Synthesis of ^{14}C -Labeled Cyclic Peroxide, 4-Ethoxy-2,3-benzodioxin-1-ol, and Its Decomposition

Seiichi MATSUGO[†], Nobuko KAYAMORI* and Tetsuya KONISHI*^{††}

Department of Medicinal Chemistry and *Department of
Radiochemistry-biology, Niigata College of Pharmacy
Kamishin'ei 5-chome Niigata-shi 950-21, Japan

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Synthesis of ^{14}C -labeled 4-ethoxy-2,3-benzodioxin-1-ol (EtO-BD) from [1(4,5,8)- ^{14}C] naphthalene and the decomposition behavior of the labeled EtO-BD are described. ^{14}C -EtO-BD is stable over months as long as kept at -40°C as a solid state, and over weeks in acetonitrile. The decomposition of EtO-BD is facilitated by the presence of H_2O giving rise to two major decomposition products, o-phthalaldehydic acid and its ethyl ester. Thus the generation of OH radical is expected on its decomposition path and is enhanced as increasing H_2O concentration. Fe(II) seems to facilitate EtO-BD decomposition through the electron transfer reaction.

Key Words : 4-ethoxy-2,3-benzodioxin-1-ol, carbon-14, cyclic peroxide, decomposition, hydroxyl radical generation

1. Introduction

Reactive oxygen radicals, especially OH radical, are considered to be the central species involved in the cell injury caused by radiation or oxygen stress¹⁾. To study the cell damaging process caused by certain oxygen radicals, a specific oxygen radical generating system is waited to be developed. The pulse radiolysis is the one which provides the pure OH radical and has been used for the analysis of the OH radical mediated reactions²⁾. However, the amount of radical generated by this method is too little to give enough reaction products to be analyzed by a routine chemical methodology. On the other hand, peroxidic compounds, such as lipid peroxides, have been known to be toxic because they generate oxygen radicals on their decomposition processes. We previously synthesized a cyclic peroxide, 4-ethoxy-2,3-benzodioxin-1-ol (EtO-BD), as a proto type of the radical releasing compound which decomposition under control accompanies the oxygen radical genera-

tion. In the previous ESR spin trapping studies using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), we showed that OH radical was actually released during the degradation of EtO-BD³⁾. Further studies on the reaction of EtO-BD with cytochrome c (cyt.c) *in vitro* revealed that cyt.c was degraded by the radicals such as $\cdot\text{OH}$ or $\cdot\text{OR}$ generated directly from the cyclic peroxide decomposition⁴⁾⁻⁶⁾.

For studying further the mechanism of biological action of cyclic peroxides, we synthesized here ^{14}C -labeled EtO-BD. Using the labeled EtO-BD, the decomposition products were identified and quantitated on the radiochromatogram. Then, decomposition behavior of EtO-BD in several reaction conditions were further studied.

2. Materials and Method

2.1 Synthesis of ^{14}C -labeled EtO-BD

Labeling of EtO-BD with ^{14}C was carried out as shown in Chart 1. Ozonization of naphthalene was followed essentially by the method of Bailey et al.⁷⁾. Briefly, 9.25 MBq (250 μCi) of [1(4,5,8)- ^{14}C]naphthalene (166.5 MBq/ μmol) was dissolved in 10 ml of 1 mM naphthalene in ethanol and quantitatively transferred

[†] Present address; Kobe University of Mercantile Marine, Fukae-Minamimachi 5-chome, Higashi-nada-ku, Kobe-shi 658, Japan.

^{††} To whom correspondence should be addressed.

Chart 1. Synthesis of ^{14}C -labeled 4-ethoxy-2,3-benzodioxin-1-ol from ^{14}C -naphthalene.

into a 500 ml flask. After the addition of 7.1 mg of cold naphthalene in 20 ml of ethanol, the solution was cooled to -70°C in a dry ice-acetone bath, then O_3 was weakly bubbled through the reaction mixture to ozonize the naphthalene at -70°C for 3 hours. Ozone was generated by a Nippon Ozone model 0-10-2 ozone generator. The reaction mixture was concentrated by a rotary evaporation *in vacuo* at 0°C to give an oily residue. Rotary evaporation procedure was repeated twice after the addition of a small portion of diethylether. Finally the residue was dissolved in 2 ml of diethylether. The solution was transferred to a 20 ml flask and the reaction flask was washed with 10 ml of diethylether containing 5 mg of authentic EtO-BD obtained as in (7). Then the solution was concentrated to about 2 ml *in vacuo* and was kept in a freezer at -40°C after adding another 5 mg of authentic EtO-BD to crystallize the labeled EtO-BD. After crystallization of EtO-BD, diethylether was filtered off and the crystal was washed with a small volume of ice cold diethylether.

