An Ergosterol Peroxide, a Natural Product That Selectively Enhances the Inhibitory Effect of Linoleic Acid on DNA Polymerase β

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Department of Applied Biological Science, Science University of Tokyo,^a Noda, Chiba 278-8510, Japan, Laboratory of Cancer Cell Biology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine,^b Nagoya 466-8550, Japan, Third Section of Research and Development, Toyo Suisan Kaisha, Ltd.,^c Minato, Tokyo 108-0075, Japan, The Institute of Physical and Chemical Research (Riken),^d Wako, Saitama 351-0198, Japan and Laboratory of Cell Biology, Aichi Cancer Center Research Institute,^e Nagoya 464-0021, Japan.

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As described previously (Mizushina Y., Tanaka N., Yagi H., Kurosawa T., Onoue M., Seto H., Horie T., Aoyagi N., Yamaoka M., Matsukage A., Yoshida S., and Sakaguchi K., *Biochim. Biophys. Acta*, 1308, 256–262, 1996), linoleic acid (LA) inhibits the activities of mammalian DNA polymerases. We found a natural product from a basidiomycete, *Ganoderma lucidum*, that enhances this effect of LA in a special manner. The structure was identified to be an ergosterol peroxide, 5,8-epidioxy-5 α ,8 α -ergosta-6,22E-dien-3 β -ol by spectroscopic analyses. The ergosterol peroxide (EPO) itself scarcely inhibited the activities of calf thymus DNA polymerase α (pol. α) or rat DNA polymerase β (pol. β). However, when EPO at 0.25 mM was present, 10 μ M or less of LA almost completely inhibited the pol. β activity, while almost complete inhibition by LA itself was achieved at 80 μ M or higher. Interestingly, under the same conditions, EPO did not affect the LA-effect on pol. α . The action mode of the EPO was discussed.

Key words DNA polymerase; enzyme inhibitor; ergosterol peroxide; linoleic acid; basidiomycete; *Ganoderma lucidum*

Eukaryotic DNA polymerases are designated as α , β , γ , δ and ϵ , each responsible for different DNA syntheses.¹⁾ The current intense interest in understanding the precise *in vivo* role of the polymerases and of the factors controlling their activity has prompted us to undertake a major search for wide inhibitors of these enzymes.^{2–10)} The search for DNA polymerase β (pol. β) inhibitors is especially urgent, because the role of pol. β *in vivo* is still obscure. Although several pol. β inhibitors have been reported, including nucleotide analogs,^{11,12)} flavonoids,¹³⁾ sulfate- or sialic acid-containing glycolipids,^{14,15)} phospholipids^{16,17)} triterpenoids^{18,19)} and fatty acids,^{20–22)} more potent agents are still needed.

We have previously reported the establishment of an assay^{20,21)} to detect DNA polymerase inhibitors and have used it to screen microbial secondary metabolites.²¹⁾ In the present study, colonies of actinomycetes, fungi and mycelia of basidiomycetes were collected from field soil near our laboratory. All the mycelia from the actinomycetes (*ca.* 1500 strains), fungi (200 strains) and basidiomycetes (200 strains) were homogenized in a Waring blender, and their acetone-soluble extracts were evaluated in a DNA polymerase assay system. We found that strong inhibitory activity for the polymerases was present in the homogenate of the fruiting caps of a basidiomycete, *Ganoderma lucidum*, and the major component was identified as linoleic acid (LA). However, the greatest activity disappeared in the final step of the purification by high-performance liquid chromatography (HPLC). We therefore investigated other compounds separated in the final step of the HPLC which could enhance the inhibitory effect of the fatty acid, and found that 5,8-epidioxy-5 α ,8 α -ergosta-6,22E-dien-3 β -ol (EPO), one of the sterol peroxides which were isolated from marine invertebrates such as tunicate, coral, sponge and sea hare by Gunatilaka *et al.*, filled that role.²³⁾ The biological activities of EPO have not been

reported yet. The addition of EPO to a polymerase reaction mixture led to a decrease in the minimum inhibitory concentration (MIC) of LA against pol. β activity, but did not affect the inhibitory effect on DNA polymerase α (pol. α). We report here the isolation and spectroscopic analyses of EPO, as well as its biochemical action with and without LA.

MATERIALS AND METHODS

Materials Nucleotides and chemically synthesized template-primers such as poly(dA) and oligo(dT)_{12–18} were purchased from Pharmacia (Uppsala, Sweden). [³H]-deoxythymidine 5'-triphosphate (dTTP) (43 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, MA). All other reagents, including LA, were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan).

