

Inhibition of Mammalian DNA Polymerases and the Suppression of Inflammatory and Allergic Responses by Tyrosol from Used Activated Charcoal Waste Generated during Sake Production

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ABSTRACT: The components adsorbed onto activated charcoal following the fermentation process of the Japanese rice wine “sake” have been studied with the aim of identifying suitable applications for this industrial food waste product. The absorbed materials were effectively extracted from the charcoal, and inhibited the activity of several mammalian DNA polymerases (pols). Subsequent purification of the extract afforded tyrosol [4-(2-hydroxyethyl)phenol] as the active component, which selectively inhibited the activity of 11 mammalian pols with IC₅₀ values in the range of 34.3–46.1 μ M. In contrast, this compound did not influence the activities of plant or prokaryotic pols or any of the other DNA metabolic enzymes tested. Tyrosol suppressed both anti-inflammatory and antiallergic effects in vivo, including 12-O-tetradecanoylphorbol-13-acetate-induced inflammatory mouse ear edema, and immunoglobulin E-induced passive cutaneous anaphylactic reaction in mice. These results suggested that this byproduct formed during the sake-brewing process could be used as an anti-inflammatory and/or antiallergic agent.

KEYWORDS: sake production waste, tyrosol, DNA polymerase, enzyme inhibitor, anti-inflammatory, antiallergy

INTRODUCTION

DNA-dependent DNA polymerase (pol) (E.C. 2.7.7.7) catalyzes the addition of deoxyribonucleotides to the 3'-hydroxyl terminus of primed double-stranded DNA (dsDNA) molecules.¹ The human genome encodes at least 15 pols that play important roles in cellular DNA synthesis.^{2,3} Eukaryotic cells contain three replicative pols (α , δ , and ϵ), one mitochondrial pol (γ), and at least 11 nonreplicative pols (β , ζ , η , θ , ι , κ , λ , μ , ν , terminal deoxynucleotidyl transferase (TdT), and REV1).^{4,5} Pols have a highly conserved structure, and their overall catalytic subunits show very little variation among species. It is noteworthy that conserved enzyme structures that are preserved over time usually perform important cellular functions that confer evolutionary advantages. On the basis of their sequence homology, eukaryotic pols can be divided into four main families, including A, B, X, and Y.⁵ Family A includes mitochondrial pol γ as well as pols θ and ν ; family B includes the three replicative pols α , δ , and ϵ , as well as pol ζ ; family X consists of pols β , λ , and μ , as well as TdT; and family Y includes pols η , ι , and κ , in addition to REV1.⁴ During the last 15 years, we have investigated the isolation and subsequent evaluation of selective inhibitors of eukaryotic pols derived from natural products, including food materials and components, and have discovered more than 100 inhibitors of the mammalian pols.^{6,7}

During our studies toward the identification of new pol inhibitors, we found that pol λ -selective inhibitors, such as curcumin-based derivatives,⁸ could suppress inflammation induced by the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA).⁹ Although several tumor promoters,

including TPA, are classified as compounds capable of promoting the formation of a tumor,¹⁰ they can also cause inflammation and are commonly used as artificial inducers of inflammation to screen for anti-inflammatory agents.¹¹ The tumor promoter TPA is frequently used to search for new types of anti-inflammatory compounds. TPA not only causes inflammation, but also influences the proliferation of mammalian cells,¹² suggesting that the molecular basis of inflammation stems from pol reactions related to cell proliferation. However, to develop a deeper understanding of this process, this relationship needs to be investigated in greater detail.

We recently found that the inhibitory activities of 20 different essential oils toward pol λ activity showed a high correlation with their in vivo antiallergic effects, such as an immunoglobulin E (IgE)-induced passive cutaneous anaphylactic (PCA) reaction in mice, resulting from mast cell degranulation.¹³ It has been suggested that these results and phenomena are indicative of the anti-inflammatory/antiallergic activities resulting from pol λ inhibition.

To identify potential bioactive compounds from industrial waste, we have focused our attention in the current study on investigating the components absorbed onto activated charcoal following its use as a decolorizing agent during the manufacture

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of the Japanese rice wine “sake”. Herein, we describe the extraction of the adsorbed components from the used activated charcoal and their subsequent evaluation as pol inhibitors. This work culminated in the isolation of a potent inhibitor of mammalian pol, which was also investigated for its anti-inflammatory and antiallergic activities. Finally, we have provided a detailed discussion of the way in which bioactive compounds can be isolated from waste products to provide potential anti-inflammatory/antiallergic agents.

MATERIALS AND METHODS

Chemicals and Materials. The used activated charcoal, which is an industrial waste product generated during the manufacture of sake (trade name: Kuromatsu-Hakushika), was obtained from Tatsuum-Honke Brewing Co. Ltd. (Nishinomiya, Japan). Tyrosol (1), which is also known as 4-(2-hydroxyethyl)phenol, 3-hydroxytyrosol (2), and 3,4-dihydroxyphenyl acetic acid (3) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). These compounds were of purchased as the analytical grade and purified by high-performance liquid chromatography. A chemically synthesized DNA template, poly(dA), was purchased from Sigma-Aldrich Inc., and a customized oligo(dT)₁₈ DNA primer was produced by Sigma-Aldrich Japan K.K. (Ishikari, Japan). The radioactive nucleotide [³H]-labeled 2'-deoxythymidine-5'-triphosphate (dTTP; 43 Ci/mmol) was obtained from Moravak Biochemicals Inc. (Brea, CA, U.S.A.). Precoated Silica-Gel 60 plates (10 × 20 cm², 0.25 mm layer thickness) for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). All of the other reagents used in the current study were purchased from Nacalai Tesque Inc. (Kyoto, Japan) as the analytical grade.

Instrumental Analyses. ¹H- and ¹³C-nuclear magnetic resonance (NMR) were recorded on a Bruker DRX600 (Bruker Biospin GmbH, Rheinstetten, Germany). Mass spectra were obtained on an esquire6000-TR (Bruker Daltonics, Billerica, MA, U.S.A.).

Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography according to the method described by Tamai et al.¹⁴ Recombinant rat pol β was purified from *Escherichia coli* Jmp β 5 according to the procedure reported by Date et al.¹⁵ The human pol γ catalytic gene was cloned into the pFastBac vector. A histidine-tagged enzyme was subsequently expressed using the BAC-TO-BAC HT Baculovirus Expression System (Life Technologies, Frederick, MD, U.S.A.) according to the manufacturer's instructions and purified over ProBound resin (Invitrogen Japan, Tokyo Japan).¹⁶ Human pols δ and ϵ were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ϵ -conjugated affinity column chromatography, respectively.¹⁷ A truncated form of human pol η (residues 1–511) tagged with His₆ at its C-terminal was expressed in *E. coli* cells and purified as previously described by Kusumoto et al.¹⁸ A recombinant mouse pol ι tagged with His₆ at its C-terminal was expressed and purified by nickel-nitriloacetic acid (Ni-NTA) column chromatography as previously described.¹⁹ A truncated form of pol κ (residues 1–560) with a His₆ residue tag attached to its C-terminal was overexpressed in *E. coli* and purified according to the method described by Ohashi et al.²⁰ Recombinant human His-pol λ was overexpressed and purified according to the method described by Shimazaki et al.²¹ Recombinant human His-pol μ was overexpressed in *E. coli* BL21 and purified by Glutathione Sepharose 4B column chromatography (GE Healthcare Bio-Science Corp., Piscataway, NJ, U.S.A.) according to the method described by Shimazaki et al.²¹ for the preparation of pol λ . Pol α from the higher plant cauliflower inflorescence was purified according to the methods reported by Sakaguchi et al.²² Recombinant rice (*Oryza sativa* L. cv. Nipponbare) pol λ tagged with His₆ at its C-terminal was expressed in *E. coli* and subsequently separated from the cells according to the method described by Uchiyama et al.²³ Calf TdT, Taq pol, T4 pol, T7 RNA polymerase, and T4 polynucleotide kinase were purchased from Takara Bio Inc. (Kyoto, Japan). The Klenow fragment of pol I from *E. coli* was purchased from Worthington Biochemical

Corp. (Freehold, NJ, U.S.A.). Purified human placenta DNA topoisomerases I and II were purchased from TopoGen Inc. (Columbus, OH, U.S.A.). Bovine pancreas deoxyribonuclease I was obtained from Stratagene Cloning Systems (La Jolla, CA, U.S.A.).

Measurement of Pol Activity. The compositions of the reaction mixtures used for calf pol α , rat pol β , plant pol α , and the prokaryotic pols have been described previously elsewhere.^{24,25} The reaction mixtures used for human pol γ , as well as human pols δ and ϵ have been described previously by Umeda et al.¹⁶ and Ogawa et al.,²⁶ respectively. The reaction mixtures used for mammalian pols η , ι , and κ were the same as that used for pol α , whereas those used for human pols λ and μ , and plant pol λ were the same as that of pol β . For the pol reactions, poly(dA)/oligo(dT)₁₈ (A/T, 2/1) and dTTP were used as the DNA template-primer and nucleotide (dNTP; 2'-deoxynucleoside-5'-triphosphate) substrates, respectively. For the TdT reactions, oligo(dT)₁₈ (3'-OH) and dTTP were used as the DNA primer and nucleotide substrates, respectively.

The compounds to be tested were dissolved in freshly distilled dimethyl sulfoxide to various concentrations, and the resulting solutions were sonicated for 30 s. For microliter aliquots were of the solutions were then mixed with 16 μ L of each enzyme (0.05 units) in 50 mM Tris-HCl (pH 7.5), containing 1 mM dithiothreitol, 50% glycerol (v/v), and 0.1 mM ethylenediaminetetraacetic acid, and the resulting mixtures were held at 0 °C for 10 min. Eight microliter aliquots of the inhibitor-enzyme mixtures were then added to 16 μ L of the enzyme standard reaction mixture and incubated at 37 °C for 60 min, except for Taq pol, which was incubated at 74 °C for 60 min. Activity in the absence of the inhibitor was considered to be 100%, and the relative activity was determined for each inhibitor concentration. One unit of pol activity was defined as the amount of each enzyme required to catalyze the incorporation of 1 nmol of dTTP into the synthetic DNA template-primers in 60 min at 37 °C under standard reaction conditions.^{24,25}

Other DNA Metabolic Enzyme Assays. The activities of T7 RNA polymerase, human DNA topoisomerases I and II, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured using standard assay protocols according to the manufacturers' specifications, as described by Nakayama and Saneyoshi,²⁷ Yonezawa et al.,²⁸ Mizushima et al.,²⁹ Soltis and Uhlenbeck,³⁰ and Lu and Sakaguchi,³¹ respectively.

Animals. Female ICR mice (6 weeks old with a body weight in the range of 25–27 g) were obtained from Japan SLC, Inc. (Hamamatsu, Japan), and maintained on a standard moderate fat (MF) diet (Oriental Yeast Co. Ltd., Osaka, Japan), with water provided ad libitum. Mice that had been bred in-house with free access to food and water were used for all of the experiments in the current study. All of the mice were maintained under a 12-h light/dark cycle and housed at room temperature (25 °C). This study was approved by the Institutional Animal Care and Use Committee of Kobe Gakuin University and was performed in accordance with the guidelines outlined in the Care and Use of Laboratory Animals of Kobe Gakuin University. The animals were anesthetized with pentobarbital before undergoing cervical dislocation.

Measurement of Anti-Inflammatory Activity. The mouse inflammatory test was performed according to Gschwendt's method.³² Briefly, an acetone solution of the compound of interest (500 μ g in 20 μ L) or 20 μ L of acetone as a vehicle control was applied to the inner part of the mouse ear. Thirty minutes after the test compound was applied, a TPA solution (0.5 μ g/20 μ L of acetone) was applied to the same part of the ear. TPA was also applied to the other ear of the same mouse as a control. After 7 h, a disk (6 mm diameter) was obtained from the ear and weighed. The inhibitory effect (IE) was then determined as the ratio of the weights of the two ear disks, as follows:

$$\text{IE} = \frac{[(\text{TPA only}) - (\text{tested compound plus TPA})]}{[(\text{TPA only}) - (\text{vehicle})]} \times 100$$

Measurement of Anti-Anaphylactic Activity. The PCA reaction was conducted according to the method described by Sato et al.³³ Mice were sensitized by an intradermal injection of 0.1 μ g of

