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Pinophilins A and B, Inhibitors of Mammalian A-, B-, and Y-Family DNA Polymerases and Human Cancer Cell Proliferation

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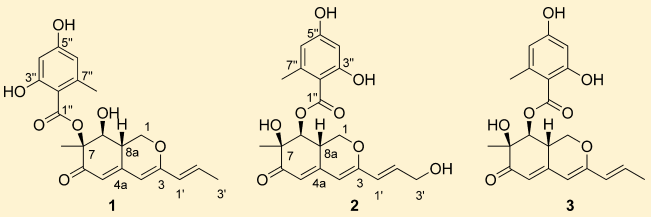
ABSTRACT: Pinophilins A (**1**) and B (**2**), new hydrogenated azaphilones, and Sch 725680 (**3**) were isolated from cultures of a fungus (*Penicillium pinophilum* Hedgcok) derived from a seaweed, and their structures were determined using spectroscopic analyses. These compounds selectively inhibited the activities of mammalian DNA polymerases (pols), A (pol γ), B (pols α, δ, and ε), and Y (pols η, ι, and κ) families, but did not influence the activities of the four X-family pols (pols β, λ, μ, and terminal deoxynucleotidyl transferase). Compound **1** was the strongest inhibitor, with IC_{50} values of 48.6 to 55.6 μM. Kinetic analysis showed that compound **1** is a noncompetitive inhibitor of both pol α and κ activities with the DNA template-primer substrate, and a competitive inhibitor with the nucleotide substrate. In contrast, compounds **1**−**3** showed no effect on the activities of plant and prokaryotic pols or any other DNA metabolic enzymes tested. The compounds suppressed cell proliferation and growth in five human cancer cell lines, but had no effect on the viability of normal human cell lines.



We have long been interested in the integrity of the eukaryotic genome and its relation to cell differentiation. DNA replication, recombination, and repair in eukaryotes are key systems for maintaining these processes,[1] in which DNA polymerases (pols) have important roles. In this regard, we have concentrated our efforts on investigating eukaryotic pols associated with these processes.[2]

Pol catalyzes the addition of deoxyribonucleotides to the 3′-hydroxy terminus of primed double-stranded DNA molecules.[3] The human genome encodes at least 15 pols that carry out cellular DNA synthesis.[4,5] Eukaryotic cells contain three replicative pols (α, δ, and ε), mitochondrial pol γ, and at least 11 nonreplicative pols β, ζ, η, θ, ι, κ, λ, μ, ν, terminal deoxynucleotidyl transferase (TdT), and REV1.[4−6] Pols have a highly conserved structure, which suggests that their overall catalytic subunits vary very little between species. Conserved structures usually indicate important, irreplaceable cellular functions, the stability of which provides evolutionary advantages. On the basis of sequence homology, eukaryotic pols can be divided into four main families, A, B, X, and Y.[7] Family A includes mitochondrial pol γ and pols θ and ν; family B includes the three replicative pols (α, δ, and ε) and pol ζ; family X consists of pols β, λ, μ, and TdT; and family Y includes pols η, ι, κ, and REV1. Pols are not only essential for DNA replication, repair, and recombination but also involved in cell division. Selective pol inhibitors are considered to be a group of potentially useful chemotherapeutic agents, because some inhibitors suppress human cancer cell proliferation and are cytotoxic.[8]

In this study, we describe the new compounds, pinophilins A (**1**) and B (**2**), isolated from a fungal strain derived from seaweed (Figure 1). We also found the hydrogenated azaphilone Sch 725680 (**3**)[9] and determined its absolute configuration. The inhibitory effects of these compounds on mammalian pol activity and human cancer cell growth were investigated in vitro.

RESULTS AND DISCUSSION

Repeated separation of an 8 L culture extract from *Penicillium pinophilum* Hedgcok using silica gel column chromatography yielded compounds **1**−**3**. The molecular formula of compound **1** was determined to be $C_{21}H_{22}O_7$ by HR-ESIMS. The IR spectrum indicated the presence of a hydroxy group (3373 cm^{-1}), a conjugated ester (1728 cm^{-1}), and a conjugated carbonyl (1648 cm^{-1}). As shown in Table 1, the ^1H and ^{13}C NMR spectra suggested that compound **1** comprises an azaphilone and 2,4-dihydroxy-6-methylbenzoic acid structural

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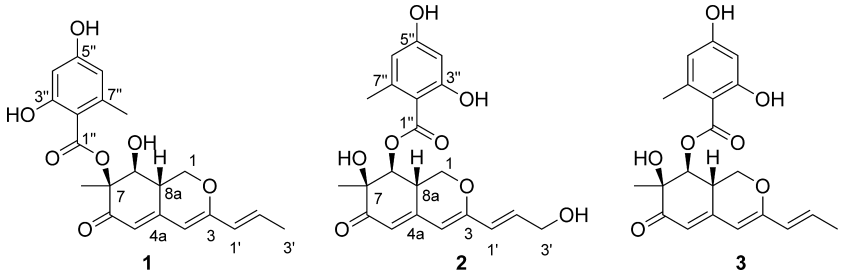

Figure 1. Structure of compounds **1** (pinophilin A), **2** (pinophilin B), and **3** (Sch 725680).