Enzymes and DNA Polymerase Assays The DNA polymerases and DNA metabolic enzymes used and the enzyme assay methods are the same as those described in previous reports.^{20–22)} EPO was dissolved in dimethyl sulfoxide (DMSO), and 4 μ l of the dissolved sample was mixed with 16 μ l of each enzyme (final 0.05 units) in 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 1 mM dithiothreitol and 50% glycerol, then kept at 0 °C for 10 min. Eight ml of each of the preincubated solutions was added to 16 μ l of each of the enzyme standard reaction mixtures, then each of the enzyme activities was measured.

Extraction and Purification of EPO from the Fruiting Caps of the Basidiomycete, *Ganoderma lucidum* The fruiting caps of the mushroom *Ganoderma lucidum* were purchased from Japan Microbe Chemical Co. (Tokyo, Japan). After homogenization in a Waring blender, the fruiting caps were extracted with acetone for 3 d. The evaporated extract

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Table 1. Fatty Acids in the Fruiting Caps of the Basidiomycete, *Ganoderma lucidum*

Fatty acid	Symbol	Composition rate (%)
<i>cis</i> -9 Tetradecenoic acid	14:1Δ9 <i>cis</i>	0.5
Hexadecanoic acid (Palmitic acid)	16:0	2.8
<i>cis</i> -9 Hexadecenoic acid (Palmitoleic acid)	16:1Δ9 <i>cis</i>	0.8
Octadecanoic acid (Stearic acid)	18:0	1.1
<i>cis</i> -9 Octadecenoic acid (Oleic acid)	18:1Δ9 <i>cis</i>	1.8
<i>cis</i> -9, <i>cis</i> -12 Octadecadienoic acid (LA)	18:2Δ9-12 <i>cis</i>	93.0

was partitioned between EtOAc and H₂O and adjusted to pH 7. The evaporated organic layer was subjected to silica gel column chromatography and then eluted with *n*-hexane/2-propanol (v/v 16:1). The active fractions were collected and then purified by preparative HPLC (on a YMC A-323 column (C₁₈-ODS) 10 i.d. × 250 mm in CH₃CN with the flow rate of 6.0 ml/min, detected with UV at 210 nm) to give a white powder.

Positive FAB-MS and high resolution (HR)-FAB-MS were run on a glycerol matrix, and were recorded on a JEOL JMS HX110 mass spectrometer. [M+H]⁺ obsd. *m/z* 429.3289; calcd. *m/z* 429.3368 for C₂₈H₄₅O₃. NMR measurements were performed on a Bruker AC-300 Plus spectrometer. ¹H and ¹³C spectra were recorded in CDCl₃ solution at 300 and 75 MHz, and the chemical shifts are given relative to tetramethylsilane (TMS); CDCl₃ solvent peaks are δ 0.00 and 77.0 ppm, respectively. ¹³C; δ (ppm) 135.4, 135.2, 132.3, 130.7, 82.1, 79.4, 6.5, 56.2, 51.6, 51.0, 44.5, 42.8, 39.7, 39.3, 36.9 (overlapped), 34.7, 33.1, 30.1, 28.6, 23.4, 20.9, 20.6, 19.6, 18.2, 17.1, 14.1, 12.9; ¹H; δ (ppm) 6.50 (1H, d, *J*=8.5 Hz), 6.25 (1H, d, *J*=8.5 Hz), 5.22 (1H, dd, *J*=7.1, 15.2 Hz), 5.14 (1H, dd, *J*=7.8, 15.2 Hz), 3.96 (1H, m), 3.49 (1H, s), 2.2—1.2 (20H, m), 1.01 (3H, d, *J*=6.6 Hz), 0.90 (3H, d, *J*=6.8 Hz), 0.88 (3H, s), 0.83 (3H, s), 0.83 (3H, d, *J*=6.9 Hz), 0.82 (3H, s), 0.81 (3H, d, *J*=6.7 Hz).

Figure 1 shows the chemical structure of the ergosterol peroxide, EPO (A) and the deoxidized form, ergosterol (B).

Fatty Acid Analysis of the Basidiomycete, *Ganoderma lucidum* The fatty acid fraction of fruiting caps of the basidiomycete *Ganoderma lucidum* was isolated by silica gel column chromatography using *n*-hexane/2-propanol (v/v 16:1). Gas chromatography analysis was performed with a Hewlett Packard 5890 Series II system equipped with a hydrogen flame ionization detector (FID) with flow rate of 1 ml/min on a Spelco Wax (df 0.2 μm) at 200—220 °C (2 °C/min).