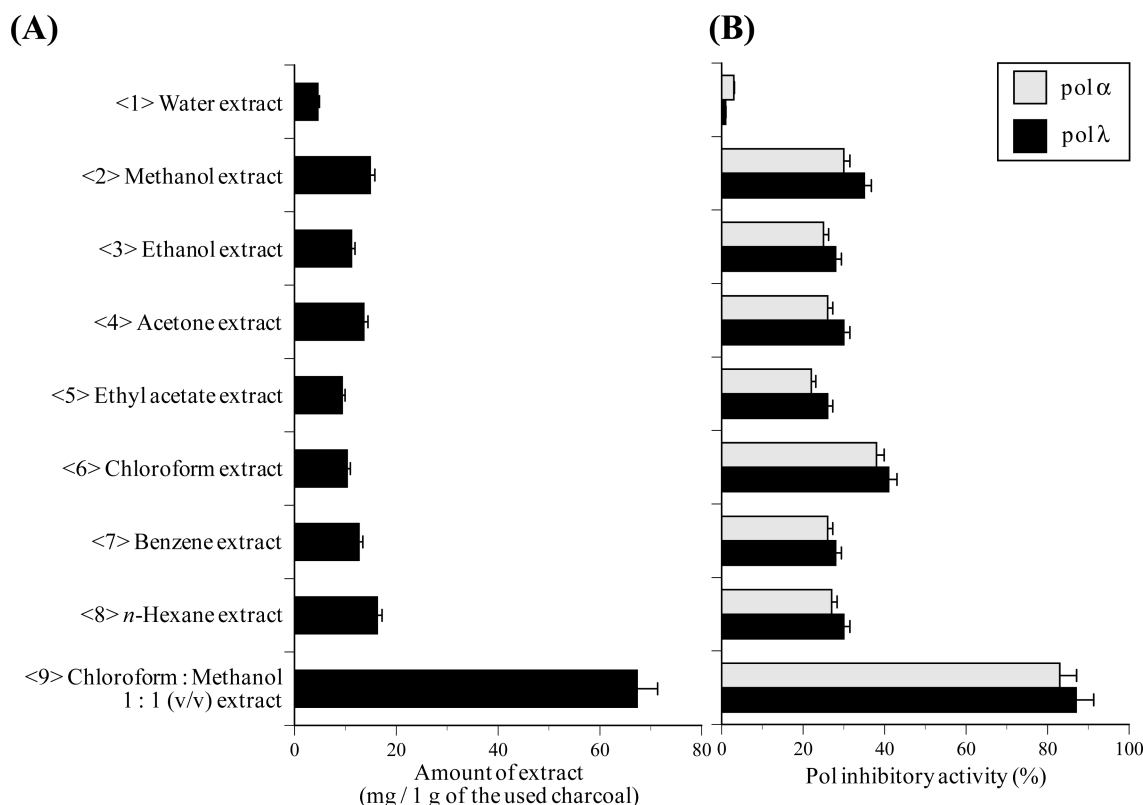


Figure 1. Extraction of the absorbed materials from the used activated charcoal generated during the sake-brewing process with different organic solvents. (A) Amount of extract collected from the used activated charcoal by the nine organic solvents. Data are shown as the mean values $\pm$ SD of three independent experiments. (B) Inhibitory activity of calf pol α and human pol λ by 100 μ g/mL of extract collected from the used activated charcoal by the nine organic solvents. Data are shown as the mean values $\pm$ SD of three independent experiments.

antidinitrophenyl (DNP) IgE in their ear. Four hours later, the mice were intravenously challenged with 0.2 mL (1 mg/mL) of DNP-labeled human serum albumin containing 2% Evans blue dye. A test compound (100 mg/kg) or saline was also administered orally 2 h prior to the antigen challenge, with saline being used as a control. The mice were subsequently sacrificed, and their ears removed and weighed 30 min after the challenge. The ears were then dissolved in 200 μ L of 1 N KOH, and the resulting solutions were incubated overnight at 37 $^{\circ}$ C. To measure the amount of Evans blue dye present in the exudates, the dissolved tissue solution was added to 400 μ L of a 5:13 (v/v) mixture of acetone and 0.6 N phosphoric acid, and the optical density of the resulting solution was measured at 620 nm. The amount of dye in the exudates was calculated from an Evans blue standard curve, and the results expressed as a percentage for the mean amount of exudate dye from the treated mice. These data were then compared with those from the controls.

Statistical Analysis of Animal Experiments. All data of animal experiments such as anti-inflammatory and antiallergic activities are expressed as the means $\pm$ SD of six independent determinations for each experiment. Statistical significance between each experimental group was analyzed using Student's *t* test, and a level of probability of 0.01 and 0.05 was used as the criterion of significance.

RESULTS AND DISCUSSION

Extraction of Absorption Components from the Used Activated Charcoal Derived from Sake-Brewing Process.

The Japanese rice wine “sake” is made from fermented rice. The fermentation process for the production of this wine involves the conversion of starch to sugar using the fungus *Aspergillus oryzae*, whereas the brewing process uses the yeast *Saccharomyces cerevisiae*. During sake production, the fermented sake (i.e., irekuchi-sake) becomes colored and off-flavored, and

is consequently treated with activated charcoal to remove the colors and off-flavors. The resulting activated charcoal-treated sake is known as “sumikuchi-sake”, and this material is subsequently packaged and distributed as a commercial product. The used activated charcoal, however, is treated as an industrial waste product and disposed of accordingly. The aim the current study was to identify bioactive compounds that had been adsorbed onto the activated charcoal during the manufacture of sumikuchi-sake, and evaluate these compounds in terms of their pol-inhibitory activities. In this way, it could be possible to recycle these byproducts and find alternative industrial/medical applications for these materials.

We investigated the extraction of the absorbed materials from the used activated charcoal using nine different organic solvent systems, including (1) water, (2) methanol, (3) ethanol, (4) acetone, (5) ethyl acetate, (6) chloroform, (7) benzene, (8) *n*-hexane, and (9) a 1:1 (v/v) mixture of chloroform and methanol (Figure 1). Solvents (1) to (8) have been listed in order of their hydrophilicity. Five-hundred milligrams of the used activated charcoal was added to 1 mL of each organic solvent, and the resulting mixtures were shaken and vortex-mixed for 10 min, before being centrifuged at 10 000g for 10 min to separate the organic extract from the charcoal. Extract from solvent system (9) was very yellow in color, and the extract from solvent (4) was only slightly yellow in color. In contrast, the extracts from all of the other solvents remained colorless. The amounts of adsorbed material extracted from 1 g of used activated charcoal by the different organic solvents are shown in Figure 2A. The amount of material extracted by solvent system (9) was more than 4-fold greater than that of