Table 1. NMR Data (400 MHz) for Compounds 1 (Pinophilin A) in $CDCl_3$ and 2 (Pinophilin B) in Methanol-d_4

	1		2	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	68.5, CH_2	4.84, dd (10.8, 5.2)	67.9, CH_2	4.48, dd (10.8, 4.8)
		3.81, dd (13.6, 10.8)		3.86, dd (13.6, 10.8)
3	160.9, C		160.1, C	
4	102.4, CH	5.53, s	103.5, CH	5.81, s
4a	152.6, C		151.7, C	
5	115.4, CH	5.81, d (1.6)	115.9, CH	5.79, d (2.0)
6	190.6, C		195.8, C	
7	89.3, C		73.5, C	
7-Me	18.4, CH_3	1.79, s	18.2, CH_3	1.31, s
8	74.6, CH	3.61, dd (10.0, 10.0)	75.1, CH	5.29, d (10.0)
8-OH		4.94, br d (10.0)		
8a	37.8, CH	2.85, dddd (13.6, 10.0, 5.2, 1.6)	34.8, CH	3.45, dddd (13.6, 10.0, 4.8, 2.0)
1′	125.2, CH	5.88, dq (15.6, 1.4)	122.5, CH	6.21, dt (15.4, 1.4)
2′	134.4, CH	6.47, dq (15.6, 7.0)	136.5, CH	6.51, dt (15.4, 4.4)
3′	15.4, CH_3	1.86, dd (7.0, 1.4)	61.1, CH_2	4.21, dd (4.4, 1.4)
1″	172.0, C		170.5, C	
2″	104.8, C		104.0, C	
3″	166.1, C		164.6, C	
4″	101.6, CH	6.27, d (2.4)	100.4, CH	6.20, d (2.4)
5″	161.4, C		162.7, C	
6″	112.1, CH	6.20, d (2.4)	111.2, CH	6.26, d (2.4)
7″	145.0, C		143.5, C	
7″-Me	24.7, CH_3	2.30, s	23.2, CH_3	2.58, s

moieties. The azaphilone skeleton was determined by long-range correlations measured in an HMBC experiment (Figure 2). The C-3′ methyl group was adjacent to a double bond on the basis of the HMBC correlations from H-3′ to C-1′ and C-2′. The geometry of the double bond at C-1′ was assigned as *E* on the basis of the coupling constant ($J_{1',2'}$ = 15.6 Hz). This double bond was conjugated to two additional double bonds, and the conjugation further extended to the C-6 carboxy group on the basis of the HMBC correlations of H-4 to C-1′ and C-5 and of H-5 to C-4. Judging from the HMBC correlations from H-5 to C-7 (δ 18.4), the C-6 carboxy group was adjacent to an oxygenated quaternary carbon (C-7). C-7 was substituted with a methyl group and an aliphatic oxymethine carbon (C-8) on the basis of the HMBC correlations from 7-CH_3 to C-6, C-7, and C-8. The aliphatic oxymethine carbon (C-8a) showing correlations with H-1, H-5, and H-8 were determined to be

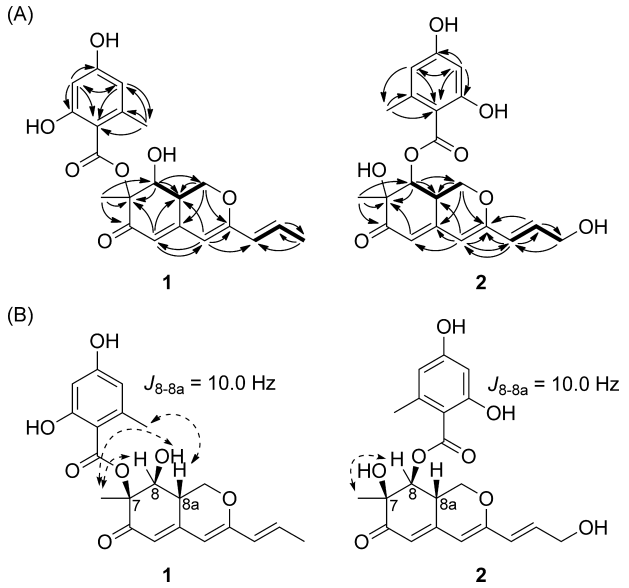

Figure 2. (A) COSY (bold lines) and selected HMBC ($^1H \rightarrow {}^{13}C$) (arrows) correlations and (B) key NOESY (dashed lines) correlations in compounds **1** (pinophilin A) and **2** (pinophilin B).