RESULTS AND DISCUSSION

Presence of a New Compound That Facilitates the Inhibitory Effect of LA on Pol. β The basidiomycete *Ganoderma lucidum* is a medicinal mushroom used in traditional Chinese medicine, and has been called *reishi* or *mannentake*. The powder of the dried *reishi* has been used as a cancer chemotherapy agent in ancient China, and was used in the Imperial Court. We found potent activity in the fruiting caps of this mushroom in that it inhibits rat pol. β activity. We then tried to extract the effective fraction. The inhibitory agent was found in the final step of the purification to be fatty acids, as elucidated by gas chromatography analysis, which were mostly composed of LA (Table 1). Since we re-

Table 2. IC₅₀ Values for the Inhibition of DNA Polymerase Activities of EPO in Each of the Purification Steps

Purification step	IC ₅₀ value (μg/ml)	
	Calf pol. α	Rat pol. β
Acetone extract	25	30
EtOAc/H ₂ O partition	20	20
Silica gel column chromatography	10	0.7
HPLC peak I (LA)	8	12
HPLC peak II (EPO)	210	450

The standard reaction mixture for calf thymus pol. α (24 μl final volume) contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM MgCl₂, 10 μM poly(dA), 5 μM (dT)₁₂₋₁₈, 10 μM [³H]-dTTP (100 cpm/pmol), 15% (v/v) glycerol and 8 μl inhibitor-enzyme mixture solution. The standard reaction mixture for rat pol. β was the same reaction mixture as used above, but also contained 150 mM KCl. The purification samples were dissolved in DMSO and sonicated for 30 s. Four μl of the sonicated sample was mixed with 16 μl of each enzyme (final 0.05 units). These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme's standard reaction mixtures. After incubation at 37 °C for 60 min, the radioactive DNA products were collected on DEAE-cellulose paper (DE81) discs as described by Lindahl *et al.*,²⁴ and radioactivity was measured in a scintillation counter. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphates into synthetic template-primers (*i.e.* poly(dA)/oligo(dT)₁₂₋₁₈, A/T=2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme. Enzyme activity in the absence of an inhibitor was taken as 100%.

ported previously that longer-chain fatty acids than C₁₈ could be potent inhibitors of eukaryotic DNA polymerases,²⁰ we speculated that LA could itself be the inhibitor. However, the active fractions of *reishi* extract in each of the purification steps before the HPLC originally seemed to be a much stronger inhibitor for pol. β than the fatty acid (Table 2), so we further attempted to purify the inhibitor. The elution pattern of the final HPLC step of the purification showed an inhibitory-activity peak (peak I in Table 2), and it was LA. However, in the presence of 25 μM LA, another new inhibitor peak (peak II in Table 2) occurred in the elution pattern. The LA level used (25 μM) was one-third of its MIC. We therefore suspected that the *reishi* powder must contain something to help or to strengthen the inhibitory effect of LA. The remainder of this report describes the purification and characterization of the newly identified compound.

Purification of the *Reishi* Compound from the Fruiting Caps, and Its Chemical Structure The active compound from the fruiting caps of the mushroom was extracted and purified as described in Materials and Methods. The molecular formula C₂₈H₄₄O₃ was determined by positive HR-FAB-MS. In the ¹³C-NMR, two of four olefinic carbon signals at δ 135.4, 135.2, 132.3 and 130.7 ppm showed the presence of two olefins in its structure. The simple doublet olefin protons at δ 6.50 (*J*=8.5 Hz) and 6.25 (*J*=8.5 Hz) showed further *cis* olefin couplings by protons. The relative down-field shifted carbon signals at δ 82.1 and 79.4 ppm suggested the peroxide residue was related to the above olefin. The doublets of dou-

blets olefin protons at δ 5.22 ($J=7.1$, 15.2 Hz) and 5.14 ($J=7.8$, 15.2 Hz) implied *trans* olefin coupled with a methin proton, respectively. In accordance with the presence of six methyls and the molecular formula, the structure of the active compound was identified as ergosterol peroxide, EPO (Fig. 1A). EPO is a derivative of ergosterol (Fig. 1B). EPO is one of the nine rare sterols which were previously isolated from marine invertebrates such as tunicate, common pillar

coral, sponge and sea hare by Gunatilaka *et al.*²³⁾

Inhibition by EPO of the Activities of DNA Polymerases with or without LA As shown in Fig. 2, the inhibition by EPO itself was too weak to consider EPO as an inhibitor of calf pol. α (Fig. 2B) or rat pol. β (Fig. 2D).

