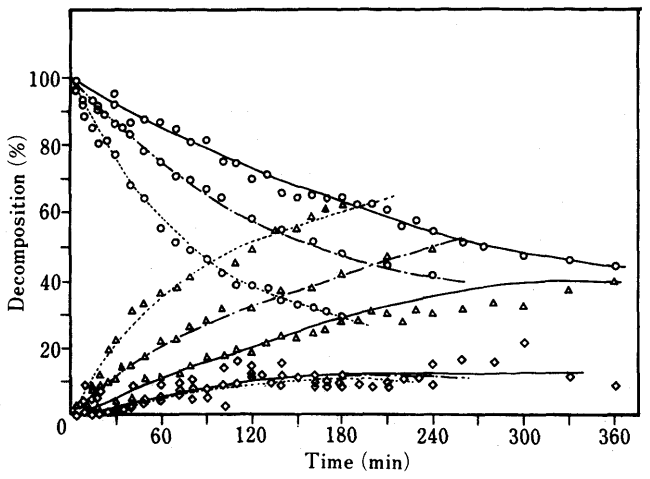

Fig. 2. Decomposition Profiles of 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1b**) in Aqueous MeCN (90, 70, 50%) Solutions at 29.5±0.5 °C

○, peroxide; △, ester; ◇, acid. —, $CH_3CN:H_2O$ (9:1); ———, $CH_3CN:H_2O$ (7:3); – – –, $CH_3CN:H_2O$ (5:5).

TABLE I. Thermal Decomposition Kinetics of 4-Methoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1a**) and 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1b**) in Various Aqueous Solvent Media[a]

Solvent MeCN : H_2O	Peroxide	Temp. (°C)	K_{obsd} ($\times 10^6 h^{-1}$)
9 : 1	**1a**	30.3	2.602±0.30
8 : 2	**1a**	30.3	6.600±0.25
7 : 3	**1a**	29.6	10.384±0.30
6 : 4	**1a**	29.6	17.287±0.50
5 : 5	**1a**	29.6	23.956±0.50
9 : 1	**1b**	29.6	1.894±0.50
8 : 2	**1b**	29.6	4.029±0.20
7 : 3	**1b**	29.6	7.460±0.30
6 : 4	**1b**	29.6	11.072±0.40
5 : 5	**1b**	29.6	15.525±0.30

a) Peroxides (**1a**, **b**) did not decompose at all in 100% MeCN even if the sample solution was heated at 60 °C for 3 h.

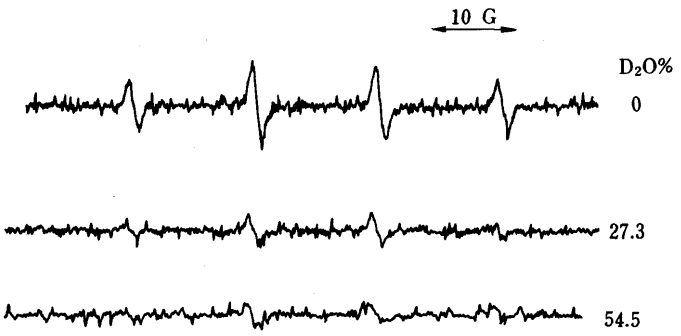

Fig. 3. Effect of D_2O on DMPO-OH Adduct Formation Mediated by the Decomposition of 4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1b**)

The reaction mixture, containing 40 μl of DMPO aqueous solution (50 mM), 60 μl of (H_2O + various volumes of D_2O) and 10 μl of **1b** in acetonitrile (10 mM), was heated for 3 min, at 40 °C. The ESR measurement conditions were as described in the experimental section.

For example, the rate constant of **1a** in MeCN–H_2O (1 : 1) was *ca.* ten times larger than that of **1a** in MeCN–H_2O (9 : 1) (Table I). Increasing the temperature up to 65 °C accelerate the decomposition reaction without producing any significant change in ester/acid ratio in 50% aqueous solution.[8]

In an aqueous Bd solution, a typical DMPO-OH adduct signal ($A_N = A_H^\beta = 14.86$ G) was detected after heat treatment at temperatures above 30 °C, when ESR spectra were recorded in the presence of DMPO as a spin trapping reagent (see also refs. 5 and 7). Although increasing the concentration of acetonitrile decreased the DMPO-OH formation (not shown), participation of H_2O in hydroxyl radical generation is not conclusive, because acetonitrile itself is a quencher of hydroxyl radical. Therefore, the effect of D_2O on the DMPO-OH formation was measured to clarify the direct participation of H_2O in Bd decomposition. As is shown in Fig. 3, DMPO-OH formation decreased as the concentration of D_2O was increased, as expected.