adjacent to C-1, C-4a, and C-8; therefore, the cyclohexenone ring was constructed. HMBC correlations from the oxymethylene H-1α and H-1β to the C-3 olefinic carbon indicated the presence of a dihydropyran moiety. Thus, the 6,7,8,8a-tetrahydro-1*H*-isochromene ring skeleton was determined. The remaining seven carbons were constructed to represent a 2,4-dihydroxy-6-methylbenzoyloxy moiety on the basis of the following HMBC correlations: from H-4″ to C-2″, C-3″, C-5″, and C-6″; from H-6″ to C-2″, C-4″, and 7″-Me; from 7″-Me to C-2″, C-6″, and C-7″. The benzoyloxy group was assigned to C-7 on the basis of the coupling of H-8 and 8-OH. The relative configuration of compound **1** was determined by $^1H-^1H$ coupling constants and NOESY correlations. The *anti* relationship of H-8/H-8a was deduced from the coupling constant ($J_{8,8a}$ = 10.0 Hz). The *syn* relationship of H-8a/7-*O*-benzoyl group was determined from NOE correlations between 7″-CH_3 and H-8a; between 7-CH_3 and 8-H; and between 7-CH_3 and 8-OH. To determine the absolute configuration, the exciton chirality method[10] was applied. The CD spectrum of compound **1** exhibited Cotton effects due to the interaction between the benzoate and conjugated trienone chromophores at 325 and 297 nm ($\Delta\varepsilon$ −8.6 and 2.8), indicating the *S* configuration[11] at C-7; therefore, the structure of compound **1** was determined to be (7*S*,8*S*,8a*S*)-8-hydroxy-7-methyl-6-oxo-3[(1*E*)-prop-1-en-1-yl]-6,7,8,8a-tetrahydro-1*H*-isochromen-7-yl 2,4-dihydroxy-6-methylbenzoate (**1** in Figure 1), and was named pinophilin A.

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The molecular formula of compound **2** was determined to be $C_{21}H_{22}O_8$ using HR-ESIMS. As shown in Table 1, the ^1H and ^{13}C NMR spectra suggested that compound **2** and Sch 725680 (**3**)[9] had similar structures and differed only at C-3′ of their side chains. A methyl group in **3** was replaced by an oxymethylene group in **2** [δ 3.22 (H-3′) and 61.1(CH_2, C-3′)]. The relative configuration of compound **2** was determined by $^1H-^1H$ coupling constants and NOESY correlations. The relative configuration of C-7, C-8, and C-8a and the geometry of the double bond were identical with those of compound **1**. The absolute configuration of compound **2** was determined by the exciton chirality method.[10] The CD spectrum of compound **2** exhibited Cotton effects at 379 and 305 nm ($\Delta\varepsilon$ 0.6 and −3.2), indicating the *S* configuration at C-8. Thus, the structure of compound **2** was determined to be (7*S*,8*S*,8a*S*)-7-hydroxy-3-[(1*E*)-3-hydroxyprop-1-en-1-yl]-7-methyl-6-oxo-6,7,8,8a-tetrahydro-1*H*-isochromen-8-yl 2,4-dihydroxy-6-methylbenzoate (**2** in Figure 1) and was named pinophilin B.

The absolute configuration of Sch 725680 (**3**) was determined by the exciton chirality method.[10] The CD spectrum of Sch 725680 exhibited Cotton effects at 378 and 307 nm ($\Delta\varepsilon$ 2.3 and −1.6), indicating the *S* configuration at C-7.

Compounds **1**−**3** were tested to determine whether they could inhibit the activity of 11 mammalian pols, such as families A (pol γ), B (pols α, δ, and ε), X (pols β, λ, μ, and TdT), and Y (pols η, ι, and κ). The purity of each compound was more than 98%, as assessed by NMR analysis. As shown in Table 2, compounds **1**−**3** were found to selectively inhibit the activity of A-, B-, and Y-family pols. These compounds did not influence the four X-family pols' activity, even at 200 μM. The compounds most strongly inhibited calf pol α and human pol κ of the B- and Y-families, respectively. Compound **1** had the strongest inhibitory effect of the three compounds, which ranked as follows: **1** > **3** > **2**. Fifty percent inhibition of these mammalian pols was observed for compound **1** at concentrations of 48.6−55.6 μM. Among the novel azaphilones, compound **1** is a ca. 1.8-fold stronger inhibitor than compound **2**. The structural backbone of compounds **1** and **2** is similar, differing by a C-3′ hydroxy group. Therefore, the hydrophobicity of this moiety may be important for inhibition.

