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Novel azaphilones, kasanosins A and B, which are specific inhibitors of eukaryotic DNA polymerases β and λ from *Talaromyces* sp.

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Abstract—Kasanosins A (**1**) and B (**2**) are novel azaphilones isolated from cultures of *Talaromyces* sp. derived from seaweed, and their structures were determined by spectroscopic analyses. These compounds selectively inhibited the activities of eukaryotic DNA polymerases β and λ (pols β and λ) in family X of pols, and compound **1** was a stronger inhibitor than compound **2**. The IC_{50} values of compound **1** on rat pol β and human pol λ were 27.3 and 35.0 μM, respectively. On the other hand, compounds **1** and **2** did not influence the activities of terminal deoxynucleotidyl transferase (TdT), which is a pol of family X, and the other families of eukaryotic pols, such as family A (i.e., pol γ), family B (i.e., pols α, δ, and ε) and family Y (i.e., pols η, ι, and κ), and showed no effect even on the activities of plant pol α, fish pol δ, prokaryotic pols, and 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. The results suggested that these novel compounds could identify the inhibition between pols β, λ, and TdT in family X.

1. Introduction

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

The human genome encodes at least 14 pols to conduct cellular DNA synthesis,[3,4] and pols have a highly conserved structure, which means that their overall catalytic subunits vary, on the whole, very little from species to species. Conserved structures usually indicate important, irreplacable functions of the cell, the maintenance of which provides evolutionary advantages. Based on sequence homology, eukaryotic pols can be further subdivided into mainly four different families, A, B, X, and Y.[5] Family A of pols contains mitochondrial pol γ, and pol θ, and family B of pols mostly contains three replicative types, pols α, δ, and ε, and pol ζ. Family X of pols has pols β, λ, μ, and terminal deoxynucleotidyl transferase (TdT), and family Y of pols has pols η, ι, κ, and REV1; however, not all functions of eukaryotic pols have been fully elucidated. Selective inhibitors of pol families are useful tools for distinguishing pols and clarifying their biological functions. We have been searching for natural compounds that selectively inhibit each of these eukaryotic pols.[6–15]

In this study, we report newly found compounds **1** and **2** that selectively inhibit only the activity of pol β and λ in family X of eukaryotic pols. These natural compounds were named kasanosin A (**1**) and kasanosin B (**2**)

Keywords: Kasanosin A; Kasanosin B; Azaphilone; DNA polymerase β; DNA polymerase λ; Family X of DNA polymerases; Enzyme inhibitor.
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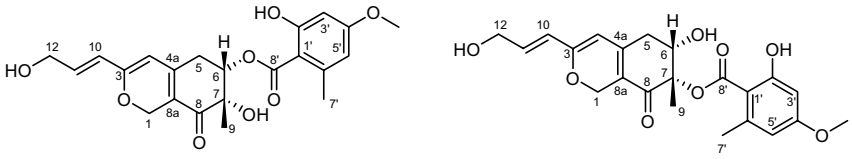

Figure 1. Structure of kasanosins A (**1**) and B (**2**).

(Fig. 1), isolated from the cultures of *Talaromyces* sp. derived from seaweed. To our knowledge, there have been no reports on such natural inhibitors specific to the pol X family, such as pols β and λ, although we previously reported selective inhibitors of only family X of pols: prunasin as a pol β inhibitor,[8] and nodulisporol and nodulisporone as pol λ inhibitors.[14]

In this paper, we report the isolation and structural determination of compounds **1** and **2**, which are azaphilone derivatives.

2. Results

2.1. Isolation and cultivation of fungus

A fungal strain, ka02k3, was isolated from seaweed in Kasai Rinkai Park, Tokyo, Japan. After treatment with 5% acetic acid, the seaweed was suspended in sterilized water. The suspension was then added to potato dextrose agar plates (Difco) and cultured at 27 °C. Ka02k3 was isolated by transferring the mycerial tips several times and identified as *Talaromyces* sp. by TechnoSuruga Laboratory Co. Ltd. (Shizuoka, Japan). A small agar plug was then transferred into a 3 L Erlenmeyer flask containing 1 L of a culture of 24 g potato dextrose broth (Difco), 6 g soytone peptone (Difco), 4 g yeast extract (Oxoid), and 100 mg NaCl. Cultures of ka02k3 (3 L) were grown for three weeks without shaking in the dark.

2.2. Extraction and purification of compounds

Fungal mycelia 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 46.0 mg crude residue. This crude extract was separated by a silica gel column chromatographed on silica gel with hexane–EtOAc (4:1–0:1) to give 9.6 mg mixture. The mixture was twice chromatographed on silica gel with toluene–EtOAc (2:1–0:1) and $CHCl_3$–methanol (96:4–19:1) to give compound **1** (1.0 mg) and compound **2** (2.9 mg) as yellow powders.