However, when LA was present in the polymerase reaction mixture, the EPO inhibitory patterns were greatly changed (Fig. 2). As described previously,²⁰⁾ LA at 80 μ M or more nearly completely inhibited the activities of pol. α and pol. β , and at less than 25 μ M, LA lost such activity (Fig. 2A and C). Figure 2C shows the dose-curves of the effect of LA with 250 μ M of EPO on pol. β activity. The inhibition was dose-dependent, with 50% inhibition of pol. β observed at a dose of 3 μ M; almost complete inhibition (more than 80–90%) was achieved at 10 to 15 μ M. However, the weak pol. α -inhibitory activity by EPO at 250 μ M was revised with 5 μ M LA (Fig. 2A). To compare the effect of EPO with other similar compounds, we tested the enhancing effect of an ergosterol which is the deoxidized form of EPO (Fig. 1B). The ergosterol, despite structural similarity, did not have any effect on the activities of pol. α or pol. β (Fig. 2A and C). When dodecanoic acid (C_{12} -fatty acid) was used instead of LA (C_{18} -fatty acid), EPO had no such effect (Fig. 2B and D). However, LA and longer chain fatty acids did have the effect (data not shown).

EPO did not influence the activities of cauliflower DNA

Fig. 2. Inhibition of pol. α (A, B) and β (C, D) Activities by EPO, LA and the Mixture of Both

Ergosterol and dodecanoic acid were used as the control agents of EPO and LA, respectively. The standard reaction mixture for calf pol. α (24 μ l final volume) contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM $MgCl_2$, 10 μ M poly(dA), 5 μ M (dT)_{12–18}, 10 μ M [³H]-dTTP (100 cpm/pmol), 15% (v/v) glycerol and 8 μ l inhibitor-enzyme mixture solution. The standard reaction mixture for rat pol. β was the same reaction mixture as used above, but also contained 150 mM KCl. EPO and LA were dissolved in DMSO and sonicated for 30 s. Four μ l of the sonicated sample was mixed with 16 μ l of each enzyme (final 0.05 units). These inhibitor-enzyme mixtures (8 μ l) were added to 16 μ l of each of the enzyme's standard reaction mixtures. After incubation at 37 °C for 60 min, the radioactive DNA products were collected on DEAE-cellulose paper (DE81) discs, as described by Lindahl *et al.*²⁴⁾ and radioactivity was measured in a scintillation counter. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphates into synthetic template-primers (*i.e.* poly(dA)/oligo(dT)_{12–18}, A/T=2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme. Enzyme activity in the absence of an inhibitor was taken as 100%.

Table 3. The effect of EPO, LA and the Mixture of Both on the Activities of Various DNA Polymerases and Other DNA-Metabolic Enzymes

Enzyme	EPO (250 μM)	LA (25 μM)	EPO (250 μM) + LA (25 μM)	EPO/(EPO+LA)
Calf pol. α	74	53	59	1.25
Rat pol. β	90	65	5	18
Plant pol. II (β like)	103	100	101	1.02
HIV-RT	160	101	175	0.91
<i>E. coli</i> pol. I (Klenow fragment)	111	98	101	1.10
Taq pol.	108	98	105	1.03
T4 pol.	110	104	106	1.04
Calf TdT	118	96	108	1.09
Bovine DNase I	104	100	106	0.98