Compounds **1**−**3** had no effect on the activity of higher plant pols (cauliflower pol α and rice pol λ) and prokaryotic pols (the Klenow fragment of *E. coli* pol I, *Taq* pol, and T4 pol). When activated DNA (i.e., DNA with gaps digested by deoxyribonuclease I) and 2′-deoxyribonucleoside 5′-triphosphate (dNTP) were used as the DNA template-primer substrate and nucleotide substrate instead of synthesized DNA [poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1)] and 2′-deoxythymidine 5′-triphosphate (dTTP), respectively, the inhibitory effects of the compound were unchanged (data not shown).

In addition, they had minimal influence on the activity of other DNA metabolic enzymes, such as calf primase pol α, human immunodeficiency virus type-1 (HIV-1) reverse transcriptase, T7 RNA polymerase, mouse IMP dehydrogenase (type II), T4 polynucleotide kinase, and bovine deoxyribonuclease I. Collectively, these results suggest that compounds **1**−**3** may be selective inhibitors of mammalian A-, B-, and Y-family pol species.

Next, to elucidate the mechanism of selective inhibition of compound **1** for mammalian pol species, the inhibitory mode of the compound against calf pol α and human pol κ, which respectively belong to the B- and Y-family pols, was investigated. Poly(dA)/oligo$(dT)_{18}$ and dTTP were used as synthetic DNA template-primer substrate and nucleotide substrate in kinetic analysis. The extent of inhibition as a function of the DNA template-primer substrate or nucleotide substrate concentration was measured (Table 3).

Table 2. IC_{50} Values of Compounds 1 (Pinophilin A), 2 (Pinophilin B), and 3 (Sch 725680) for Mammalian Pols, Various Pols, and Other DNA Metabolic Enzymes[a]

	IC_{50} values (μM)		
enzyme	**1**	**2**	**3**
Mammalian Pols			
[A-family of pol]			
human pol γ	55.6 ± 3.3	93.7 ± 5.6	67.3 ± 4.0
[B-family of pols]			
calf pol α	49.1 ± 2.9	89.5 ± 5.3	63.2 ± 3.8
human pol δ	54.5 ± 3.2	96.4 ± 5.8	68.0 ± 4.1
human pol ε	52.0 ± 3.1	93.9 ± 5.6	65.6 ± 3.9
[X-family of pols]			
rat pol β	>200	>200	>200
human pol λ	>200	>200	>200
human pol μ	>200	>200	>200
calf TdT	>200	>200	>200
[Y-family of pols]			
human pol η	50.9 ± 3.1	92.1 ± 5.5	63.1 ± 3.8
mouse pol ι	50.2 ± 3.0	91.0 ± 5.4	63.8 ± 3.8
human pol κ	48.6 ± 2.9	88.9 ± 5.3	59.8 ± 3.6
Plant Pols			
cauliflower pol α	>200	>200	>200
rice pol λ	>200	>200	>200
Prokaryotic Pols			
E. coli pol I	>200	>200	>200
Taq pol	>200	>200	>200
T4 pol	>200	>200	>200
Other DNA Metabolic Enzymes			
calf primase of pol α	>200	>200	>200
HIV-1 reverse transcriptase	>200	>200	>200
T7 RNA polymerase	>200	>200	>200
mouse IMP dehydrogenase (type II)	>200	>200	>200
T4 polynucleotide kinase	>200	>200	>200
bovine deoxyribonuclease I	>200	>200	>200

[a]Each compound was incubated with each enzyme (0.05 units). Data are shown as the means ± SE of three independent experiments.