We showed previously that the DMPO-OH signal increased at higher temperatures concomitantly with Bd decomposition, and the presence of a hydroxyl radical scavenger such as ethyl alcohol caused disappearance of the signals.[5]

The above experimental results suggest that water in the medium favors the formation of the ester **2** and hydroxyl radical, and thus presumably participates in the decomposition of **1**. In order to know how the water molecule is involved in the peroxide decomposition, we examined the decomposition of **1a** in MeCN–$H_2{}^{18}O$ (v/v, 1 : 1) solvent. If water in the medium participates directly in the decomposition of **1a**, ^{18}O should be incorporated into the ester **2a**. In the gas chromatography–mass spectrometry (GC-MS) analysis of the ester **2a**, the signal corresponding to $M^+ - Me$ was observed as a doublet at *m/z* 151 and 149 although M^+ of **2a** could not be detected under the measuring conditions; the relative intensity was 46 : 54. Further, the signal corresponding to $M^+ - OMe$ was observed as a doublet at *m/z* 133 and 135. Since the signal due to $M^+ - CHO(^{18}O)$ might also appear at *m/z* 135, the fraction corresponding to ^{18}O labeled ion (labeled $M^+ - OMe$) in the *m/z* 135 signal was calculated by subtracting the fraction corresponding to $M^+ - CHO(^{18}O)$ from the value at *m/z* 135 according to Eq. 1.

$$135_{labeled}(M^+ - OMe) = 135_{labeled} - 136_{labeled} \times 135_{control}/136_{control} \quad (1)$$

Finally, $135_{labeled}(M^+ - OMe) : 133_{labeled}(M^+ - OMe) = 44.7 : 55.3$ was obtained. Thus, it is clear that ^{18}O label incorporation in ester **2a** is *ca.* 50%. On the other hand, the deformyl ion, $M^+ - CO$, appeared as a singlet at 136, indicating the absence of ^{18}O label in the fragment ion. Thus, ^{18}O is concluded to be incorporated exclusively into the formyl position of the ester **2a** (Fig. 4, Table II).

Based on these experimental results, a plausible decomposition pathway of **1** in an aqueous medium is as follows.

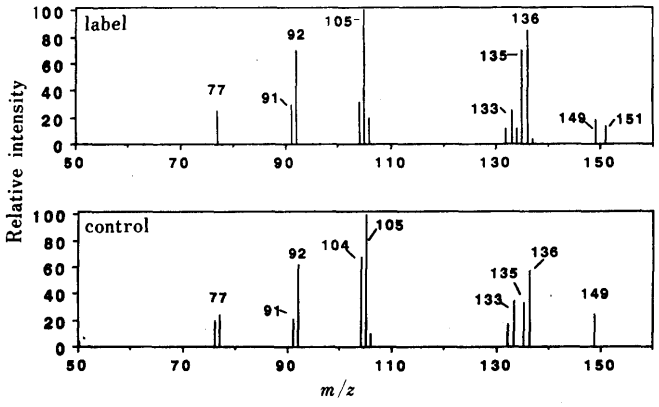

Fig. 4. GC-MS of Methyl 2-Formyl-benzoate **2a** Obtained by the Decomposition of **1a** in MeCN–$H_2{}^{18}O$ (v/v = 1 : 1, Label) at Room Temperature and **2a** Obtained by the Decomposition of **1a** in MeCN–H_2O (v/v = 1 : 1, Control) at Room Temperature

TABLE II. GC-MS Data for **2a** Obtained during the Decomposition of **1a** in MeCN–$H_2{}^{18}O$ (1 : 1) and MeCN–H_2O (1 : 1)

	Peaks
Labeling experiment **2a**	151 ($M^+ - Me$, 13.33), 135[a] ($M^+ - CHO(^{18}O)$, $M^+ - OMe$, 69.30), 149 ($M^+ - Me$, 16.27), 133 ($M^+ - OMe$, 26.67), 136 ($M^+ - CO(^{18}O)$, 84.44), 105 ($M^+ - CO(^{18}O) - OMe$, 100)
Control experiment **2a**	149 ($M^+ - Me$, 26.67), 135 ($M^+ - CHO$, 34.67), 136 ($M^+ - CO$, 61.33), 133 ($M^+ - OMe$, 37.86), 105 ($M^+ - COOMe$, 100)

a) This peak corresponds to the sum of labeled $M^+ - CH^{18}O$, non-labeled $M^+ - CHO$ and labeled $M^+ - OMe$.

