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Hymenoic acid, a novel specific inhibitor of human DNA polymerase λ from a fungus of *Hymenochaetaceae* sp.

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Abstract—Hymenoic acid (**1**) is a natural compound isolated from cultures of a fungus, *Hymenochaetaceae* sp., and this structure was determined by spectroscopic analyses. Compound **1** is a novel sesquiterpene, *trans*-4-[(1′*E*,5′*S*)-5′-carboxy-1′-methyl-1′-hexenyl]cyclohexanecarboxylic acid. This compound selectively inhibited the activity of human DNA polymerase λ (pol λ) in vitro, and 50% inhibition was observed at a concentration of 91.7 μM. Compound **1** did not influence the activities of the other seven mammalian pols (i.e., pols α, γ, δ, ε, η, ι and κ), but also showed no effect even on the activity of pol β, which is thought to have a very similar three-dimensional structure to the pol β-like region of pol λ. This compound also did not inhibit the activities of prokaryotic pols and other DNA metabolic enzymes tested. These results suggested that compound **1** could be a selective inhibitor of eukaryotic pol λ. This compound had no inhibitory activities against two N-terminal truncated pol λ, del-1 pol λ (lacking nuclear localization signal (NLS), BRCA1 C-terminus (BRCT) domain [residues 133–575]), and del-2 pol λ (lacking NLS, BRCT domain and proline-rich region [residues 245–575]). The compound **1**-induced inhibition of intact pol λ activity was non-competitive with respect to both the DNA template-primer and the dNTP substrate. On the basis of these results, the pol λ inhibitory mechanism of compound **1** is discussed.

1. Introduction

The human genome encodes 16 DNA polymerases (pols) to conduct cellular DNA synthesis.[1] Eukaryotic cells reportedly contain three replicative types: pols α, δ, and ε, mitochondrial pol γ, and at least twelve repair types: pols β, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ and REV1.[2] We have searched for natural compounds that selectively inhibit each of these eukaryotic pols, to use as tools and molecular probes to distinguish pols and to clarify their biological and in vivo functions.[3–10] In this study, we report a newly found compound that selectively inhibits only the activity of pol λ. The natural compound is a novel sesquiterpene, hymenoic acid (**1**), produced by a fungus (*Hymenochaetaceae* sp.) (Fig. 1). To our knowledge, there have been no reports about such natural inhibitors specific to X-family pols such as β, λ and μ, except for solanapyrone A as a pol β- and λ-inhibitor,[7] and prunasin as a pol β-inhibitor,[5] which we reported previously. The compound differed from solanapyrone A in that it inhibited only pol λ among the pols examined to date.[7] No such pol λ-specific inhibitors have been reported.

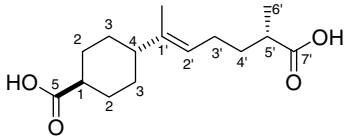

Figure 1. Structure of compound **1** (hymenoic acid).

Keywords: Hymenoic acid; Sesquiterpene; DNA polymerase λ; Family X of DNA polymerases; Enzyme inhibitor.
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Pol λ is a recently described eukaryotic pol belonging to the pol X family comprising enzymes involved in DNA repair processes, whose main member is pol β.[11] Human pol λ (63.4-kDa) consists of a nuclear localization signal (NLS) (residues 1–35), a BRCA1 C-terminus (BRCT) domain (residues 36–132), a proline-rich region (residues 133–244) and a pol β-like region containing a helix–hairpin–helix (HhH) and pol X motif (residues 245–575). The C-terminal part of pol λ (residues 244–575) is composed of a catalytic core which is similar to pol β.[12] A truncated pol λ, in which the BRCT motif was deleted from the N-terminal region (i.e., the C-terminal region including the pol β-like region), has pol activity.

In this paper, we report the isolation and structural determination of a new sesquiterpene, hymenoic acid (**1**). We discuss the inhibitory action of compound **1** on various eukaryotic pols and DNA metabolic enzymes and its binding relation to the enzyme structure of pol λ.

2. Results

2.1. Isolation and cultivation of fungus

The fungal strain, HJ31A, was isolated from a coral collected from Hachijo Island, Japan. The fungus was selected by culturing coral fragments on potato dextrose agar plates (Difco) and was transferred several times. The culture was incubated at 25 °C. This fungus was identified as *Hymenochaetaceae* sp. by TechnoSuruga Laboratory Co. Ltd (Shizuoka, Japan). A small agar plug was then transferred into a 2 L Erlenmeyer flask containing 1 L of a culture 24 g potato dextrose broth (Difco). Cultures of HJ31A strain (5 L) were grown for 49 days without shaking in the dark.

