# Degradation mediated OH radical generation from synthetic cyclic peroxides: ESR studies

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Hydroxy radical generation on thermal decomposition of synthetic cyclic peroxides was determined by ESR using DMPO as a spin-trapping reagent. Both the 6- and 7-membered cyclic peroxides, 4-alkoxy-2,3-benzodioxan-1-ols and 4-alkoxy-2,3-benzodioxepin-1-ols, respectively, which were synthesized according to our idea of a radical-releasing drug, gave rise to DMPO-OH signals on heat treatment, i.e. under their degradation conditions. The signals were completely abolished with higher concentrations of OH scavenger.

*Cyclic peroxide* *OH radical generation* *Spin-trapping ESR* *Synthetic peroxide* *Peroxide thermodegradation*

## 1. INTRODUCTION

It has become apparent that the carcinostatic action of some kinds of anticancer drugs, such as adriamycin [1] or the therapeutic effect of radiation [2] are mediated by activated oxygen radicals such as the OH radical. On the other hand, the biological toxicity of hydroperoxidic compounds, typically lipid peroxides, has widely been recognized [3] and the activated oxygen species which are produced on their degradation are also determined as the toxic species involved. Based on these observations, we previously proposed an approach to cancer necrosis by organic peroxides which can be locally decomposed at the tumor site by physical means such as radiation or heating to generate activated oxygen radicals [4,5]. As a candidate of such peroxides, we synthesized cyclic peroxides, 4-alkoxy-2,3-benzodioxan-1-ols, because it is considered that the cyclic peroxides are rather stabler to handle compared to non-cyclic peroxides and are expected to generate activated oxygen radicals on degradation. Our previous studies on the thermal degradation process of benzodioxans [4,5] indicated that activated oxygen radicals such as ·OR or ·OH are possibly generated during degradation and are the species responsible for the observed antibacterial activity of benzodioxans.

To confirm the generation of activated oxygen radicals during decomposition of cyclic peroxides, ESR studies using spin-trapping technique were carried out here on the thermal decomposition of 6- and 7-membered cyclic peroxides, benzodioxans and benzodioxepins, respectively. Our results show that OH radicals are generated on decomposition of both peroxides. This also suggests the involvement of the OH radical in bioactivities of naturally occurring cyclic peroxides.

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*Abbreviations:* HPLC, high performance liquid chromatography; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; BHT, 2,6-di-*tert*-butyl-*p*-cresol; BHQ, 2,5-di-*tert*-butylhydroquinone

## 2. MATERIALS AND METHODS

4-Alkoxy-2,3-benzodioxan-1-ols and 4-alkoxy-2,3-benzodioxepins were synthesized by ozonolysis of naphthalene or indene, respectively, in corresponding alcohol solvents according to [6,7] and the purities were checked as in [4].

Degradation kinetics were followed by HPLC

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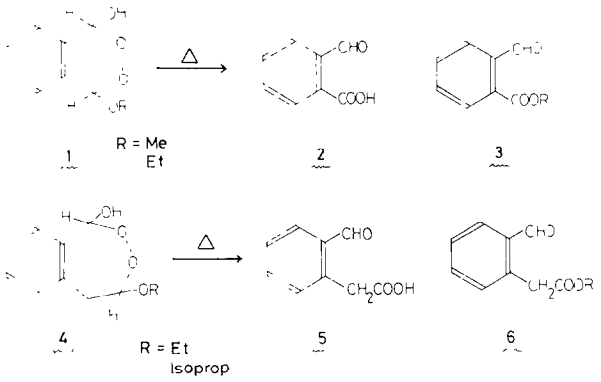
using a Nippon Bunko Twincle model equipped with an LS410K reverse phase column (Toyo Soda). Acetonitrile: water (80:20, v/v) was used as eluent. Elution peaks were detected at 254 nm using a model Uvidec 100 III UV detector (Nippon Bunko). 10 $\mu$l of a 1 mM solution of benzodioxan or dioxepin was injected for analysis before or after heat treatment.

ESR spectra were determined in the presence of DMPO as spin trap using a Varian model X-4 ESR spectrometer. Measuring conditions were as follows: 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 kHz.

DMPO was purchased from Aldrich, dissolved in deionized and distilled $H_2O$, and then purified by passing through a charcoal column before use. All other reagents were from Wako.