The collected data were expressed as double reciprocal plots and showed that compound **1** inhibited pol α activity in a noncompetitive manner with respect to the DNA template-primer substrate, but in a competitive manner with the nucleotide substrate. For the DNA template-primer substrate, the apparent Michaelis constant (K_m) was unchanged at 7.80 μM, whereas a decrease of 55.6, 35.7, 26.3, and 20.8 pmol/h in maximum velocity (V_{max}) was observed in the presence of compound **1** at 0, 10, 20, and 30 μM, respectively. The V_{max} for the nucleotide substrate was unchanged at 29.2 pmol/h, whereas the K_m for the nucleotide substrate increased from 1.56 to 8.33 μM in the presence of 0 to 30 μM compound **1**. The inhibition constant (K_i), obtained from Dixon plots, was found to be 13.8 μM for the DNA template-primer substrate and 9.60 μM for the nucleotide substrate. Because the K_i value for the nucleotide substrate was approximately 1.4-fold less than that for the DNA template-primer substrate, it was concluded that

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Table 3. Kinetic Analysis of the Inhibitory Effects of Compound 1 (Pinophilin A) on Mammalian Pols α and κ as a Function of DNA Template-Primer Dose and Nucleotide Substrate Concentration

enzyme	substrate	compound 1 (μM)	K_m[a] (μM)	V_{max}[a] (pmol/h)	K_i[b] (μM)	inhibitory mode
calf pol α	DNA template-primer[c]	0	7.80	55.6	13.8	noncompetitive
		10		35.7		
		20		26.3		
		30		20.8		
	nucleotide substrate[d]	0	1.65	29.2	9.60	competitive
		10	2.78			
		20	5.00			
		30	8.33			
human pol κ	DNA template-primer[c]	0	1.54	52.6	18.1	noncompetitive
		10		19.2		
		20		11.8		
		30		8.47		
	nucleotide substrate[d]	0	2.00	41.7	9.85	competitive
		10	3.33			
		20	5.56			
		30	8.33			

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

compound **1** had a greater affinity for the nucleotide substrate binding site than for the DNA template-primer substrate binding site of the pol α protein.

Similarly, pol κ inhibition by compound **1** was noncompetitive with the DNA template-primer substrate because there was no change in the apparent K_m (1.54 μM), while the V_{max} decreased from 52.6 to 8.47 pmol/h for DNA template-primer substrate in the presence of 0 to 30 μM compound **1** (Table 3). The induced inhibition of pol κ activity by compound **1** was competitive with respect to the nucleotide substrate (V_{max} was unchanged at 41.7 pmol/h). The K_m for the nucleotide substrate was 4.2-fold greater in the presence of 30 μM compound **1**. From Dixon plots, the K_i value was 18.1 μM for the DNA template-primer substrate and 9.85 μM for the nucleotide substrate. Therefore, compound **1** had a 1.8-fold greater affinity for the nucleotide substrate binding site than for the DNA template-primer substrate binding site of the pol κ protein.

When activated DNA and four dNTPs were used as the DNA template-primer substrates and nucleotide substrates, respectively, the mode of mammalian pol α and κ inhibition by compound **1** was the same as that with synthetic DNA template-primer substrate (data not shown). The mode of inhibition for pols α and κ by compound **2** was the same as that displayed by compound **1** (data not shown). The results suggested that these compounds bind directly to the nucleotide substrate binding site of pols α and κ, but may bind to or interact with a site distinct from the DNA template-primer substrate binding site.

Furthermore, the inhibitory effects of isolated compounds **1**–**3** on five human cancer cell lines were investigated. As shown in Table 4, these compounds suppressed cell proliferation of all cancer cell lines tested. The compounds were ranked **1** > **3** > **2** in terms of growth inhibitory effect. The compounds at 50% growth inhibition (GI_{50}) values prevented the incorporation of tritiated thymidine into cancer cell lines, such as BALL-1 and HCT116, and arrested the cell cycle at the S-phase (data not shown). The effect of these compounds on human cancer cell growth showed a similar inhibitory trend to that on mammalian A-, B-, and Y-family pols (Table 2). Since

Table 4. Inhibitory Effect of Pinophilin A (1), Pinophilin B (2), Sch 725680 (3), and Ara-C on the Proliferation of Human Cancer and Normal Cells[a]

human cell line	GI_{50} value (μM)			
	1	**2**	**3**	Ara-C
Cancer Cells				
A549	52.5 ± 4.7	93.1 ± 8.3	65.7 ± 5.9	0.20 ± 0.02
BALL-1	50.2 ± 4.5	90.4 ± 8.1	62.0 ± 5.5	0.08 ± 0.01
HCT116	51.3 ± 4.6	92.5 ± 8.3	64.6 ± 5.8	0.12 ± 0.01
HeLa	55.6 ± 5.0	99.0 ± 8.7	68.8 ± 6.1	0.18 ± 0.02
NUGC-3	54.7 ± 4.9	96.8 ± 8.6	66.4 ± 5.9	0.25 ± 0.03
Normal Cells				
HDF	>200	>200	>200	0.25 ± 0.02
HUVEC	>200	>200	>200	0.23 ± 0.02