2.2. Extraction and purification of pol λ inhibitor from fungi

The fungal mycelia of HJ31A strain (*Hymenochaetaceae* sp.) were removed from the culture broth by filtering through cheesecloth. The filtrate was extracted with CH_2Cl_2. The organic layer was evaporated in vacuo to obtain 189 mg crude residue. This crude extract was separated by silica gel column chromatography with toluene/EtOAc (1:1–1:4, with 2% acetic acid) to give an active fraction, which inhibited pol activity (i.e., minimum inhibitory concentration was less than 1 mg/ml). The active fraction was concentrated in vacuo to yield compound **1** (11.5 mg).

2.3. Structure determination of pol λ inhibitor

The molecular formula of **1** was determined by high resolution electrospray ionization mass spectrometry to be $C_{15}H_{24}O_4$. ^1H and ^{13}C NMR data for **1** are summarized in Table 1. COSY and HMBC spectra suggested that **1** has 1,4-disubstituted cyclohexane and 6-substituted-2-methyl-5-heptanonyl moiety as partial structures (Fig. 2A). The IR absorption at 1705 cm^{-1} and two carbon resonances at δ 180.8 and 180.1 (C-7′ and C-5, respectively) showed the presence of two carboxy groups. Taken together, the structure of **1** was determined to be 4-(-5′-carboxy-1′-methyl-1′-hexenyl)cyclohexanecarboxylic acid.

Table 1. ^1H and ^{13}C NMR spectral data of compound **1** (hymenoic acid)

Position	1	
	δ_H (*J* Hz)	δ_C
1	2.20 (tt, 12.1, 3.8)	44.4
2	2.05–1.99 (m)	30.5
	1.48–1.37 (m)	
3	1.78–1.70 (m)	32.1
	1.34–1.21 (m)	
4	1.83 (tt, 11.8, 3.1)	47.9
5		180.1
1′		141.4
1′-Me	1.58 (s)	14.4
2′	5.16 (t, 7.0)	123.3
3′	2.06–1.99 (m)	26.4
4′	1.72–1.62 (m)	35.0
	1.48–1.37 (m)	
5′	2.44–2.33 (m)	40.2
6′	1.13 (d, 7.0)	17.7
7′		180.8

Recorded in CD_3OD for the residual solvent peak as an internal standard, and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; t, triplet; tt, triplet of triplets; m, multiplet.

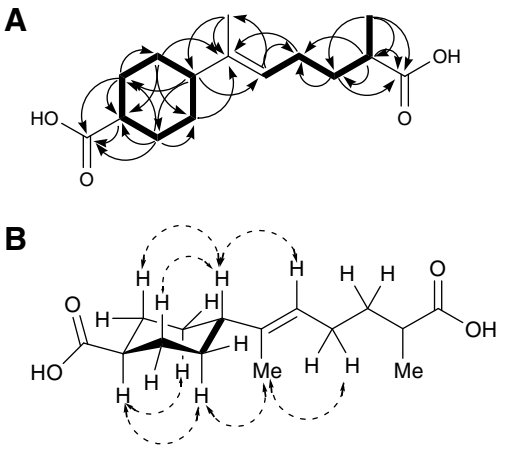


Figure 2. (A) Selected COSY (bold lines) and HMBC ($^1H \rightarrow {}^{13}C$) (arrows) correlations and (B) key NOESY (dashed lines) correlations in compound **1** (hymenoic acid).

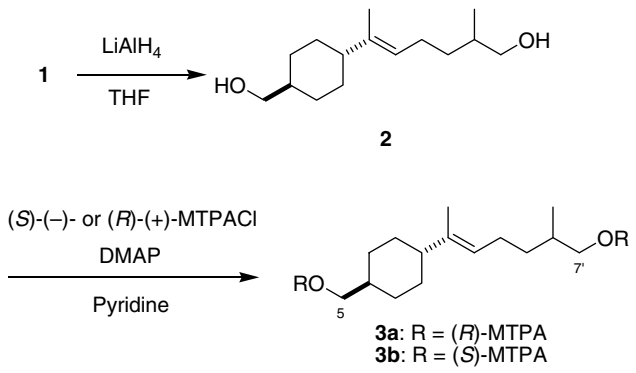
The relative stereochemistry of **1** was determined by NOESY analysis (Fig. 2B). NOESY correlations between axial H-1 and axial H-3 protons, and those between axial H-4 and axial H-2 protons indicated a 1,4-*trans* relationship between the substituents bonded to the cyclohexane ring. The geometry of the double bond at C-1′ was determined to be in an *E*-orientation by NOESY correlations between H-4 and H-2′, and between 1′-Me and H-3′ protons.