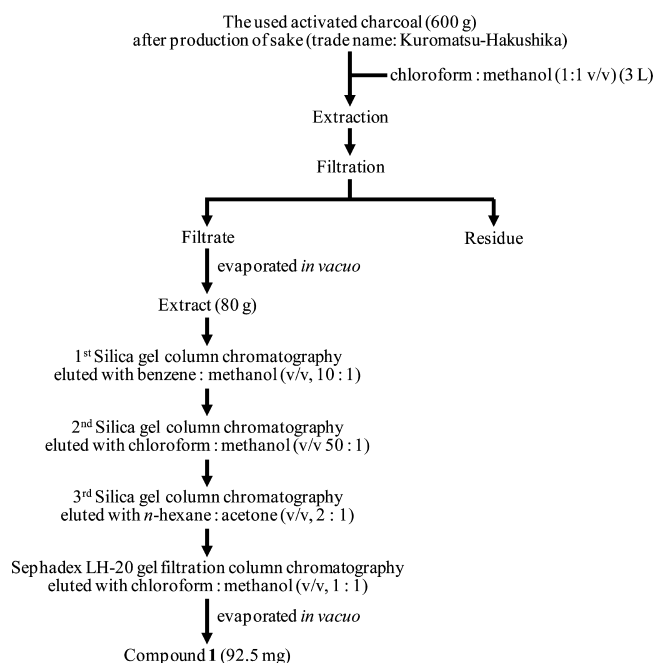


Figure 2. Method for purification of compound 1, a mammalian pol inhibitor, from the used activated charcoal generated during the sake-brewing process.

any other organic solvent. Solvent system (9) therefore exhibited the best extraction efficiency of all of the solvents tested toward the used activated charcoal. The inhibitory activities of the extracts of nine organic solvent toward mammalian pols was investigated, using pol α and pol λ as a representative DNA replicative pol and DNA repair/recombination-related pol, respectively.^{4,5} The inhibitory activity of calf pol α and human pol λ against 100 $\mu\text{g/mL}$ of each extract is shown in Figure 1B. Among the nine extracts tested, solvent system (9) showed the strongest inhibition of both pols α and λ , with inhibitory activities of 83.0 and 87.2%, respectively. The extracts of solvent system (2) to (8) slightly inhibited the pol activity, but solvent system (1) had no effect on the activity, suggesting that water-soluble (i.e., hydrophilic) components of the absorbed materials from the used activated charcoal did not have the inhibitory activities of mammalian pols.

Isolation of a Mammalian Pol Inhibitor from the Absorption Extract of the Used Activated Charcoal. The materials extracted from the used activated charcoal with solvent system (9) exhibited potent inhibitory activity toward mammalian pol, and the decision was taken to purify this extract to isolate and identify the compound responsible for this inhibitory activity. Briefly, a large portion of used activated charcoal (600 g) was extracted with a 1:1 (v/v) mixture of chloroform and methanol (3 L), and the resulting extract was evaporated to dryness to give a residue (80 g), which was subjected to purification by column chromatography over silica gel eluting with a 10:1 (v/v) mixture of benzene and methanol. The active fractions (1.15 g) were then combined and purified by column chromatography over silica gel eluting with a 50:1 (v/v) mixture of chloroform and methanol. Once again, the active fractions (310 mg) were combined and purified by column chromatography over silica gel eluting with a 2:1 (v/v) mixture of *n*-hexane and acetone. Finally, the active fractions (123 mg) were combined and purified by column chromatography over Sephadex LH-20 eluting with a 1:1 (v/v) mixture of

chloroform and methanol to give compound 1 as a white powder (92.5 mg), which was confirmed to be an inhibitor of mammalian pol (Figure 2). The yield and 50% inhibitory concentration (IC_{50}) for human pol λ activity of the purification steps are shown in Table 1. Since the extract of solvent system

Table 1. Purification Steps of Compound 1 from the Used Activated Charcoal after Production of Sake

step of purification	yield (mg)	IC_{50} value for pol λ ($\mu\text{g/mL}$)
extract of chloroform: methanol (1:1 v/v)	80 000	55.3
active fraction of first silica gel column chromatography	1150	66.8
active fraction of second silica gel column chromatography	310	18
active fraction of third silica gel column chromatography	123	6.5
active fraction of LH-20 gel filtration column chromatography (compound 1)	92.5	4.7

(9) was stronger pol λ inhibitor than the active fraction of first silica gel column chromatography, the extract of solvent system (9) should contain several pol inhibitory compounds.

Determination of the Structure of Compound 1 Purified from the Used Activated Charcoal Derived from the Sake-Brewing Process. Compound 1 purified from the used activated charcoal was identified as tyrosol [4-(2-hydroxyethyl)phenol] by spectroscopic analyses, including 1D/2D NMR and MS. These spectroscopic data were consistent with those reported previously in the literature for the same compound.³⁴ Two tyrosol (1) analogues were also prepared, including 3-hydroxytyrosol (2) and 3,4-dihydroxyphenyl acetic acid (3) (Figure 3), and all three of these compounds were used in the next part of the study.

Effects of Tyrosol (1) and Its Analogues (2 and 3) on the Activities of Various Pol and Other DNA Metabolic Enzymes. Tyrosol (1), 3-hydroxytyrosol (2), and 3,4-dihydroxyphenyl acetic acid (3) were evaluated in terms of

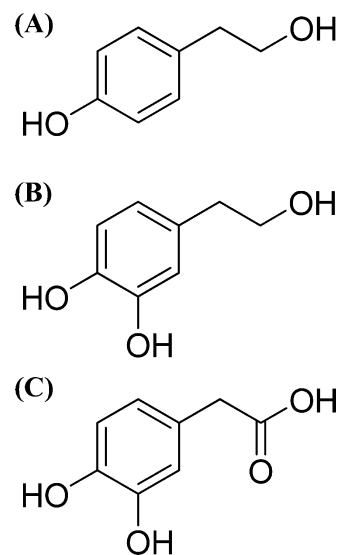


Figure 3. Structure of compound 1, which was obtained from the absorption extract of the used activated charcoal generated during the sake-brewing process, and two related compounds. Compound 1, tyrosol [4-(2-hydroxyethyl)phenol]; compound 2, 3-hydroxytyrosol; and compound 3, 3,4-dihydroxyphenyl acetic acid.