As the cyclic peroxide **1a** has a partial structure resembling to a hemiacetal, the ring structure may easily open to afford the non-cyclic hydroperoxide **4** by analogy with carbohydrates. This structure could be the precursor of **1a**. So, in an aqueous medium, the cyclic peroxide **1a** is in equilibrium with its non-cyclic hydroperoxide **4**. Since **3** does not incorporate any ^{18}O of $H_2{}^{18}O$ at neutral pH, water in the medium would initially add to the aldehyde portion of **4** to produce the hydrated hydroperoxide **5**. Hydrogen abstraction by **8**, **9**, or hydroxyl radical (spontaneously produced to a slight extent by the O–O homolytic scission of **1a**) gives the carbon-centered radical **6**. Successive decomposition of **6** generates hydroxyl radical to afford the hydrated ester **7**.[9] Dehydration of **7** results in the formation of the ester **2a** as a final product. Here, the possibility of losing H_2O or $H_2{}^{18}O$ from **7** is expected to be about the same. Namely, 50% loss of the label would be expected. This was actually the case in our present study.

Direct attack of H_2O on cyclic peroxide was reported[10] only in strongly alkaline solution, but not at neutral pH. Further, hydrolysis and methanolysis of endoperoxide have also been reported only under acidic conditions.[11] Thus, a direct attack of water to **1a** under our mild reaction conditions is unlikely. This view is consistent with the non-linear correlation of the $k_{decomp.}$ of **1** to the molar concentration of water. If direct attack of water played a central role in the decomposition of **1**, a linear correlation would be expected between water content and the decomposition rate, which is not the case. As for the formation of **3**, we did not detect any ^{18}O incorporation into **3** under the same conditions as those where we observed *ca.* 50% ^{18}O incorporation in **2a**, so it would be reasonable to consider a different pathway for the formation of **3**, namely, O–O homolytic scission. The O–O homolytic scission of **1a** gives the biradical **8**, which equilibrates with **9**. Intramolecular hydrogen abstraction would afford hemiacetal **10**, which excludes methanol to afford **3** as a final product. The mechanism proposed here well explains the 50% incorporation of ^{18}O in **2a** at its formyl position and is also consistent with the observation that hydroxyl radical is generated from **1** in an aqueous medium[7] (see also Fig. 3).

Antibacterial Activity of Bd Active oxygen species such as hydroxyl radical are well known to possess cell-killing activity. Thus, it is of interest to know if hydroxyl radical-generating cyclic peroxides have biological effects. We examined the antibacterial activities of **1b** toward fifteen different gram-positive or gram-negative bacterial strains by the broth dilution method. It was found that **1b** had moderate but reproducible antibacterial activities toward *Pseudomonas* strains and *Proteus rettgeri* (Table III).

The antibacterial activity of **1b** was dependent on the preincubation time, namely, the antibacterial activity became lower if *Ps. maltophilia* was inoculated with a lag period after the addition of peroxide in the culture medium (Table IV); presumably some of the peroxide (**1b**) would

Table III. Antibacterial Activity Spectrum of **1b**

Strains	MIC (μg/ml)
Alcaligenes faecalis	100
Pseudomonas aeruginosa	100
Ps. maltophilia 2491	25
Ps. pseudoalcaligenes	50
Ps. putida	200
Ps. stutzeri	50
Staphylococcus aureus ATCC 25923	100
Bacillus subtilis ATCC 6633	400
Escherichia coli K12	200
E. coli 15	400
Enterobacter cloacae	400
Proteus rettgeri	25
Pr. vulgaris	100
Salmonella typhimurium	200
Shigella sonnei	400