Compound **1** was an optically active compound, since the optical rotation value of **1** was $[\alpha]_D^{22}$ +16.3 (*c* 1.0, CH_3OH). To elucidate the absolute configuration at

C-5′, compound **1** was reduced to the corresponding diol **2**, which was converted to its Mosher esters **3** (Scheme 1). The absolute configuration at C-5′, where a methyl group was located, was elucidated on the basis of chemical shift differences and signal patterns of the two geminal protons at C-5′.[13,14] Reduction of **1** with $LiAlH_4$ in THF gave **2**. Esterification of **2** with (*S*)- and (*R*)-MTPACl gave bis-(*R*)- and bis-(*S*)-MTPA esters (**3a** and **3b**, respectively). Absolute configuration at C-5′ was deduced by differences in the chemical shift and the signal pattern of the two geminal protons at C-7′ in **3a** and **3b**.[13,14] CH_2 protons at C-7′ in **3a** appeared as adjacent signals at δ 4.16 (Fig. 3A). On the other hand, CH_2 protons at C-7′ in **3b** appeared as two separate signals at δ 4.24 and δ 4.08 (Fig. 3B). These results indicates that the absolute configuration at C-5′ was *S*. Thus, compound **1** was determined to be *trans*-4-[(1′*E*,5′*S*)-5′-carboxy-1′-methyl-1′-hexenyl]cyclohexanecarboxylic acid, and named hymenoic acid.

2.4. Effects of compound 1 on the activities of mammalian DNA polymerases and other DNA metabolic enzymes

First, the isolated compound **1** was investigated as to whether it inhibits the activities of nine mammalian pols, pols α to κ. As shown in Figure 4A, compound **1** at 200 μM inhibited the activity of human pol λ, but had no influence at all on the activities of nuclear replicative pols such as calf pol α, human pol δ and human pol ε, human mitochondrial replicative pol γ, or repair-related pols such as rat pol β, human pols η, ι and κ. This compound also inhibited the activity of plant (i.e., rice) pol λ to the same extent as it inhibited human pol λ. Compound **1** had no inhibitory effect on fish (i.e., cherry salmon) pol δ, insect (i.e., fruit fly) pols α, δ and ε, plant (i.e., cauliflower) pol α, or prokaryotic pols such as the Klenow fragment of *Escherichia coli* pol I, T4 pol and Taq pol. The compound did not inhibit the activities of other DNA metabolic enzymes, such as calf primase of pol α, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, human telomerase, T7 RNA polymerase, mouse inosine 5′-monophosphate (IMP) dehydrogenase (type II), human topoisomerases I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I either (Fig. 4B). These results suggested that compound **1** could selectively inhibit the activity of eukaryotic pol λ.



Scheme 1. Synthesis of MTPA esters (**3a** and **3b**).

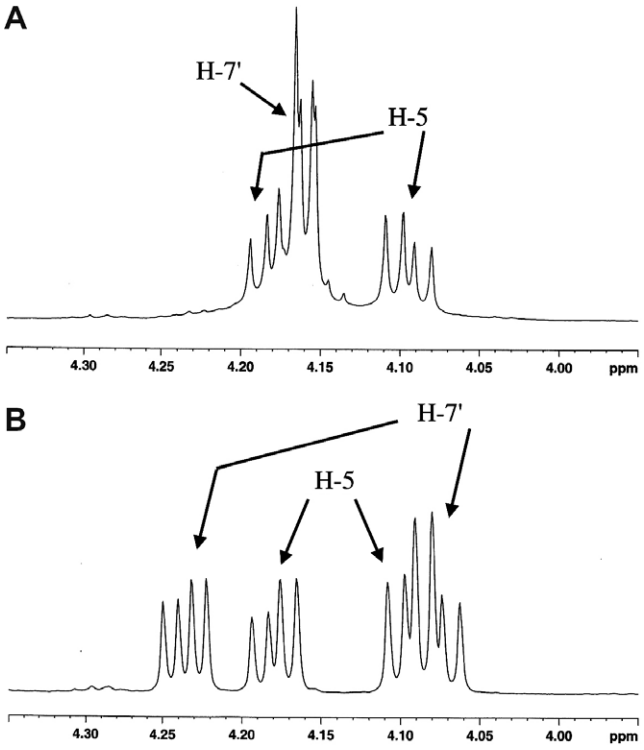


Figure 3. (A) Proton signals of H-5 and H-7′ in bis-(*R*)-MTPA ester (**3a**) and (B) proton signals of H-5 and H-7′ in bis-(*S*)-MTPA ester (**3b**).