Table 2. IC₅₀ Values of Tyrosol (1) and Its Analogues (2 And 3) towards Various Pols and other DNA Metabolic Enzymes^a

enzymes	IC ₅₀ values (μM)				
	tyrosol (1)	3-hydroxytyrosol (2)	3,4-dihydroxyphenylacetic acid (3)	aphidicolin	curcumin
[1] mammalian pols					
[A-family]					
human pol γ	42.0 ± 2.5	>200	>200	>200	>200
[B-family]					
calf pol α	40.7 ± 2.4	>200	>200	19.6 ± 1.0	>200
human pol δ	42.6 ± 2.6	>200	>200	13.3 ± 0.7	>200
human pol ε	42.8 ± 2.5	>200	>200	16.2 ± 0.8	>200
[X-family]					
rat pol β	36.0 ± 2.1	>200	>200	>200	>200
human pol λ	34.3 ± 2.0	>200	>200	>200	7.0 ± 1.0
human pol μ	37.5 ± 2.2	>200	>200	>200	>200
calf TdT	38.1 ± 2.2	>200	>200	>200	>200
[Y-family]					
human pol η	44.4 ± 2.6	>200	>200	>200	>200
mouse pol ι	46.1 ± 2.7	>200	>200	>200	>200
human pol κ	45.2 ± 2.5	>200	>200	>200	>200
[2] plant pols					
cauliflower pol α	>200	>200	>200	25.0 ± 1.3	>200
rice pol λ	>200	>200	>200	>200	>200
[3] prokaryotic pols					
<i>E. coli</i> pol I	>200	>200	>200	>200	>200
<i>Taq</i> pol	>200	>200	>200	>200	>200
T4 pol	>200	>200	>200	>200	>200
[4] other DNA metabolic enzymes					
human topo I	>200	>200	>200	>200	>200
human topo II	>200	>200	>200	>200	>200
mouse IMP dehydrogenase (type II)	>200	>200	>200	>200	>200
T4 polynucleotide kinase	>200	>200	>200	>200	>200
bovine deoxyribonuclease I	>200	>200	>200	>200	>200

^aCompounds 1–3 and positive controls (aphidicolin and curcumin) were incubated with each pol (0.05 U) and other DNA metabolic enzymes. Enzyme activity in the absence of the compound was considered to be 100%. Data are shown as the mean values ± SD of three independent experiments.

their inhibitory activities toward various pols and other DNA metabolic enzymes. The purities of these compounds were determined to be greater than 98% by NMR analysis (data not shown).

Tyrosol (1) exhibited inhibitory activity toward 11 mammalian pols, including pols belonging to family A (human pol γ), family B (calf pol α, human pol δ, and human pol ε), family X (rat pol β, human pol λ, and human pol μ), and family Y (human pol η, mouse pol ι, and human pol κ),^{4,5} with IC₅₀ values of 42.0, 40.7–42.8, 34.3–38.1, and 44.4–46.1 μM, respectively (Table 2). Tyrosol (1) strongly inhibited the activities of X-family pols, with human pol λ being the most sensitive of all 11 of the mammalian pols tested toward this compound. Aphidicolin, a known inhibitor of pols α, δ, and ε,¹ and curcumin, a specific inhibitor of pol λ,³⁵ showed stronger pol inhibitory activity than tyrosol (1).

In contrast, 3-hydroxytyrosol (2) and 3,4-dihydroxyphenyl acetic acid (3) had no effect on any of these pols, indicating that the presence of a hydroxyl group at the 3-position tyrosol was having an adverse impact on its inhibitory activity toward mammalian pols, and that the whole structure of tyrosol (1) may be essential to its inhibitory activity. When activated DNA (bovine deoxyribonuclease I-treated DNA) was used as the DNA template-primer substrate instead of synthesized DNA [poly(dA)/oligo(dT)₁₈ (A/T = 2/1)], and dNTP was used as

the nucleotide substrate instead of dTTP, the inhibitory effects of these compounds did not change (data not shown).

None of these three compounds exhibited any influence over the activities of the plant pols, including cauliflower pol α and rice pol λ. Furthermore, none of these compounds exhibited inhibitory activity toward prokaryotic pols, such as the Klenow fragment of *E. coli* pol I, *Taq* pol and T4 pol, or DNA metabolic enzymes, such as T7 RNA polymerase, human DNA topoisomerases I and II, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase, and bovine deoxyribonuclease I (Table 2). On the basis of these results, tyrosol (1) can be classified as a selective inhibitor of mammalian pols. Given that tyrosol (1), which is an absorption component of the activated charcoal waste generated during the manufacture of sake, is an inhibitor of the mammalian pol species is of great interest.

Specific assays were used to determine whether the tyrosol (1)-induced inhibition was dependent on the binding of tyrosol (1) to DNA or to the enzyme. The extent of the interaction between tyrosol (1) and dsDNA was investigated based on the thermal transition of dsDNA by measuring the melting temperature (*T*_m) of dsDNA in the presence of an excess amount of tyrosol (1) (200 μM) using a spectrophotometer equipped with a thermoelectric cell holder. A thermal transition of *T*_m was not observed within the compound concentration range used in the assay. In contrast, a typical intercalating compound used as a positive control (ethidium bromide, 15

μM) produced a clear thermal transition (data not shown). To determine whether the inhibitory effect of tyrosol (1) was caused by nonspecific adhesion to mammalian pols or its selective binding to specific sites, we proceeded to investigate whether an excessive amount of nucleic acid [poly(rC)] or protein [bovine serum albumin (BSA)] abrogated the effects of tyrosol (1). Poly(rC) and BSA had little to no effect on the pol-inhibitory activity of tyrosol (1) (data not shown), which suggested that this compound was binding selectively to the pol molecule. These observations indicated that tyrosol (1) was not acting as a DNA intercalating agent, and that it was exerting its inhibitory activity by binding directly to selected pol enzymes.

Effect of Tyrosol (1) and Its Analogues (2 and 3) on TPA-Induced Inflammation In Vivo. In our previous pol inhibitor studies, we observed a relationship between pol λ inhibitors and their ability to suppress TPA-induced inflammation.^{6,7,9,35,36} With this in mind, we used an in vivo mouse ear inflammatory test in the current study to examine the anti-inflammatory activities of tyrosol (1), 3-hydroxytyrosol (2), and 3,4-dihydroxyphenyl acetic acid (3). The application of TPA (0.5 μg) to the mouse ear induced edema resulted in a 241% increase in the weight of the ear disk 7 h after application. As shown in Figure 4, pretreatment with 500 μg of tyrosol (1) had