[a]Human cancer cell lines were A549 (lung cancer cells), BALL-1 (acute lymphoblastoid leukemia cells), HCT116 (colon carcinoma cells), HeLa (cervix cancer cells), and NUGC-3 (stomach cancer cell). Normal human cell lines were HDF (human dermal fibroblasts) and HUVEC (human umbilical vein endothelial cells). Human cancer cell lines and normal cell lines were incubated with each compound for 24 and 72 h, respectively. The cell viability was determined by WST-1 assay.[37] Data are shown as the means ± SE of five independent experiments.

the GI_{50} values of compounds **1**–**3** for cancer cell growth was almost the same as the IC_{50} values for pol inhibition, these compounds might be able to penetrate the nucleus of cancer cells and inhibit the activity of A-, B-, and Y-family pols, which may lead to cell growth suppression.

Cytarabine (cytosine arabinoside or arabinofuranosyl cytidine: Ara-C) is a major cancer drug that inhibits both DNA and RNA polymerases and the nucleotide reductase enzymes needed for DNA synthesis. Ara-C strongly suppressed cell proliferation of both human cancer cell lines and normal human cell lines tested, and the inhibitory effect on all cancer cells was comparable to that of normal cells (Table 4). On the other hand, compounds **1**–**3** had no effect on the cell proliferation and growth of normal human cells such as HDF (human dermal fibroblasts) and HUVEC (human umbilical vein

endothelial cells). Therefore, we concluded that these compounds might be selective agents against cancer cell growth.

Many azaphilones have been described to exhibit inhibitory activities against various therapeutic targets, such as the inhibition of acyl-CoA: cholesterol acyltransferase,[12] cholesteryl ester transfer protein,[13] platelet-derived growth factor,[14] endothelin receptor,[15] gp120-CD4,[16] monoamine oxidase,[17] and phospholipase A2.[18] Some azaphilones have also been reported to block tumor progression.[19] In this report, we found that azaphilones **1**–**3** displayed novel bioactivities such as mammalian pol inhibition and human cancer cell growth suppression. Further study will be conducted to clarify the relationship between the inhibition of pols and cell growth by these azaphilones.

Compounds **1**–**3** are assumed to suppress human cancer cell proliferation by inhibiting DNA replication because of the correlation between cancer cell growth suppression and the inhibition of B-family pols, which are essential for DNA replication during cell proliferation. Normal human cell growth might not be influenced by these compounds because their cell proliferation and DNA replication rates are significantly slower than those of cancer cells. These compounds may be developed as chemotherapeutic agents based on their specific inhibitory activities for A-, B-, and Y-family pols.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco micro melting point apparatus. Optical rotations were recorded on a JASCO P-1010 digital polarimeter at room temperature. UV/vis spectra were obtained using a JASCO V-650 DS spectrophotometer in MeOH at 25 °C. The concentrations of compound **1** and **2** were 2.6×10^{-6} and 1.0×10^{-5} M, respectively. CD spectra were recorded using a JASCO J-720 CD spectrometer at a concentration of 1.0×10^{-4} M in MeOH at 25 °C. Both UV/vis and CD spectra were measured between 200 and 600 nm using 10 mm path-length quartz cuvettes. Infrared spectra (IR) were recorded on a JASCO FT/IR-410 spectrometer and were reported as wave numbers (cm^{-1}). ^1H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (Avance DRX-400), using $CDCl_3$ (with TMS for ^1H NMR and $CDCl_3$ for ^{13}C NMR as an internal reference) solution or methanol-d_4 (using residual undeuterated solvent for ^1H NMR and methanol-d_4 for ^{13}C NMR as an internal reference), unless otherwise noted. Chemical shifts are expressed in δ (ppm) relative to TMS or residual solvent resonance, and coupling constants (J) are expressed in Hz. Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under conditions of high resolution, using poly(ethylene glycol) as the internal standard. Analytical TLC was carried out on precoated silica gel 60 F_{254} plates (Merck, Germany).

Materials. The chemically synthesized DNA template, poly(dA), was purchased from GE Healthcare Bio-Sciences (Little Chalfont, UK). The oligo$(dT)_{18}$ DNA primer was customized by Sigma-Aldrich Japan K.K. (Hokkaido, Japan). The radioactive nucleotide, [^3H]-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol), was obtained from MP Biomedicals LLC (Solon, OH, USA). All other reagents were of analytical grade and were purchased from Wako Chemical Industries (Osaka, Japan).