2.5. Inhibitory effect of compound 1 on full-length or fragments of human pol λ

Figure 5 shows the inhibition dose–response curves of compound **1** against intact or truncated human pol λ. Full-length pol λ (residues 1–575) and the N-terminal deleted versions, del-1 pol λ (133–575) and del-2 pol λ (245–575), were prepared. Since a C-terminal pol β-like region containing pol X motif in pol λ is essential for DNA polymerization activity,[12] these truncated pol λ have pol activity. The inhibition of intact pol λ was dose-dependent, with 50% inhibition observed at doses of 91.7 μM. On the other hand, compound **1** did not influence the activity of two truncated pol λ, del-1 (lacking NLS and BRCT domain) and del-2 (lacking NLS, BRCT domain and proline-rich region). These results suggested that the N-terminal region of pol λ might be important for inhibition of the activity of DNA polymerization by compound **1**.

2.6. Mode of inhibition of pol λ by compound 1

Next, to elucidate the mechanism by which compound **1** inhibited full-length pol λ (residues 1–575), the extent of inhibition as a function of the DNA template-primer or dNTP substrate concentration was studied (Table 2). In kinetic analysis, poly(dA)/oligo$(dT)_{12-18}$ and deoxythymidine 5′-triphosphate (dTTP) were used as the synthesized DNA template-primer and deoxynucleoside 5′-triphosphate (dNTP) substrate, respectively. Double reciprocal plots of the results showed that the com-

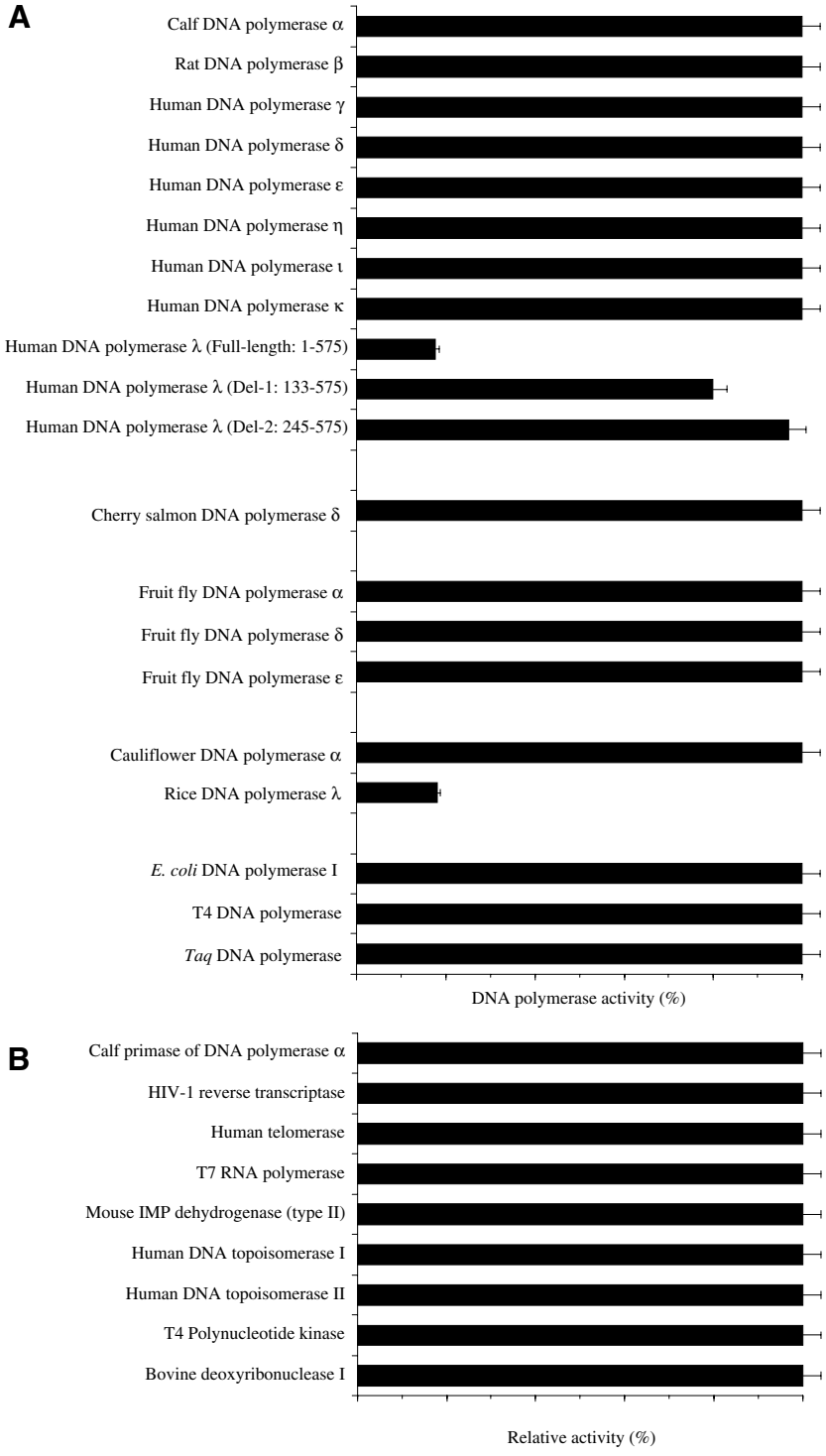


Figure 4. Effect of compound **1** (hymenoic acid) on the activities of various DNA polymerases and other DNA metabolic enzymes. (A) Pols from mammals and various species, (B) other DNA metabolic enzymes. Compound **1** (200 μM each) was incubated with each enzyme (0.05 U). % of relative activity. Enzymatic activity was measured as described previously.[3,4,6,22] Enzyme activity in the absence of the compound was taken as 100%. Data are shown as means ± SEM of four-independent experiments.