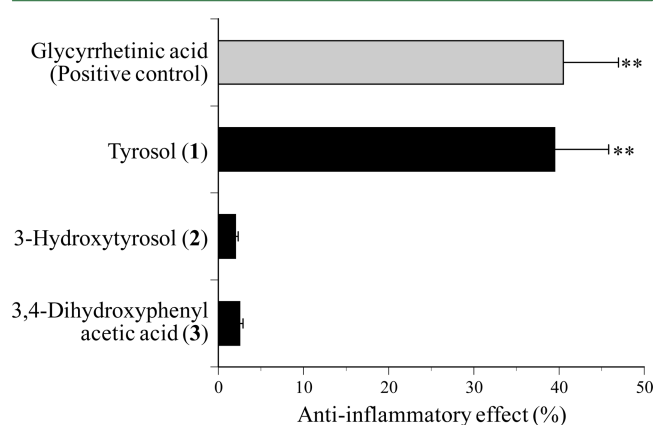


Figure 4. Anti-inflammatory activities of tyrosol (1) and its analogues (2 and 3) toward TPA-induced edema in a mouse ear model. Each compound (500 μg) was applied individually to one ear of a mouse, and TPA (0.5 μg) was applied 30 min later to both ears. Edema was evaluated after 7 h. The inhibitory effect is expressed as the percentage of edema. Data are shown as the mean values $\pm$ SD of six independent experiments. ** $P < 0.01$ versus controls.

a 55% anti-inflammatory effect, whereas 3-hydroxytyrosol (2) and 3,4-dihydroxyphenyl acetic acid (3) had no effect. The in vivo anti-inflammatory effects of these compounds therefore displayed a similar trend to that observed for their in vitro inhibitory effects toward mammalian pols (Table 2). The anti-inflammatory effect of glycyrrhetinic acid, which is a known anti-inflammatory agent, was very similar to that of tyrosol (1), and it was therefore concluded that tyrosol (1) was at least as effective as this benchmark compound.

Effect of Tyrosol (1) and Its Analogues (2 and 3) on Anti-Allergic Activity In Vivo. The IgE-mediated PCA reaction can be used as an in vivo tool to study the mechanism of the immediate hypersensitivity reaction. As shown in Figure 5, tyrosol (1) inhibited the PCA reaction in mice by 79.4%, when it was used at a dose of 100 mg/kg. In contrast, 3-hydroxytyrosol (2) and 3,4-dihydroxyphenyl acetic acid (3) had very little impact on the reaction. The antiallergic effects of

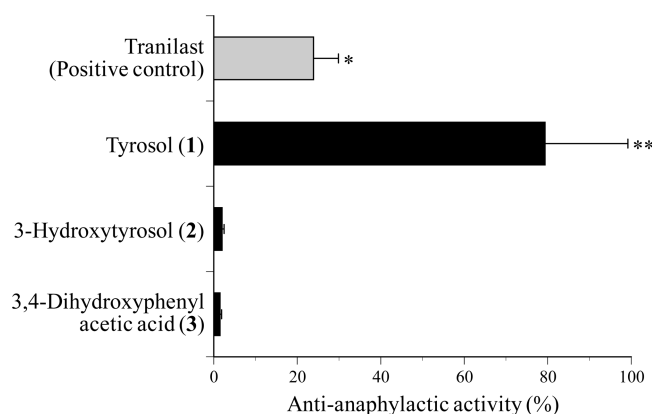


Figure 5. Antiallergic activities of tyrosol (1) and its analogues (2 and 3) measured by mouse PCA reaction. Each compound (100 mg/kg) was orally administered to the mice, and the IgE-dependent PCA reaction was investigated. Inhibition of anaphylactic activity by the vehicle control was taken as 0%. Data are shown as the mean values $\pm$ SD of six independent experiments. ** $P < 0.01$ and * $P < 0.05$ versus controls.

these compounds were therefore of the same order of magnitude as their in vitro inhibitory effects toward the mammalian pols (Table 2). Tranilast, which is a commonly used antiallergic drug that targets mast cell degranulation and inhibits the PCA reaction, caused 23.9% inhibition in this PCA reaction assay at a dose of 100 mg/kg (Figure 5). The inhibitory effect of tyrosol (1) was 3.3-fold greater than that of tranilast, which suggested that tyrosol could potentially be used as an antiallergic compound. Antiallergic effect by PCA experiment using rat of dorsal skin was same tendency as that using mouse ear (the inhibitory effect of 100 mg/kg tyrosol (1) was 2.6-fold greater than that of 100 mg/kg tranilast).

In addition to causing inflammation, TPA influences cell proliferation and exhibits tumor-promoting activity.¹² Anti-inflammatory agents are therefore expected to suppress DNA replication/repair/recombination in nuclei in relation to the action of TPA. Given that pol is related to repair/recombination,^{1–3} our finding that it is a molecular target of tyrosol (1), which selectively inhibited mammalian pol activity (Table 2), is in agreement with this expected mechanism of anti-inflammatory/antiallergic action. Recently, we found that there is the positive correlation between inflammatory cytokines induction, such as TNF- α by lipopolysaccharide and expression of pol λ ,³⁷ which has been implicated in 5'-deoxyribose-5-phosphate (dRP)-lyase activity,³⁸ V(D)J recombination,³⁹ translesion synthesis,⁴⁰ and base excision repair,⁴¹ in cultured mouse macrophage RAW264.7 cells; thus, not only DNA polymerization activity, but also the protein expression of pol λ is likely to be important in inflammation. However, detailed mechanisms describing the way in which tyrosol (1) inhibits mammalian pols, such as pol λ , as well as the way in which it elicits its anti-inflammatory effects, remain unclear, and further studies are currently underway in our laboratory to elucidate these mechanisms.

Sake-byproduct is formed during fermentation of *Orzya sativa* to produce the Japanese rice wine "sake". This contains fermentation products of fungus (*Aspergillus oryzae*) and yeast (*Saccharomyces cerevisiae*) using rice tissues such as endosperm, bran, and germ, and this byproduct is an unused resource containing novel bioactive compounds. Therefore, we focused on the absorption extract, collected from the used activated

charcoal, of sake-byproduct. Activated charcoal is a form of carbon that is processed in such a way as to introduce multiple small, low-volume pores that are designed to increase the surface area available for adsorption or chemical reactions. These pores can also bind to low molecular weight compounds containing benzene rings, although very little is known about the molecular mechanism involved in adsorption of different materials by activated charcoal. Tyrosol (**1**) is produced from tyrosine during the fermentation of yeast,⁴² and is therefore present in the fermented sake. Tyrosol (**1**) is a derivative of phenethyl alcohol, which is known as a natural phenolic antioxidant. As an antioxidant, tyrosol (**1**) can protect cells against injuries caused by oxidation.⁴³ To the best of our knowledge, this study represents the first reported account of tyrosol (**1**) being used to inhibit the activities of mammalian polys. These results suggest that the used activated charcoal generated as a waste product during sake production could be used as a good source of tyrosol (**1**) for its anti-inflammatory/antiallergic activities. This compound could also be used as an effective nutritional supplement because of its anti-inflammatory and antiallergic effects, which could lead to significant improvements in human health. The tyrosol (**1**)-enriched fraction of the used activated charcoal generated during the sake-brewing process, or purified tyrosol (**1**), could potentially be used in functional foods or cosmetics to deliver anti-inflammatory/antiallergic properties.