Table IV. Effect of Preincubation of **1b** Containing Culture Medium on MIC for *Ps. maltophila* 2491

Preincubation (h)	MIC (μg/ml) **1b**
0	25
20	100
46	200

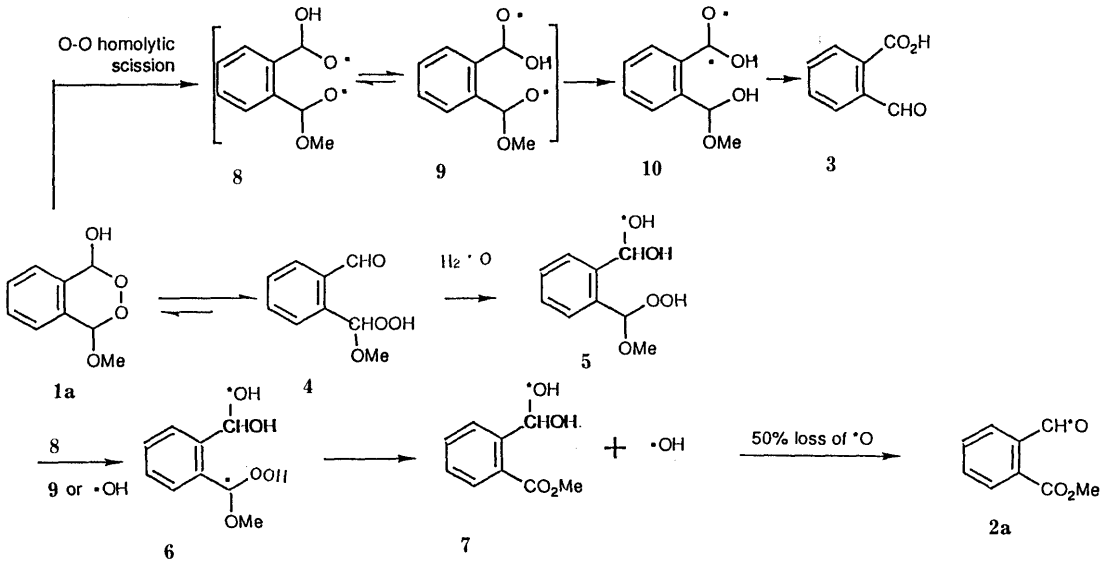

Chart 1

have decomposed. In addition, the antimicrobial activity of **1a**, which is less stable in an aqueous medium than **1b**, were rather low and more variable (not shown).

Therefore, it appears that the antibacterial activity of **1b** is related to the peroxide decomposition, and the radicals generated, including hydroxyl radical, are the reactive species responsible for the antibacterial activity.

Based on the above experimental results, we have reached the following conclusions.

1) In an aqueous medium, the decomposition of 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1**) proceeds through two different pathways, namely that involving water participation and the O–O homolytic path.

2) Hydrogen abstraction from the hydrated non-cyclic hydroperoxide **5** exclusively affords the ester **2** accompanied with the hydroxyl radical.

3) The synthetic cyclic peroxide **1b** showed moderate antibacterial activities especially toward *Pseudomonas* strains, and the antibacterial activity is related to the generation of active oxygen radicals by the water-mediated decomposition of **1b**.

Experimental

Melting points were recorded on a Yanagimoto melting point apparatus. Proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were measured with a JEOL JNM-FX-200 spectrometer using tetramethylsilane as an internal standard. Infrared (IR) spectra were measured with a Jasco A-3 spectrometer. GC-MS was measured with a Hitachi RMU-7M mass spectrometer. ESR spectra were obtained using a Varian model X-4 ESR spectrometer. DMPO (Aldrich) was purified by passing it through a charcoal column and dissolved in distilled water to prepare a 0.5 M solution. Compound **1b** was dissolved in MeCN to prepare a 50 mM solution. The reaction mixtures containing 40 μl of DMPO solution, 60 μl of (H_2O+D_2O) and 10 μl of Bd solution were heated at 40 °C for 3 min. ESR spectra were measured under the following conditions: field setting, 3380 G; modulation amplitude, 0.5 × 1 G; time constant, 0.28 × 0.3 s; scan range, 200 G; microwave power, 10 mW; modulation frequency, 100 MHz.