pound **1**-induced inhibition of intact pol λ activity was non-competitive with respect to both the DNA template-primer and the dNTP substrate. For the DNA template-primer, the apparent Michaelis constant (K_m) was unchanged at 2.38 μM, whereas 75.0, 58.6 and 48.9% decreases in maximum velocity (V_{max}) were observed in the presence of 20, 40 and 60 μM of compound **1**, respectively. The K_m for the dNTP substrate was unchanged at 1.18 μM, and the V_{max} for the dNTP substrate decreased from 52.6 to 27.0 pmol/h in the presence of 60 μM of compound **1**. Inhibition constant (K_i) values, obtained from Dixon plots, were found to

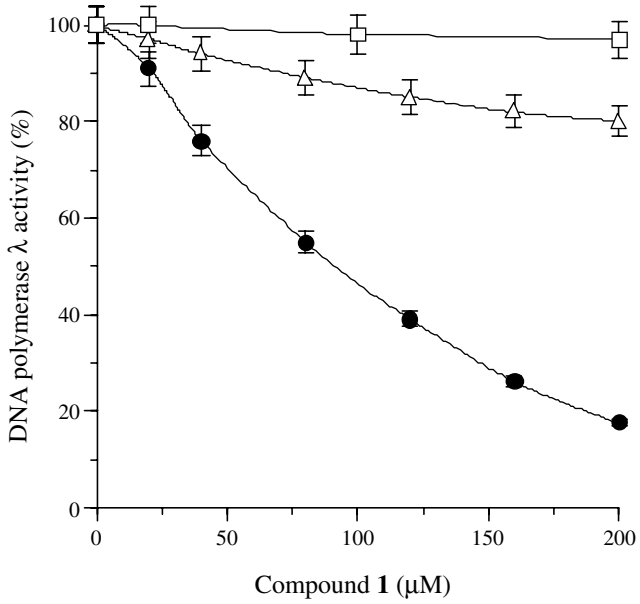


Figure 5. Dose–response curves of compound **1** (hymenoic acid) for human pol λ inhibition. The enzymes used (0.05 U each) were full-length (residues 1–575) human pol λ (closed circle), del-1 (residues 133–575) of human pol λ (open triangle), and del-2 (residues 245–575) of human pol λ (open square). Pol activities were measured as described in Section 4. Pol activity in the absence of the compound was taken as 100%. Data are shown as means ± SEM of three-independent experiments.

Table 2. Kinetic analysis of the inhibitory effects of Compound **1** (hymenoic acid) on the activity of human DNA polymerases λ (full-length, residues 1–575) as a function of the DNA template-primer dose and the nucleotide substrate concentration

DNA substrate	Compound **1** (μM)	K_m[a]	V_{max}[a] (μM)	K_i[b] (pmol/h)	Inhibitory mode[a] (μM)
Template-primer[c]	0	2.38	83.3	52.8	Non-competitive
	20		62.5		
	40		48.8		
	60		40.8		
Nucleotide[d] substrate	0	1.18	52.6	44.7	Non-competitive
	20		40.0		
	40		32.3		
	60		27.0		

[a] These data were obtained from Lineweaver–Burk plot.
[b] These data were obtained from Dixon plot.
[c] That is, poly(dA)/oligo(dT)$_{12-18}$.
[d] That is, dTTP.

be 52.8 and 44.7 μM for the DNA template-primer and dNTP substrate, respectively. Because the K_i value was smaller for the dNTP substrate than for the DNA template-primer, the affinity of compound **1** is greater for the enzyme–nucleotide substrate binary complex than for the enzyme–DNA template-primer complex. When activated DNA (i.e., DNA with gaps digested by bovine dexoyribonuclease I) and four dNTPs were used as the DNA template-primer and dNTP substrates, respectively, the mode of inhibition of pol λ by compound **1** was the same as that with the synthesized DNA template-primer (data not shown).

3. Discussion

As described in this report, we found a novel potent inhibitor specific to human pol λ from a fungus, *Hymenochaetaceae* sp. This natural compound was found to be a sesquiterpene, hymenoic acid (**1**).