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Notes

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REFERENCES

- (1) Kornberg, A.; Baker, T. A. *DNA Replication*, 2nd ed.; Freeman, W. D., Ed.; University Science Books: Sausalito, CA, 1992; Chapter 6, pp 197–225.
- (2) Bebenek, K.; Kunkel, T. A. *DNA Repair and Replication*. In *Advances in Protein Chemistry*; Yang, W., Ed.; Elsevier: San Diego, CA, 2004; pp 137–165.
- (3) Hubscher, U.; Maga, G.; Spadari, S. Eukaryotic DNA polymerases. *Annu. Rev. Biochem.* **2002**, *71*, 133–163.
- (4) Lange, S. S.; Takata, K.; Wood, R. D. DNA polymerases and cancer. *Nat. Rev. Cancer* **2011**, *11*, 96–110.

- (5) Loeb, L. A.; Monnat, R. J., Jr. DNA polymerases and human disease. *Nat. Rev. Genet.* **2008**, *9*, 594–604.
- (6) Mizushina, Y. Specific inhibitors of mammalian DNA polymerase species. *Biosci. Biotechnol. Biochem.* **2009**, *73*, 1239–1251.
- (7) Mizushina, Y. Screening of novel bioactive compounds from food components and nutrients. *J. Jpn. Soc. Nutr. Food Sci.* **2011**, *64*, 377–384.
- (8) Mizushina, Y.; Kamisuki, S.; Kasai, N.; Ishidoh, T.; Shimazaki, N.; Takemura, M.; Asahara, H.; Linn, S.; Yoshida, S.; Koiwai, O.; Sugawara, F.; Yoshida, H.; Sakaguchi, K. Petasiphenol: A DNA polymerase λ inhibitor. *Biochemistry* **2002**, *41*, 14463–14471.
- (9) Mizushina, Y.; Hirota, M.; Murakami, C.; Ishidoh, T.; Kamisuki, S.; Shimazaki, N.; Takemura, M.; Perpelescu, M.; Suzuki, M.; Yoshida, H.; Sugawara, F.; Koiwai, O.; Sakaguchi, K. Some anti-chronic inflammatory compounds are DNA polymerase λ -specific inhibitors. *Biochem. Pharmacol.* **2003**, *66*, 1935–1944.
- (10) Hecker, E. *Carcinogenesis*; Raben Press: New York, 1978; pp 11–48.
- (11) Fujiki, H.; Sugimura, T. *Adv. Cancer Res.*; Academic Press, Inc.: London, UK, 1987; pp 223–264.
- (12) Nakamura, Y.; Murakami, A.; Ohto, Y.; Torikai, K.; Tanaka, T.; Ohgashi, H. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by a superoxide generation inhibitor 1'-acetoxychavicol acetate. *Cancer Res.* **1995**, *55*, 4832–4839.
- (13) Mitoshi, M.; Kuriyama, I.; Nakayama, H.; Miyazato, H.; Sugimoto, K.; Kobayashi, Y.; Jippo, T.; Kanazawa, K.; Yoshida, H.; Mizushina, Y. Effects of essential oils from herbal plants and citrus fruits on DNA polymerase inhibitory, cancer cell growth inhibitory, antiallergic, and antioxidant activities. *J. Agric. Food Chem.* **2012**, *60*, 11343–11350.
- (14) Tamai, K.; Kojima, K.; Hanaichi, T.; Masaki, S.; Suzuki, M.; Umekawa, H.; Yoshida, S. Structural study of immunoaffinity-purified DNA polymerase α -DNA primase complex from calf thymus. *Biochim. Biophys. Acta* **1988**, *950*, 263–273.
- (15) Date, T.; Yamaguchi, M.; Hirose, F.; Nishimoto, Y.; Tanihara, K.; Matsukage, A. Expression of active rat DNA polymerase β . *E. coli. Biochem.* **1998**, *27*, 2983–2990.
- (16) Umeda, S.; Muta, T.; Ohsato, T.; Takamatsu, C.; Hamasaki, N.; Kang, D. The D-loop structure of human mtDNA is destabilized directly by 1-methyl-4-phenylpyridinium ion (MPP⁺), a parkinsonism-causing toxin. *Eur. J. Biochem.* **2000**, *267*, 200–206.
- (17) Oshige, M.; Takeuchi, R.; Ruike, R.; Kuroda, K.; Sakaguchi, K. Subunit protein-affinity isolation of *Drosophila* DNA polymerase catalytic subunit. *Protein Expr. Purif.* **2004**, *35*, 248–256.
- (18) Kusumoto, R.; Masutani, C.; Shimmyo, S.; Iwai, S.; Hanaoka, F. DNA binding properties of human DNA polymerase η : Implications for fidelity and polymerase switching of translesion synthesis. *Genes Cells* **2004**, *9*, 1139–1150.
- (19) Braithwaite, E. K.; Kedar, P. S.; Stumpo, D. J.; Bertocci, B.; Freedman, J. H.; Samson, L. D.; Wilson, S. H. DNA polymerases β and λ mediate overlapping and independent roles in base excision repair in mouse embryonic fibroblasts. *PLoS One* **2010**, *5*, No. e12229.
- (20) Ohashi, E.; Murakumo, Y.; Kanjo, N.; Akagi, J.; Masutani, C.; Hanaoka, F.; Ohmori, H. Interaction of hREV1 with three human Y-family DNA polymerases. *Genes Cells* **2004**, *9*, 523–531.
- (21) Shimazaki, N.; Yoshida, K.; Kobayashi, T.; Toji, S.; Tamai, T.; Koiwai, O. Over-expression of human DNA polymerase λ in *E. coli* and characterization of the recombinant enzyme. *Genes Cells* **2002**, *7*, 639–651.
- (22) Sakaguchi, K.; Hotta, Y.; Stern, H. Chromatin-associated DNA polymerase activity in meiotic cells of lily and mouse. *Cell Struct. Funct.* **1980**, *5*, 323–334.
- (23) Uchiyama, Y.; Kimura, S.; Yamamoto, T.; Ishibashi, T.; Sakaguchi, K. Plant DNA polymerase λ , a DNA repair enzyme that functions in plant meristematic and meiotic tissues. *Eur. J. Biochem.* **2004**, *271*, 2799–2807.
- (24) Mizushina, Y.; Tanaka, N.; Yagi, H.; Kurosawa, T.; Onoue, M.; Seto, H.; Horie, T.; Aoyagi, N.; Yamaoka, M.; Matsukage, A.; Yoshida, S.; Sakaguchi, K. Fatty acids selectively inhibit eukaryotic DNA

polymerase activities in vitro. *Biochim. Biophys. Acta* **1996**, 1308, 256–262.