## 3. RESULTS AND DISCUSSION

Our previous studies on the thermal decomposition of benzodioxans in several solvent systems revealed that *o*-phthalaldehydic acid and the corresponding ester (**2** and **3** of scheme 1) were the major decomposition products in aqueous medium [4,5]. Therefore, it was suggested that the elimination step of the OH radical is involved in their degradation pathway. Seven-membered cyclic peroxides, benzodioxepins, also gave rise to the same type of degradation products as shown in scheme 1 [5].




Scheme 1. Structures of 4-alkoxy-2,3-benzodioxin-1-ols (**1**), 4-alkoxy-2,3-benzodioxepin-1-ols (**4**) and their degradation products.

To confirm the radical generation in the degradation process of these cyclic peroxides, ESR studies were carried out using DMPO as a spin-trapping reagent, which is frequently used to detect OH radicals in biological systems [8].

Neither purified DMPO nor cyclic peroxides gave rise to any signals, but when DMPO was mixed with EtO-benzodioxan at room temperature

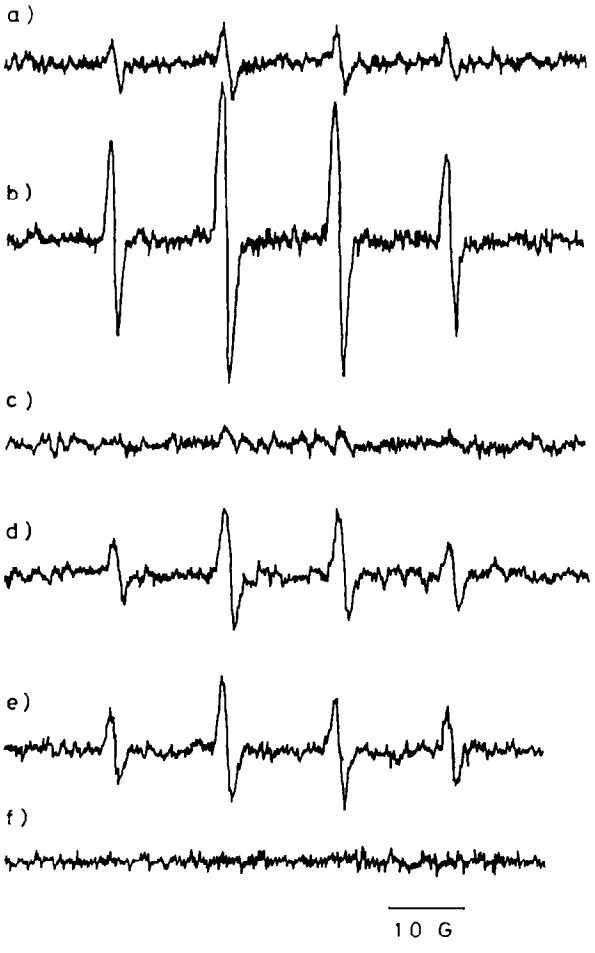



Fig.1. ESR spectra of DMPO-adduct generated on degradation of cyclic peroxides. 10 $\mu$l of 50 mM peroxides in acetonitrile was mixed with 50 $\mu$l of 500 mM DMPO aqueous solution. 30 $\mu$l of the above mixture was taken in a capillary and then the ESR spectra were measured. ESR conditions are given in the text. a, DMPO + EtO-benzodioxan at 15°C; b, (a) at 60°C for 2 min; c, DMPO + Eto-benzodioxepin at 15°C; d, (c) at 60°C for 2 min; e, isopropoxy-benzodioxepin at 60°C for 2 min; f, DMPO at 60°C for 2 min.

(15°C) it gave rise to small ESR signals consisting of a 1:2:2:1 quartet. The equal nitrogen and hydrogen hyperfine coupling constants ($A_N = A_H^\beta$ = 14.86 G) in these spectra are characteristic of the OH spin adduct of DMPO reported in [8]. The signal did not increase significantly after standing for 30 min at room temperature, but was enhanced remarkably when the mixture was heated at 60°C for 2 min as shown in fig.1. However, signals due to other radical species such as ·OR could not be detected under these conditions. Seven-membered cyclic peroxides, benzodioxepins, on the other hand, did not show any significant signals at room temperature, but the same signal corresponding to the typical DMPO-OH adduct was produced after heat treatment at 60°C for 2 min (fig.1). The differences in the spectral intensity obtained from these two types of cyclic peroxides are due to their thermal stability in aqueous solution. It is obvious that EtO-benzodioxepin is more stable than EtO-benzodioxan from the thermal decomposition profiles of these cyclic peroxides shown in fig.2.