Isolation and Cultivation of Fungi from Seaweed. Wild seaweed was collected along the coast of Kasai Marine Park, Edogawa-ku, Tokyo, Japan, treated with 5% aqueous HOAc, and suspended in sterilized H_2O. The suspension was placed on potato dextrose agar (PDA) plates (Difco & BBL, NJ, USA), and the plates were cultured for 1–2 weeks at 28 °C. Each grown mycelium on the plate was transferred onto individual PDA plates and cultured at 28 °C for 10 days. Each cultured fungus was transferred onto a new PDA plate and cultured at 28 °C for 10 days. Transformation and culture on PDA plates was repeated 2 to 5 times to obtain pure mycelium strains. The isolated and purified fungal strains were stored at −80 °C.

The isolated fungal strains were cultured in liquid potato dextrose medium, and each mycelium was extracted using CH_2Cl_2. After evaporation of the solvent, the extracts were screened for inhibitory activity against mammalian pols. The fungus containing pol inhibitors was identified as *Penicillium pinophilum* Hedgcok by Techno Suruga Laboratory Co., Ltd. (Shizuoka, Japan).

Extraction and Purification of Compounds. The isolated fungal strain, *P. pinophilum* Hedgcok, from a seaweed (*Ulva fasciata*) collected in Kasai, Tokyo, Japan, was cultured by transferring a small agar piece from the cultured plate into four 2-L Erlenmeyer flasks containing potato dextrose broth (24 g) (Difco and BBL) in H_2O (1 L). The culture (4 L) was kept under static conditions in the dark for 14 days. The cultured broth was filtered through cheesecloth to remove fungal mycelia. The filtrate was extracted using CH_2Cl_2. The organic layer was evaporated in vacuo to obtain a crude extract (160 mg), which was separated by silica gel column chromatography with $CHCl_3$–MeOH (100:0–0:100) to give fractions 1–4. Fraction 2 was purified by silica gel column chromatography with toluene–EtOAc (12:1–10:1) to yield Sch 725680 (**3**) and compound **1** (3.8 mg) as a yellow solid, and fraction 4 was purified by silica gel column chromatography with toluene–EtOAc (4:1–3:1) to yield compound **2** (4.6 mg) as a yellow solid.

*Pinophilin A, (7S,8S,8aS)-8-hydroxy-7-methyl-6-oxo-3[(1E)-prop-1-en-1-yl]-6,7,8,8a-tetrahydro-1H-isochromen-7-yl 2,4-dihydroxy-6-methylbenzoate (**1**):* yellow solid; mp 213–215 °C (dec); $[\alpha]^{23}_D$ −114 (c 0.065, MeOH); UV (MeOH) λ_{max} (log ε) 267 (3.86), 316 (4.18), 335 (4.21) nm; CD (c 1.0×10^{-4}, MeOH) $\Delta\varepsilon$ (nm) −8.6 (325), +2.8 (297); IR (film) ν_{max} 3373, 2919, 2828, 1728, 1648, 1585 cm^{-1}; HR-ESIMS m/z 387.1430 $[M + H]^+$ (calcd for 387.1438); ^{13}C and ^1H NMR, see Table 1.

*Pinophilin B, (7S,8S,8aS)-7-hydroxy-3-[(1E)-3-hydroxyprop-1-en-1-yl]-7-methyl-6-oxo-6,7,8,8a-tetrahydro-1H-isochromen-8-yl 2,4-dihydroxy-6-methylbenzoate (**2**):* yellow solid; mp 212–214 °C (dec); $[\alpha]^{23}_D$ +92.4 (c 0.62, MeOH); UV (MeOH) λ_{max} (log ε) 266 (4.15), 345 (4.30) nm; CD (c 1.0×10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) +0.6 (379), −3.2 (305); IR (film) ν_{max} 3376, 2923, 2853, 1725, 1651, 1585 cm^{-1}; HR-ESIMS m/z 403.1390 $[M + H]^+$ (calcd for 403.1387); ^{13}C and ^1H NMR, see Table 1.

*Sch 725680 (**3**):* $[\alpha]^{23}_D$ +103 (c 0.27, MeOH); UV (MeOH) λ_{max} (log ε) 266 (4.12), 343 (4.24) nm; CD (c 1.0×10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) +2.2 (378), −1.6 (307); IR, ^{13}C and ^1H NMR, and MS data were consistent with reported values.[9]