(25) Mizushima, Y.; Yoshida, S.; Matsukage, A.; Sakaguchi, K. The inhibitory action of fatty acids on DNA polymerase β . *Biochim. Biophys. Acta* **1997**, 1336, 509–521.

(26) Ogawa, A.; Murate, T.; Suzuki, M.; Nimura, Y.; Yoshida, S. Lithocholic acid, a putative tumor promoter, inhibits mammalian DNA polymerase β . *Jpn. J. Cancer Res.* **1998**, 89, 1154–1159.

(27) Nakayama, C.; Saneyoshi, M. Inhibitory effects of 9- β -D-xylofuranosyladenine 5'-triphosphate on DNA-dependent RNA polymerase I and II from cherry salmon (*Oncorhynchus masou*). *J. Biochem. (Tokyo)* **1985**, 97, 1385–1389.

(28) Yonezawa, Y.; Tsuzuki, T.; Eitsuka, T.; Miyazawa, T.; Hada, T.; Uryu, K.; Murakami-Nakai, C.; Ikawa, H.; Kuriyama, I.; Takemura, M.; Oshige, M.; Yoshida, H.; Sakaguchi, K.; Mizushima, Y. Inhibitory effect of conjugated eicosapentaenoic acid on human DNA topoisomerases I and II. *Arch. Biochem. Biophys.* **2005**, 435, 197–206.

(29) Mizushima, Y.; Dairaku, I.; Yanaka, N.; Takeuchi, T.; Ishimaru, C.; Sugawara, F.; Yoshida, H.; Kato, N. Inhibitory action of polyunsaturated fatty acids on IMP dehydrogenase. *Biochimie* **2007**, 89, 581–590.

(30) Soltis, D. A.; Uhlenbeck, O. C. Isolation and characterization of two mutant forms of T4 polynucleotide kinase. *J. Biol. Chem.* **1982**, 257, 11332–11339.

(31) Lu, B. C.; Sakaguchi, K. An endo-exonuclease from meiotic tissues of the basidiomycete *Coprinus cinereus*: Its purification and characterization. *J. Biol. Chem.* **1991**, 266, 21060–21066.

(32) Gschwendt, M.; Kittstein, W.; Furstenberger, G.; Marks, F. The mouse ear edema: A quantitatively evaluable assay for tumor promoting compounds and for inhibitors of tumor promotion. *Cancer Lett.* **1984**, 25, 177–185.

(33) Sato, H.; Kobayashi, Y.; Hattori, A.; Suzuki, T.; Shigekawa, M.; Jippo, T. Inhibitory effects of water-soluble low-molecular-weight β -(1,3–1,6) D-glucan isolated from *Aureobasidium pullulans* 1A1 strain black yeast on mast cell degranulation and passive cutaneous anaphylaxis. *Biosci. Biotechnol. Biochem.* **2012**, 76, 84–88.

(34) Chu, Y. H.; Chen, C. J.; Wu, S. H.; Hsieh, J. F. Inhibition of xanthine oxidase by *Rhodiola crenulata* extracts and their phytochemicals. *J. Agric. Food Chem.* **2014**, 62, 3742–3749.

(35) Mizushima, Y.; Hirota, M.; Murakami, C.; Ishidoh, T.; Kamisuki, S.; Shimazaki, N.; Takemura, M.; Perpelescu, M.; Suzuki, M.; Yoshida, H.; Sugawara, F.; Koiwai, O.; Sakaguchi, K. Some anti-chronic inflammatory compounds are DNA polymerase λ -specific inhibitors. *Biochem. Pharmacol.* **2003**, 66, 1935–1944.

(36) Mizushima, Y.; Takeuchi, T.; Kuramochi, K.; Kobayashi, S.; Sugawara, F.; Sakaguchi, K.; Yoshida, H. Study on the molecular structure and bio-activity (DNA polymerase inhibitory activity, anti-inflammatory activity and anti-oxidant activity) relationship of curcumin derivatives. *Curr. Bioactive Compounds* **2007**, 3, 171–177.

(37) Mizushima, Y.; Nishiumi, S.; Nishida, M.; Yoshida, H.; Azuma, T.; Yoshida, M. Inhibition of repair-related DNA polymerases by vitamin Ks, their related quinone derivatives and associated inflammatory activity (Review). *Int. J. Oncol.* **2013**, 42, 793–802.

(38) Ramadan, K.; Shevelev, I. V.; Maga, G.; Hubscher, U. DNA polymerase λ from calf thymus preferentially replicates damaged DNA. *J. Biol. Chem.* **2002**, 277, 18454–18458.

(39) Nick McElhinny, S. A.; Havener, J. M.; Garcia-Diaz, M.; Juarez, R.; Bebenek, K.; Kee, B. L.; Blanco, L.; Kunkel, T. A.; Ramsden, D. A. A gradient of template dependence defines distinct biological roles for family X polymerases in nonhomologous end joining. *Mol. Cell* **2005**, 19, 357–366.

(40) Maga, G.; Villani, G.; Crespan, E.; Wimmer, U.; Ferrari, E.; Bertocci, B.; Hubscher, U. 8-Oxo-guanine bypass by human DNA polymerases in the presence of auxiliary proteins. *Nature* **2007**, 447, 606–608.

(41) Markkanen, E.; van Loon, B.; Ferrari, E.; Parsons, J. L.; Dianov, G. L.; Hubscher, U. Regulation of oxidative DNA damage repair by DNA polymerase λ and MutYH by cross-talk of phosphorylation and ubiquitination. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, 109, 437–442.

(42) Ghosh, S.; Kebaara, B. W.; Atkin, A. L.; Nickerson, K. W. Regulation of aromatic alcohol production in *Candida albicans*. *Appl. Environ. Microbiol.* **2008**, 74, 7211–7218.

(43) Giovannini, C.; Straface, E.; Modesti, D.; Coni, E.; Cantafora, A.; De Vincenzi, M.; Malorni, W.; Masella, R. Tyrosol, the major olive oil biophenol, protects against oxidized-LDL-induced injury in Caco-2 cells. *J. Nutr.* **1999**, 129, 1269–1277.