The time course of peroxide-dependent DMPO-OH adduct formation was studied at 60°C in the presence or absence of OH radical scavenger (fig.3). The benzodioxan-mediated DMPO-OH signal increased remarkably after heat treatment for 6 min, then leveled off or rather decreased.

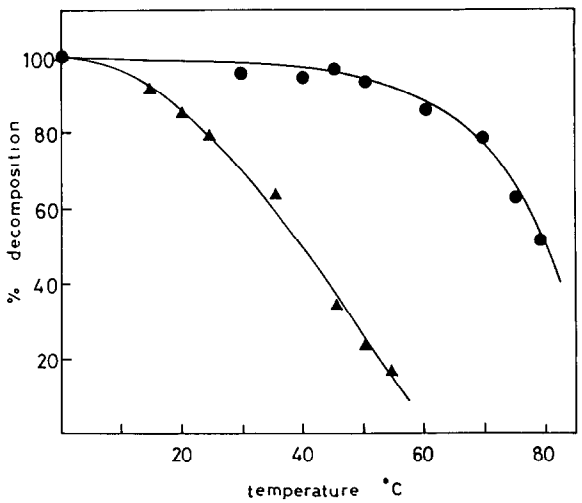



Fig.2. Thermal degradation profiles of EtO-benzodioxan and EtO-benzodioxepin. Cyclic peroxides were heat-treated for 30 min in $H_2O$-acetonitrile (50:50, v/v) at each temperature indicated and analyzed by HPLC. EtO-benzodioxan (▲), EtO-benzodioxepin (●).

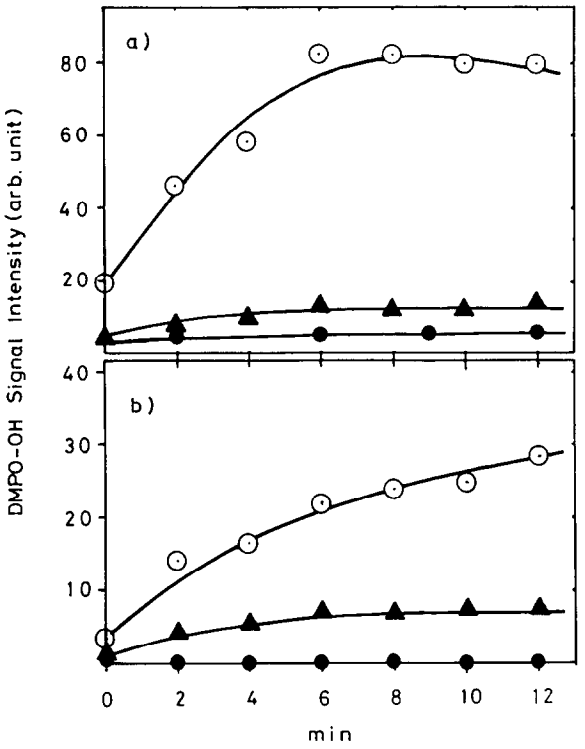



Fig.3. Time course of DMPO-OH formation at 60°C and the effect of OH scavengers. (a) EtO-benzodioxan (⊙), with 14 mM Na-benzoate (▲), EtO-benzodioxan at 15°C (●). (b) EtO-benzodioxepin (⊙), with 1.4 mM EtOH (▲), DMPO without peroxides (●).

However, in the presence of 14 mM Na-benzoate as a specific OH radical scavenger [9], OH-adduct formation was considerably inhibited. The rate of increase of the signal at room temperature was not significant, as expected from the degradation profile of EtO-benzodioxan shown in fig.3a.