Pol λ belongs to the pol X family,[11,15,16] which includes pols β, λ, μ, terminal deoxynucleotidyltransferase, yeast pol IV, mitochondrial pol β, nuclear pol β from protozoans and 20-kDa aflican swine fever virus pol X.[17–23] Family X of pols is composed of a NLS, a BRCT domain, a proline-rich region, and a pol β-like region containing two HhHs and a pol X motif (Fig. 6). However, pol β (residues 1–335) lacks an NLS, a BRCT domain and a proline-rich region. Human pol λ shares 54%, 47% and 30% homology to human pol β, μ and yeast pol IV, respectively. Interestingly, compound **1** inhibited only the activities of full-length pol λ (residues 1–575) among the eukaryotic pols and other DNA metabolic enzymes tested, although it did not inhibit the activities of the N-terminal deleted pol λ, such as del-1 (residues 133–575) and del-2 of pol λ (residues 245–575), or pol β (Fig. 5). The compound **1**-induced inhibition of intact pol λ was non-competitive with respect to both the DNA template-primer and the dNTP substrate (Table 2), indicating that compound **1** did not directly bind to either the DNA template-binding site or the dNTP substrate-binding site of pol λ. Since both the DNA template-primer and dNTP substrate-binding sites are present at pol X motif in the pol β-like region of the C-terminal pol λ,[11] compound **1** must not directly bind to the pol β-like core. These results suggested that compound **1** might bind to the N-terminal region of pol λ (residues 1–132) including the NLS and the BRCT domain directly, and subsequently inhibited enzymes having the pol β-like region of pol λ (Fig. 6).

Although the biochemical function of pol λ is unclear as yet, pol λ appears to work in a similar manner to pol β.[22] Pol β, which is widely known to have roles in the short-patch base excision repair (BER) pathway,[24–29] plays an essential role in neural development.[30] Recently, pol λ was found to contain 5′-deoxyribose-5-phosphate (dRP) lyase activity, but no apurinic/apyrimidinic (AP) lyase activity,[28] and to be able to substitute pol β in in vitro base excision repair (BER), suggesting that pol λ also participates in BER. Northern blot analysis indicated that transcripts of pol β were abundantly expressed in the testis, thymus and brain in rats,[31] but pol λ was efficiently transcribed mostly in the testis.[11] The reason why the testis and thymus require pol β activity has been suggested; both organs have DNA repair and recombination systems for meiotic crossing over and immunoglobulin production,[32,33] and the systems require the polymerase. The roles of pol β in the brain are unknown as yet; therefore, pol λ as well as pol β may also have a role in the testis. Since the DNA repair system at meiotic prophase requires pol β activity, the system must contain a process similar to BER. The system may also require pol λ activity, and pol λ may be an essential enzyme for nucleotide excision repair (NER). In this connection, the fact that the molecular target of the bio-antimutagen