A small portion of ^{14}C -EtO-BD thus obtained was dissolved in 1 ml of acetonitrile. Radioactivity of the solution was measured by a liquid scintillation counter (Aloka LSC-700) and the same volume was applied to HPLC for determining the amounts of EtO-BD to calculate the specific activity.

[1(4,5,8)- ^{14}C]naphthalene was purchased from Amersham. HPLC grade acetonitrile and other reagents were purchased from Kanto or Wako Chemical Co. Ltd.

1.2 Studies on the thermal decomposition of EtO-BD

The analysis of the decomposition products of EtO-BD and also the determination of the decomposition kinetics of EtO-BD were carried out by a HPLC (Nippon Bunko Model TWIN-KLE) equipped with a reverse phase column (Toyo Soda ODS 120 A, 210 mm $\times$ 4.6 mm I.D.) and a radio HPLC detector (Aloka RLC-601, glass-scintillator). Typically 1 ml of 1 mM ^{14}C -EtO-BD in acetonitrile or acetonitrile/water (50:50, v/v) was incubated in a water bath at a defined temperature. An aliquot of the reaction solution (10–25 μl) was taken as occasion required, applied on HPLC and eluted with acetonitrile/ H_2O (8:2, v/v) at a flow rate of 0.6 ml/min. After the retention time and the molar coefficient for the decomposition products were determined, further studies on the decomposition kinetics in various conditions were carried out by HPLC without using the labeled compound.

3. Results and Discussion

^{14}C -EtO-BD synthesized as described in the method section was dissolved in acetonitrile

Fig. 1 Radiochemical purity of synthesized ^{14}C -EtO-BD.

and the radiochemical purity was determined by HPLC (Fig. 1). Only single radioactive peak appeared in coincidence with the EtO-BD absorption peak. The radiochemical purity calculated was 92.40%. Since the radioactivity applied in HPLC was quantitatively recovered in the eluate, the specific radioactivity was calculated to be $20.87 \text{ kBq}/\mu\text{mol}$ ($0.564 \mu\text{Ci}/\mu\text{mol}$) EtO-BD and the overall radiochemical yield was 5.4%.

To identify the decomposition products, 1 mM of ^{14}C -EtO-BD (specific activity: $61.79 \text{ kBq}/\text{mg}$ ($1.67 \mu\text{Ci}/\text{mg}$) in acetonitrile/water (50:50, v/v) was incubated at 40.7°C for 3 hours. A typical HPLC pattern and its radiochromatogram are shown in Fig. 2. Two major radioactive peaks corresponding to *o*-phthalaldehydic acid (acid) and *o*-phthalaldehydic acid ethyl ester (ester), respectively, are detected in addition to the EtO-BD peak. Trace amounts of *o*-phthalaldehyde (aldehyde) was also detected. Figure 2 shows the decomposition kinetics of EtO-BD under this condition. The decomposition of EtO-BD follows the pseudo-first order kinetic and its rate constant (k) was 0.1684 h^{-1} .

To clarify the stability of synthesized ^{14}C -EtO-BD, we examined the decomposition of 1 and 20 mM of ^{14}C -EtO-BD in acetonitrile stored at -40°C or room temperature (Fig. 3).

Fig. 2 (a) HPLC pattern and its radiochromatogram of its degradation products. ^{14}C -EtO-BD (1 mM, 1 600 dpm) in acetonitrile/water (50:50) was incubated at 40.7°C for 3 hours. (b) Time course of ^{14}C -EtO-BD ($\circ$) decomposition and the formation of decomposition products; *o*-phthalaldehydic acid (acid, $\blacktriangle$), *o*-phthalaldehydic acid-ethyl ester (ester, $\diamond$) and *o*-phthalaldehyde (aldehyde, $\triangle$).