The rate of increase of the benzodioxepin-dependent DMPO-OH signal was much slower than that of benzodioxan, but did not level off even after 12 min (fig.3b). DMPO-OH formation was also remarkably inhibited in the presence of EtOH (1.4 mM) which is also an OH scavenger. Obviously, purified DMPO itself did not give rise to any signal after heating at 60°C for 12 min in both acetonitrile or $H_2O$.

Inhibition of DMPO-OH adduct formation by EtOH was concentration dependent (fig.4), although no significant formation of hydroxyethyl radical adduct [10] could be detected under our experimental conditions even at higher concentration

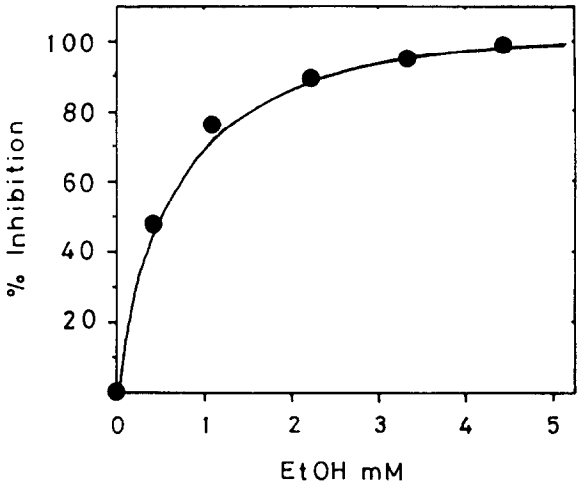



Fig.4. Effect of EtOH concentration on DMPO-OH formation. Reaction mixtures containing 10 μl of 50 mM EtO-benzodioxan, 50 μl of 500 mM DMPO and 20 μl of EtOH-$H_2O$ mixture were heated for 3 min at 60°C and then the ESR spectra were obtained.

(4.5 mM) of EtOH. Other radical scavengers, BHT and BHQ, also inhibited spin adduct formation. The inhibitory activities of scavengers are summarized in table 1.

Very few studies have been reported so far on the degradation and biological effects of cyclic peroxides, although certain cyclic peroxides were detected in the secondary peroxidation products of lipid [11]. The present studies showing the OH radical-generating nature of cyclic peroxides suggest that the cyclic peroxides also participate in the cell damaging process by lipid peroxides.

Table 1

Effects of radical scavengers on cyclic peroxide-dependent DMPO-OH formation

| Additions | Relative signal intensity | |
|---|---|---|
| | 15°C | 60°C, 2 min |
| Control | 7.0 (100%) | 34.5 (100%) |
| EtOH (1.4 mM) | 1.0 ( 14.3%) | 8.0 ( 23.2%) |
| Na-benzoate (14 mM) | 2.1 ( 30.0%) | 5.5 ( 15.9%) |
| BHT (14 mM) | 1.6 ( 22.9%) | 6.5 ( 18.8%) |
| BHQ (14 mM) | 2.0 ( 28.6%) | 10.2 ( 29.6%) |

DMPO, EtO-benzodioxin mixture was heat-treated for 2 min at 60°C in the presence of each radical scavenger

Recently, several compounds which have a cyclic peroxide linkage have been isolated from natural sources, such as quinghaosu [12], plakinic acids [13] and others [14]. It also turned out that they possess a variety of biological activities such as antimalarial [12], antifungal [13] and inhibition of cell division [14]. Although the mechanism of these activities remains unclear, the OH radical-releasing nature of cyclic peroxides found here suggests that the biological activities found in naturally occurring peroxidic compounds are also mediated by activated oxygen radicals generated directly or secondarily on their decomposition. In fact, one of the naturally occurring peroxides, plakortin, was reported to lose its antibacterial activity after standing for a long time in solution [15], probably due to the decomposition of the unstable peroxide linkage.

This study revealed that the cyclic peroxides are rather stable at room temperature, but are decomposed in a temperature-dependent manner with generation of ·OH on degradation in aqueous medium. This temperature-dependent nature of radical generation suggests the possible use of this kind of peroxide as a hyperthermic sensitizer, which is expected to potentiate the hyperthermic treatment of cancer [16]. Furthermore, it is quite important for this kind of peroxide to be a direct source of OH radicals for study of the precise mechanism of oxygen toxicity in biology, because thus far only pulse radiolysis has been a viable method to generate free OH radicals.

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