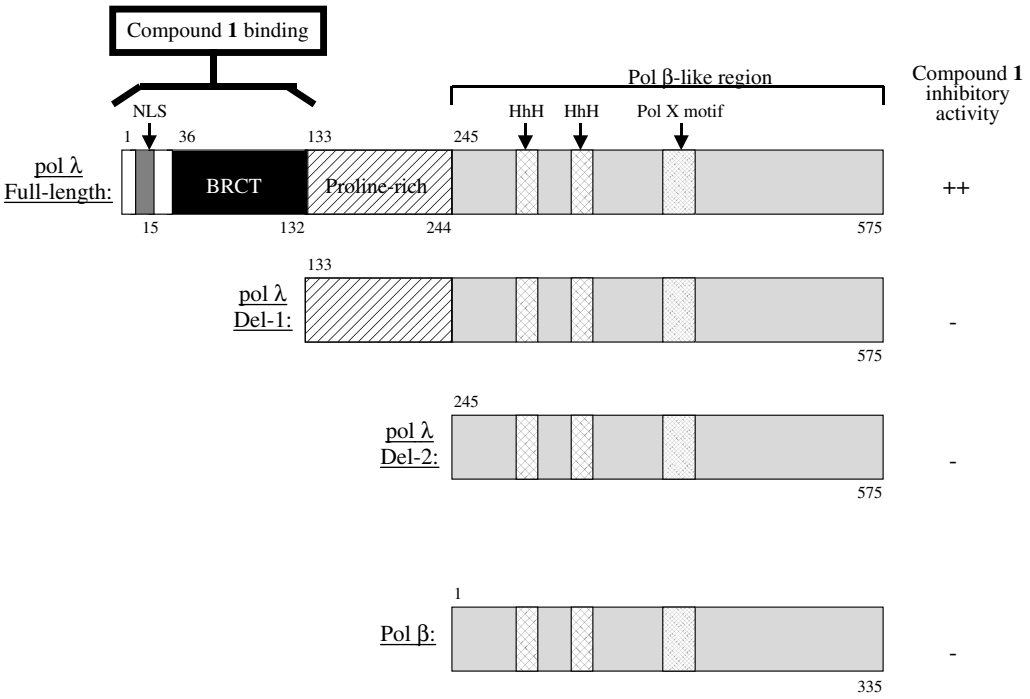


Figure 6. Schematic representation of pol λ and β of the pol X family. The NLS, BRCT domain, proline-rich region, HhH and pol X motif are indicated. The pol β-like region includes two HhHs and a pol X motif. The inhibitory activity of compound **1** against these pols is indicated below, '++' is IC_{50} value = <100 μM, '+' is IC_{50} value = 100 to 200 μM, and '−' is IC_{50} value = >200 μM.

was a pol λ inhibitor is of great interest. Bio-antimutagen may lead to blockage of the mis-match error in BER, NER and translesion synthesis of DNA-damaged cells. To determine why a bio-antimutagen is a pol λ-specific inhibitor, we are at present analyzing the structure and function of pol λ using an inhibitor.

Compound **1** could potently inhibit only the activity of human and plant pol λ, and the inhibitory effect of compound **1** on pol λ from other species was not tested; therefore, we will try to investigate it in further study. Since pol species specificity was extremely high, this sesquiterpene could be a useful molecular tool as a pol λ-specific inhibitor in studies to determine the precise roles of pol λ in vitro, and also might be useful to develop a drug design strategy for cancer chemotherapy agents for clinical radiation therapy or cancer chemotherapy.

4. Experimental

4.1. Materials

Nucleotides and chemically synthesized DNA template-primers such as poly(dA), oligo(dT)$_{12–18}$, and [^{3}H]dTTP (43 Ci/mmol) were purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan).

4.2. DNA polymerase and other DNA metabolic enzymes assays

Pols from mammals, a fish (i.e., cherry salmon), an insect (i.e., fruit fly) and a plant (i.e., cauliflower) were purified, and prokaryotic pols and other DNA metabolic enzymes were purchased as described in our previous report.[3,4,6] The intact and truncated human pol λ (full-length, del-1 and del-2 of pol λ) was constructed as described by Shimazaki et al.[34] The activities of all pols and other DNA metabolic enzymes were measured as described in previous reports.[3,4,6,35] The substrates of the pols were poly(dA)/oligo(dT)$_{12–18}$ and dTTP as the DNA template-primer and dNTP substrate, respectively. Compound **1** was dissolved in dimethylsulfoxide (DMSO) at various concentrations and sonicated for 30 s. The sonicated samples (4 μl) were mixed with 16 μl of each pol enzyme (final amount, 0.05 U) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol and 0.1 mM EDTA, and kept at 0 °C for 10 min. These inhibitor–enzyme mixtures (8 μl) were added to 16 μl of each standard enzyme reaction mixture, and incubation was carried out at 37 °C for 60 min, except for Taq pol, which was incubated at 74 °C for 60 min. Activity without the inhibitor was considered to be 100%, and the remaining activity at each concentration of the inhibitor was determined relative to this value. One unit of pol activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dNTP (i.e., dTTP) into the synthetic DNA template-primer (i.e., poly(dA)/oligo(dT)$_{12–18}$, A/T = 2/1) in 60 min at 37 °C under normal reaction conditions for each enzyme.[3,4]

4.3. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (Avance DRX-400). Samples were prepared in CD_3OD, and the residual solvent peak (δ 3.30) and δ 49.0 (ppm) from CD_3OD were used as

internal references for ^1H and ^{13}C NMR spectra, respectively. Chemical shifts were expressed in δ (ppm) relative to the reference, and coupling constants (J) were expressed in Hz.

Optical rotations were recorded on a JASCO P-1010 digital polarimeter at room temperature.

Infrared spectra (IR) were recorded on a JASCO FT/IR-410 spectrometer, and were reported as wave numbers (cm^{-1}).

Electrospray ionization mass spectra (ESIMS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under conditions of high resolution (HR) or low resolution (LR), using poly (ethylene glycol) as an internal standard.

4.4. Structure determination

4.4.1. Hymenoic acid (*trans*-4-[(1′*E*,5′*S*)-5′-carboxy-1′-methyl-1′-hexenyl]cyclohexanecarboxylic acid) (1). Yellow oil; $[\alpha]_D^{22}$ +16.3 (*c* 1.0, CH_3OH); IR (film) ν_{max} 2934, 2861, 1705, 1518, 1454, 1419, 1258, 1041, 925, 759, 670 cm^{-1}; HR-ESIMS *m*/*z* found 267.1601 $[M-H]^-$, calcd for $C_{15}H_{23}O_4$: 267.1616; ^1H and ^{13}C NMR data, see Table 1.