Fig. 3 Time course of ^{14}C -EtO-BD decomposition stored in a freezer at -40°C (1 mM $\cdots\bullet\cdots$, 20 mM $\text{---}\bigcirc\text{---}$) and room temperature (1 mM $\cdots\blacktriangle\cdots$, 20 mM $\text{---}\blacktriangle\text{---}$).

EtO-BD was quite stable at -40°C either at 1 or 20 mM concentration and any significant decomposition was observed over a month period. At room temperature EtO-BD was relatively labile and the decomposition was promoted at the lower concentration. EtO-BD in solid state was more stable and no significant decomposition was detected over several months at -40°C .

For further studies on the decomposition behavior of EtO-BD by HPLC, we calculated the molar concentration/ $A_{254\text{nm}}$ peak height ratio for each decomposed products of EtO-BD under the analytical condition using their specific radioactivity, and the values obtained were used for calibrating the molar concentration of each decomposition products in the following experiments; the values used were 0.045 pmol/mm for acid, 0.020 pmol/mm for ester, 0.409 pmol/mm for EtO-BD and 7.9×10^{-3} pmol/mm for aldehyde. The calibration curves for EtO-BD, acid, ester and aldehyde were sufficiently linear in the concentration range used in the following experiments, for example up to 0.5 μmol of EtO-BD.

Temperature dependence of EtO-BD decomposition at its 16 mM concentration in 100% acetonitrile or acetonitrile/water (50:50) were shown in Fig. 4. In 100% acetonitrile EtO-BD scarcely decomposed up to 60°C . On the other hand, in the presence of H_2O , the decomposition rate of EtO-BD markedly enhanced above the temperature approximately 30°C and the decomposition rate increased with the rise of

Fig. 4 Temperature dependence of EtO-BD decomposition. EtO-BD was heat-treated for 30 min in 100% CH_3CN or $\text{CH}_3\text{CN}/\text{H}_2\text{O}$ (50:50).

temperature. The labeled decomposition products determined through these degradation conditions using ^{14}C -EtO-BD were acid and ester as same as shown in Fig. 2 and there was no significant temperature dependent change in their molar ratio. Aldehyde was always trace. Almost the same results were obtained when 1 mM of EtO-BD was used. The rate constant for the decomposition was slightly dependent on EtO-BD concentration. It was larger in 1 mM EtO-BD than in 16 mM at the temperature above 60°C in 100% acetonitrile.

The activation energy ($\Delta E^\ddagger$) for the decomposition of 16 mM of EtO-BD obtained from the Arrhenius plot were 31.20 kcal/mol in acetonitrile and 17.59 kcal/mol in acetonitrile/water (50:50). As increasing the water contents, both the decomposition temperature (above the temperature the decomposition is markedly accelerated) and $\Delta E^\ddagger$ decreased (not shown).

Table 1 shows the molar ratio of the decomposition products after incubating EtO-BD in various reaction condition at 40°C for 3 hours. Results show that EtO-BD decomposition is highly dependent on the presence of H_2O in

Table 1 The molar ratios of the decomposed products of EtO-BD in the several reaction conditions

	Molar ratio (%)				k (h^{-1})
	EtO-BD	Acid	Ester	Aldehyde	
1 mM EtO-BD, $40.5 \pm 0.2^\circ\text{C}$, 180 min					
CH ₃ CN : H ₂ O = 9 : 1	96.7	2.0	1.0	<0.1	0.0164
7 : 3	76.3	7.8	14.6	<0.1	0.0909
1 : 1	58.2	13.1	28.7	<0.1	0.1684
CH ₃ CN : H ₂ O = 9 : 1					
+0.1 mM Fe(III)	96.8	2.0	1.2	<0.1	0.0089
+0.1 mM Fe(II)	88.4	6.5	5.0	<0.1	0.0221
+0.5 mM	5.2	65.1	29.7	<0.1	
+0.8 mM	1.9	66.5	31.2	<0.1	
+1.0 mM	2.0	65.0	33.0	<0.1	1.2356
1 mM EtO-BD, 0°C , 180 min					
CH ₃ CN : H ₂ O = 9 : 1					
+1.0 mM Fe(II)	84.7	12.6	7.1	<0.1