Degradation kinetics were followed by high performance liquid chromatography (HPLC) using a Nippon Bunko Twincle model equipped with ODS-120A reverse-phase column (Toyo Soda). Acetonitrile–water (v/v, 80 : 20) was used as an eluent. Elution peaks were detected at 254 nm using a model UVidec 100 III UV detector (Nippon Bunko). Alliquots of 10 μl of 1 mM aqueous reaction solution of 4-alkoxy-1,4-dihydro-2,3-benzodioxin-1-ols (**1a, b**) were injected for analysis before or after heat treatment at 30 °C.

Synthesis of 4-Methoxy-1,4-dihydro-2,3-benzodioxin-1-ol (1a) A solution of naphthalene (1.28 g, 0.01 mol) in 200 ml of distilled methanol was ozonized at −70 °C for 1 h. The reaction mixture was evaporated *in vacuo* at 0 °C to give an oily residue, which was then dissolved in 10 ml of ether solution and allowed to stand for 24 h at −20 °C. A white powder was precipitated, and was further washed with 1 ml of cold ether to afford analytically pure **1a** as a white powder. **1a** (1.76 g, 0.0092 mol), 126—128 °C (dec.) (lit. 126—127 °C[12]). ^{1}H-NMR ($CDCl_3$) δ: 3.66 (s, 3H), 5.07 (br s, 1H, OH), 5.53 (s, 1H), 5.95 (s, 1H), 7.28—7.42 (m, 4H). ^{13}C-NMR ($CDCl_3$) δ: 56.14 (q), 93.94 (d), 99.83 (d), 126.93 (d), 126.98 (d), 129.12 (d), 130.14 (s), 131.82 (s). IR (KBr): 3350, 3050, 2970, 2920, 1600, 1460, 1350, 1330, 1285 cm^{-1}. *Anal.* Calcd for $C_9H_{10}O_4$: C, 59.33; H, 5.53. Found: C, 59.45; H, 5.81.

4-Ethoxy-1,4-dihydro-2,3-benzodioxin-1-ol (**1b**) was obtained analogously in 88% yield using ethanol instead of methanol.

1b: 130—132 °C (dec. point). ^{1}H-NMR ($CDCl_3$) δ: 1.26 (t, 3H, J=7.0 Hz), 3.55 (q, 2H, J=7.0 Hz), 5.04 (br s, 1H, OH), 5.69 (s, 1H), 5.91 (s, 1H), 7.30—7.42 (m, 4H). ^{13}C-NMR ($CDCl_3$) δ: 15.07 (q), 64.65 (t), 93.94 (d), 98.70 (d), 126.96 (d, 2C), 129.06 (d, 2C), 130.41 (s), 131.90 (s). IR (KBr) 3400, 3070, 2990, 1600, 1450, 1340, 1320, 1260, 1200 cm^{-1}. *Anal.* Calcd for $C_{10}H_{12}O_4$: C, 61.21; H, 6.17. Found: C, 60.89; H, 5.96.

o-Formylbenzoic acid (**3**) was purchased from Tokyo Kasei Co., Ltd. and was used for the identification of the decomposition product. Methyl *o*-formylbenzoic acid (**2a**) and ethyl *o*-formyl benzoic acid (**2b**) were synthesized by the esterification of **3** with the corresponding alcohols. They were identical with the decomposition products **2a** and **2b**, respectively, on their IR and NMR spectral comparison.

Decomposition of 1a in MeCN–$H_2{}^{18}O$ The peroxide **1a** (20 mg) was dissolved in 1 ml of dry MeCN, to which 1 ml of $H_2{}^{18}O$ (^{18}O content 99.8%) was added. The mixed solution was kept at room temperature for 24 h. GC analysis revealed the reaction mixture contained **2a** (88%) and **3** (*ca.* 5%) under these reaction conditions. The reaction mixture was analyzed by GC-MS and the results were listed on Table II and Fig. 4.

Determination of Minimum Inhibitory Concentrations (MICs) MIC was determined by the broth dilution method. Serial two-fold dilutions of **1b** with nutrient broth (Difco) were inoculated with test organisms and incubated at 37 °C for 18 h (except *Ps. stuzeri*; 48 h). The minimum concentration of serial dilutions which inhibited bacterial growth was regarded as the MIC.

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