% of relative activity. The standard reaction mixture for calf pol. α , *E. coli* pol. I (Klenow fragment), Taq pol., T4 pol., and calf thymus TdT (24 μl final volume) contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM MgCl_2 , 10 μM poly(dA), 5 μM (dT)₁₂₋₁₈, 10 μM [³H]-dTTP (100 cpm/pmol), 15% (v/v) glycerol and 8 μl of an inhibitor-enzyme mixture solution. The standard reaction mixture for rat pol. β and plant pol. II (β -like) was the same reaction mixture as used above, but also contained 150 mM KCl. The standard reaction mixture for HIV-RT contained 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM MgCl_2 , 10 μM poly(rA), 5 μM (dT)₁₂₋₁₈, 10 μM [³H]-dTTP (100 cpm/pmol), 15% (v/v) glycerol, 80 mM KCl and 8 μl of an inhibitor-enzyme mixture solution. EPO and LA were dissolved in DMSO and sonicated for 30 s. Four μl of the sonicated sample was mixed with 16 μl of each enzyme (0.05 units). These inhibitor-enzyme mixtures (8 μl) were added to 16 μl of each of the enzyme's standard reaction mixtures. After incubation at 37 °C for 60 min, except Taq pol., which was incubated at 74 °C for 60 min, the radioactive DNA products were collected on DEAE-cellulose paper (DE81) discs as described by Lindahl *et al.*,²⁴ and radioactivity was measured in a scintillation counter. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleoside triphosphates into synthetic template-primers (*i.e.* poly(dA)/oligo(dT)₁₂₋₁₈, A/T=2/1) in 60 min at 37 °C under the normal reaction conditions for each enzyme. Bovine DNase I activity was measured as described previously.²⁵ Enzyme activity in the absence of an inhibitor was taken as 100%.

Fig. 3. Kinetic Analysis of the Inhibition of pol. β (0.05 units) by EPO, LA and the Mixture of Both

The effects of the mixture of EPO and LA on the K_m and V_{max} values of the DNA template-primer and nucleotide substrate were determined, and the results are displayed as Lineweaver-Burk plots. (A) Pre-incubation with pol. β in the presence of 100 μM EPO (●), 5 μM LA (▲), 250 μM EPO and 5 μM LA (◆) and 100 μM EPO and 25 μM LA (▼). The pol. β activity was then assayed using the concentrations of poly(dA)/oligo(dT) (2/1) indicated as the template/primer. The pol. β activity in the absence of the inhibitors (■). (B) The pol. β activity was assayed with the concentrations of substrate (dTTP) indicated, after the pre-incubation of pol. β without (■) or with 100 μM EPO (●), 5 μM LA (▲), 250 μM EPO and 5 μM LA (◆) and 100 μM EPO and 25 μM LA (▼).

polymerase II (plant pol. II), the prokaryotic DNA polymerases, *i.e.*, the Klenow fragment of DNA polymerase I (pol. I), T4 DNA polymerase (T4 pol.) and Taq polymerase (Taq pol.), the DNA metabolic enzyme bovine deoxyribonuclease I (DNase I), calf thymus terminal deoxynucleotidyl transferase (TdT) or the human immunodeficiency virus type 1 reverse transcriptase (HIV-RT) at all (Table 3). LA with EPO also did not influence the activities of pol. α , plant pol. II, the prokaryotic DNA polymerases, DNase I, TdT or HIV-RT (Table 3), suggesting that LA in coexistence with EPO changes to a potent inhibitor specific to the mammalian pol. β *in vitro*. An excess amount of LA did not affect these non-pol. β type EPO-effects (data not shown). EPO appears to contribute only to the pol. β inhibition by LA, but does not affect the inhibition by LA of other enzymes.

Mode of DNA Polymerase β Inhibition by EPO To elucidate the mechanism of the EPO effect on the pol. β inhibition, the extent of inhibition as a function of DNA template-primer or nucleotide substrate concentration was studied (Fig. 3). In the kinetic analysis, poly(dA)/oligo(dT)₁₂₋₁₈,

A/T=2/1 and nucleotide dTTP were used as the DNA template-primer and substrate, respectively. Double reciprocal plots of the results show that the inhibition of pol. β by the mixture of EPO and LA was intermediate, between competitive and non-competitive reactions with either the DNA template (Fig. 3A) or substrate (Fig. 3B). The K_m (Michaelis constant) for the DNA template increased from 0.53 to 0.83 μM in the presence of EPO and LA (Fig. 3A), while its V_{max} (maximum velocity) decreased from 50 to 22 pmol/h in the presence of EPO and LA (Fig. 3A). Similarly, the apparent K_m for the substrate dTTP was increased by 2.5-fold in the presence of EPO and LA (Fig. 3B), whereas a 48% decrease in the V_{max} was observed in the presence of EPO and LA (Fig. 3B). Since LA competitively inhibited pol. β , as described previously,²⁰ and since EPO itself weakly and non-competitively inhibited the pol. β activity (data not shown), these two reaction modes may be mixed. As described previously,²⁰⁻²² LA may interact with or affect both of the binding sites on pol. β , thereby decreasing its affinity for the DNA template and substrate. EPO may therefore enhance the

inhibitory effect of LA by interacting with an other domain distinct from the template-binding and catalytic sites on pol. β .

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