Chart 2. Possible path for EtO-BD decomposition in aqueous solution.

the reaction medium and the ester formation becomes dominant as H_2O concentration increased. This indicates that H_2O participated in EtO-BD decomposition. It is generally accepted that the peroxide decomposes through $\text{O}-\text{O}$ homolytic scission. If this is the case for EtO-BD decomposition, molar ratio of the

decomposition products, ester/acid, might be insensitive to the amounts of H_2O in the reaction medium. Thus, it is suggested that in an aqueous medium two pathways exist for EtO-BD decomposition as shown in Fig. 5: (1) EtO-BO initiates with $\text{O}-\text{O}$ homolytic scission, (2) by the water addition; a ring-opened hydro-

peroxide is formed, then it decomposes with O-O homolytic scission with the generation of OH radical to afford ester. In this mechanism the generation of OH radical is expected preferentially in an aqueous medium. This is supported by our previous observation that OH radical was detected on EtO-BD decomposition in an aqueous medium through the ESR study using DMPO as a spin trap²⁾. Recently we confirmed that ^{18}O was incorporated exclusively into the formyl position of the ester when EtO-BD was decomposed in acetonitrile/ H_2^{18}O (v/v, 50:50) (to be published elsewhere). This also supports the existence of the above decomposition path.

Further, the effects of metal ions on EtO-BD decomposition were examined and the results are given in Table 1. Fe(III) at 0.1 mM did not affect the EtO-BD decomposition. On the other hand, Fe(II) promoted EtO-BD decomposition at the same concentration, that is, 1/10 molar ratio for EtO-BD. In the presence of an equimolar of Fe(II) EtO-BD was almost completely decomposed within 3 hours. It is noted that the ratio of the amount of acid and ester formed is 2:1 when the Fe(II) mediated decomposition proceeded. Therefore it is suggested that Fe(II) facilitates EtO-BD decomposition through a different mechanism from that in an aqueous medium such as the electron transfer reaction shown in Fig. 5. Since the amount of Fe(II) required for EtO-BD decomposition was quite small on molar base, $\text{Fe(II)} \rightarrow \text{Fe(III)} \rightarrow \text{Fe(II)}$ recycling system is expected to be involved in the reaction mechanism in analogy to Ref. 8). Further studies are needed to clarify the mechanism of Fe(II) catalyzed EtO-BD decomposition in which the molar

ratio of acid and base is always two.

Based on the results described above, the following points are clarified;

- (1) ^{14}C -EtO-BD is stable as long as kept at -40°C as a solid state or in acetonitrile.
- (2) Essentially only two ^{14}C -labeled products, acid and ester, were identified on ^{14}C -EtO-BD decomposition.
- (3) The decomposition path of EtO-BD is highly dependent on the presence of H_2O and Fe(II) .
- (4) Medium H_2O increases molar fractions of ester in the decomposition products of EtO-BD, thereby increases OH radical generation.
- (5) Fe(II) facilitates EtO-BD decomposition through the electron transfer reaction.

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要 旨

^{14}C 標識環状過酸化物 4-Ethoxy-2, 3-benzodioxin-1-ol の合成と分解

松郷誠一†, 柏森伸子*, 小西徹也*

新潟薬科大学医薬品化学教室, *同大学放射薬品化学教室

950-21 新潟市上新栄町5-13-2

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環状過酸化物 4-EtO-2, 3-benzodioxin-1-ol(EtO-BD)の ^{14}C 標識体を合成し, その分解を検討した。その結果, ① ^{14}C -EtO-BDは固体状で -40°C で保存すれば長期間分解しない。②無水アセトニトリル中でもほとんど分解しないが, 水の存在では分解が促進され, EtO-BDの分解に水分子が関与することが示唆される。③ Fe(II) 存在下の分解は, 含水アセトニトリル中とは異なり, 電子移動により進行する, などの諸点が明らかとなった。

† 現所属: 神戸商船大学, 658 神戸市東灘区深江南町5-1-1。