4.4.2. (5*E*)-6-[*trans*-4-(Hydroxymethyl)cyclohexyl]-2-methylhept-5-en-1-ol (2). To a solution of **1** (18.0 mg, 67.5 μmol) in THF (10 ml) was added $LiAlH_4$ (25.0 mg, 675 μmol) at rt, and the mixture was stirred at rt for 1 h. The reaction was quenched by the addition of EtOAc and Na_2SO_4, filtrated through Celite and washed with EtOAc. The filtrate was concentrated, and the residue was purified by silica gel chromatography (toluene/EtOAc = 1:1) to yield **2** (10.2 mg, 57%) as a colorless oil. LR-ESIMS *m*/*z* 266.9 $[M+Na]^+$, ^1H NMR (400 MHz, $CDCl_3$); δ 6.81 (1H, t, J = 6.8 Hz), 3.51 (1H, dd, J = 10.2, 5.8 Hz), 3.46 (2H, d, J = 6.0 Hz), 3.43 (1H, dd, J = 10.2, 6.3 Hz), 2.11–1.93 (2H, m), 1.87–1.81 (2H, m), 1.83–1.73 (1H, m), 1.76–1.70 (2H, m), 1.67–1.60 (1H, m), 1.58 (3H, s), 1.49–1.39 (2H, m), 1.23 (2H, dq, J = 11.9, 3.1 Hz), 1.19–1.11 (1H, m), 0.98 (2H, dq, J = 11.9, 3.1 Hz), 0.93 (3H, d, J = 6.6 Hz); ^{13}C NMR (100 MHz, $CDCl_3$); δ 140.0, 122.6, 68.7, 68.3, 47.2, 40.3, 35.4, 33.2, 31.1 (2C), 29.6 (2C), 25.1, 16.5, 14.4.

4.4.3. Bis-(*R*)-MTPA ester (3a). To an solution of **2** (3 mg, 12.5 μmol) and DMAP (0.2 mg, 1.64 μmol) in pyridine (1 ml) was added (*S*)-(+)-MTPACl (23 μL, 123.2 μmol) at rt, and the mixture was stirred at rt for 20 h. The mixture was concentrated and the residue was purified by silica gel chromatography (toluene/EtOAc = 2:1) to yield **3a** (1.1 mg) as a colorless oil. LR-ESIMS *m*/*z* 695.0 $[M+Na]^+$, ^1H NMR (400 MHz, $CDCl_3$); δ 7.55–7.49 (4H, m), 7.44–7.38 (6H, m), 4.18 (1H, dd, J = 10.7, 6.3 Hz), 4.16 (2H, d, J = 6.1 Hz), 4.09 (1H, dd, J = 10.7, 6.5 Hz), 3.57–3.54 (6H, m), 2.06–1.92 (2H, m), 1.89–1.80 (1H, m), 1.81–1.74 (2H, m), 1.78–1.68 (1H, m), 1.72–1.65 (2H, m), 1.70–1.60 (1H, m), 1.67–1.60 (1H, m), 1.54 (3H, s), 1.43–1.32 (1H, m), 1.29–1.13 (2H, m), 1.03 (2H, m), 0.92 (3H, d, J = 6.8 Hz).

4.4.4. Bis-(*S*)-MTPA ester (3b). To an solution of **2** (3 mg, 12.5 μmol) and DMAP (0.2 mg, 1.64 μmol) in pyridine (1 ml) was added (*R*)-(−)-MTPACl (47 μL, 252 μmol) at rt, and the mixture was stirred at rt for 3 h. The mixture was concentrated and the residue was purified by silica gel chromatography ($CHCl_3$/MeOH = 99:1) to yield **3b** (9.5 mg, quant.) as a colorless oil. LR-ESIMS *m*/*z* 695.0 $[M+Na]^+$, ^1H NMR (400 MHz, $CDCl_3$); δ 7.55–7.49 (4H, m), 7.44–7.38 (6H, m), 4.24 (1H, dd, J = 10.7, 5.6 Hz), 4.18 (1H, dd, J = 10.7, 6.1 Hz), 4.09 (1H, dd, J = 10.7, 6.6 Hz), 4.08 (1H, dd, J = 10.7, 6.4 Hz), 3.57–3.54 (6H, m), 2.06–1.92 (2H, m), 1.89–1.80 (1H, m), 1.81–1.74 (2H, m), 1.78–1.68 (1H, m), 1.72–1.65 (2H, m), 1.70–1.60 (1H, m), 1.67–1.60 (1H, m), 1.54 (3H, s), 1.43–1.33 (1H, m), 1.26–1.14 (2H, m), 1.03 (2H, m), 0.92 (3H, d, J = 6.8 Hz).

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