Preparation of Enzymes. Pol α was purified from calf thymus by immuno-affinity column chromatography, as described by Tamai et al.[20] Recombinant rat pol β was purified from *E. coli* JMpβ5, as described by Date et al.[21] The human pol γ catalytic gene was cloned into pFastBac (Invitrogen Japan K.K., Tokyo Japan). Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD, USA) and was purified with ProBoundresin (Invitrogen Japan K.K.).[22] Human pols δ and ε were purified by nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pols δ and ε-conjugated affinity column chromatography, respectively.[23] A truncated form of human pol η (residues 1–511), tagged with His_6 at the C-terminus, was expressed in *E. coli* cells and purified as described by Kusumoto et al.[24] A recombinant mouse pol ι, tagged with His_6 at its C-terminus, was expressed and purified using Ni-NTA column chromatography according to the method for pol η preparation.[24] A truncated form of pol κ (residues 1–560) tagged with His_6 at its C-terminus was overexpressed in *E. coli* and purified as described by Ohashi et al.[25] Recombinant human His-pol λ was overexpressed and purified according to a method described by Shimazaki et al.[26] Recombinant human His-pol μ was overexpressed in *E. coli* BL21 and purified using Glutathione Sepharose 4B (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA) column chromatography according to the method for pol λ preparation.[26] Pol α from a higher plant, cauliflower inflorescence, was purified according to the method outlined by

Sakaguchi et al.[27] Recombinant rice (*Oryza sativa* L. cv. Nipponbare) pol λ tagged with His_6 at the C-terminus was expressed in *E. coli* and purified as described by Uchiyama et al.[28] Calf thymus TdT and bovine pancreas deoxyribonuclease I were obtained from Stratagene Cloning Systems (La Jolla, CA, USA). The Klenow fragment of pol I from *E. coli* and human immunodeficiency virus type-1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). *Taq* pol, T4 pol, T7 RNA polymerase, and T4 polynucleotide kinase were purchased from Takara Bio (Tokyo, Japan).

DNA Polymerase Assays. The reaction mixtures for pol α, pol β, plant pols, and prokaryotic pols have been described previously.[29,30] Those for pol γ and pols δ and ε were as described by Umeda et al.[22] and Ogawa et al.[31] The reaction mixtures for pols η, ι, and κ were the same as for pol α, and the reaction mixture for pols λ and μ was the same as for pol β. For pols, poly(dA)/oligo$(dT)_{18}$ (A/T = 2/1) and dTTP were used as the DNA template-primer substrate and nucleotide (i.e., dNTP) substrate, respectively. For TdT, oligo$(dT)_{18}$ (3′-OH) and dTTP were used as the DNA primer and nucleotide substrate, respectively. For HIV-1 reverse transcriptase, poly(rA)/oligo$(dT)_{18}$ (A/T = 2/1) and dTTP were used as the template-primer and nucleotide substrate, respectively.

Each compound was dissolved in distilled DMSO at various concentrations and sonicated for 30 s. Aliquots of 4 μL of sonicated samples were mixed with 16 μL of each enzyme (final amount 0.05 units) 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 each 16 μL of enzyme standard reaction mixtures, 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 100%, and the activity at each inhibitor concentration 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 synthetic DNA template-primers in 60 min at 37 °C under normal reaction conditions for each enzyme.[29,30]

Other DNA Metabolic Enzymes Assays. The activities of calf primase of pol α, T7 RNA polymerase, mouse IMP dehydrogenase, T4 polynucleotide kinase, and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer's specifications, as described by Tamiya-Koizumi et al.,[32] Nakayama and Saneyoshi,[33] Mizushina et al.,[34] Soltis et al.,[35] and Lu and Sakaguchi,[36] respectively.

Cell Culture and Measurement of Cell Viability. Cultured human cancer cell lines A549 (lung cancer cells), BALL-1 (acute lymphoblastoid leukemia cells), HCT116 (colon carcinoma cells), HeLa (cervix cancer cells), and NUGC-3 (stomach cancer cell) and normal cell lines, HDF (human dermal fibroblasts) and HUVEC (human umbilical vein endothelial cells), were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human cancer cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 mg/mL). Normal human cells were cultured in Eagle's minimum essential medium supplemented with 4.5 g of glucose per liter plus 10% fetal calf serum, 5 mM L-glutamine, 50 units/mL penicillin, and 50 units/mL streptomycin. The cells were cultured at 37 °C in a humidified incubator containing 5% CO_2/95% air. For the cell growth assay, cells were plated at 1×10^4 cells per well in 96-well microplates and cultured for 12 h, and various concentrations of the isolated compounds were subsequently added. The compounds were dissolved in DMSO at a concentration of 10 mM as a stock solution. The stock solutions were diluted to the appropriate final concentrations with growth medium to 0.5% DMSO just before use. After the cells were cultured with the compounds for 24 h, 10% WST-1 solution was added to each medium, and the wells were incubated for 4 h. The number of viable cells in each well was then counted using a microplate reader (Vmax-K, Japan Molecular Devices, Tokyo, Japan) at 450 nm and a reference wavelength of 630 nm (WST-1 assay).[37]

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