2.3. Structure determination of isolated compounds

The molecular formula of compound **1** was determined to be $C_{22}H_{24}O_8$ by a high resolution electron spray ionization mass spectrometer (HR-ESIMS). The IR spectrum indicated the presence of a hydroxyl group (3376 cm^{-1}), a conjugated ester group (1722 cm^{-1}), and a conjugated ketone group (1643 cm^{-1}). The ^1H and ^{13}C NMR spectra suggested that **1** has an azaphilone and 2-hydroxy-4-methoxy-6-methylbenzoic acid as partial structures (Table 1). The azaphilone skeleton was mainly determined by ^1H–^{13}C long ring correlations measured in a HMBC experiment (Fig. 2A). HMBC correlations from H-1 to C-3, C-4a, and C-8a showed 3,4,6-trisubstituted 2*H*-pyran moiety. The ^{13}C signal of the carbonyl carbon at 196.8 (C-8) revealed the conjugated ketone moiety. HMBC correlations from H-6 to C-4a, C-5, C-7, and C-8 as well as from H-4 to C-4a and C-5 indicated a second six-membered ring fused to the 2*H*-pyran. The methyl group (C-9) substituted on an oxygenated quaternary carbon (C-7) was located two bonds away from an aliphatic methine carbon (C-6) and the ketone (C-8), judging from HMBC correlations from H-9 to C-6, C-7, and C-8. Long-range coupling from the aliphatic methane proton (H-6) to the carbonyl carbon (C-8′) of 2-hydroxy-4-methoxy-6-methylbenzoic acid identified ester linkage between C-6 and C-8′. The presence of 3-hydroxy-1-propenyl moiety was established by ^1H–^1H COSY correlation between H-10/H-11 and H-11/H-12. According to HMBC correlations from H-4 to C-10, from H-10 to C-3, and from

Table 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR spectral data for compounds **1** (kasanosin A) and **2** (kasanosin B)

Position	**1**		**2**	
	δ_C	δ_H (mult, *J* in Hz)	δ_C	δ_H (mult, *J* in Hz)
1	64.8	4.89 (m)	65.2	4.85 (m)
3	161.3		160.6	
4	105.7	5.53 (s)	105.6	5.52 (s)
4a	149.2		149.2	
5	32.6	3.01 (m)	34.6	2.70 (dd, 17.8, 8.3)
		2.81 (m)		2.60 (dd, 17.8, 5.0)
6	78.0	5.37 t (3.6)	73.9	3.99 (dd, 8.3, 5.0)
7	75.4		86.0	
8	196.8		196.7	
8a	115.7		116.9	
9	20.1	2.17 (s)	16.7	1.67 (s)
10	123.3	6.22 (dt, 15.4, 1.8)	123.4	6.21 (dt, 15.4, 1.6)
11	138.2	6.51 (dt, 15.4, 4.7)	137.7	6.49 (dt, 15.4, 4.7)
12	62.6	4.21 (dd, 4.7, 1.8)	62.6	4.21 (dd, 4.7, 1.6)
1′	115.4		116.1	
2′	161.4		160.9	
3′	97.7	6.25 (d, 2.1)	97.5	6.24 (d, 2.1)
4′	160.2		159.8	
5′	110.2	6.21 (dd, 2.1, 0.7)	109.8	6.20 (dd, 2.1, 0.7)
6′	140.1		139.0	
7′	23.0	1.40 (s)	19.5	2.15 (s)
8′	169.5		169.5	
OMe	56.3	3.69 (s)	56.2	3.68 (s)

Recorded in CD_3OD and chemical shifts are expressed as δ ppm. s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet.

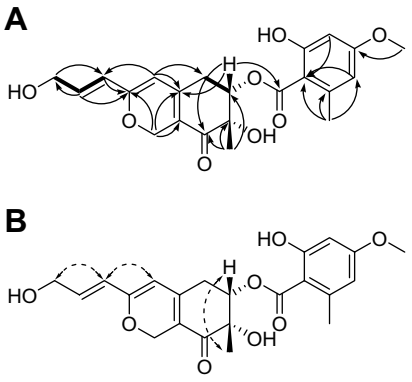

Figure 2. (A) Selected COSY (bold lines) and HMBC ($^1H \rightarrow {}^{13}C$) (arrows) correlations and (B) key NOESY (dashed lines) correlations in compound **1** (kasanosin A).

H-11 to C-3, the 3-hydroxy-1-propenyl moiety was attached to C-3 of the azaphilone skeleton. The relative configuration of **1** was determined by ^1H–^1H coupling constants and NOESY correlations (Fig. 2B). The syn relation for H-6/H-9 was deduced from NOE correlation between H-6 and H-9. The *E*-configuration of the double bond at C-10 was determined from the coupling constant (J_{10-11} = 15.4 Hz) and NOE correlation between H-10 and H-12. Therefore, the structure of compound **1** was determined to be 5,6,7,8-tetrahydro-7-hydroxy-3-[(1*E*)-3-hydroxy-1-propenyl]-7-methyl-oxo-1*H*-2-benzopyran-6-yl 2-hydroxy-4-methoxy-6-methylbenzoate, and was named kasanosin A.

Compound **2** possessed the molecular formula $C_{22}H_{24}O_8$, as established from high resolution electron spray ionization mass spectrometer (HR-ESI–MS). The spectral data of compound **2** were similar to those of kasanosin A (**1**). The IR spectrum indicated the presence of a hydroxyl group (3367 cm^{-1}), a conjugated ester group (1722 cm^{-1}), and a conjugated ketone group (1643 cm^{-1}). ^1H and ^{13}C NMR spectra suggested that **2** has the same azaphilone skeleton and 2-hydroxy-4-methoxy-6-methylbenzoic acid as partial structures (Table 1). The difference between **1** and **2** was the ester linkage of 2-hydroxy-4-methoxy-6-methylbenzoic acid moiety and the azaphilone skeleton at C-7. NOE correlation between H-6 and H-9 was observed in the NOESY spectrum, indicating the syn relation for H-6/H-9. *E*-configuration at the C-10 double bond determined the coupling constant (J_{10-11} = 15.4 Hz) and NOE correlation between H-10 and H-12. Thus, the structure of compound **2** was determined to be 5,6,7,8-tetrahydro-6-hydroxy-3-[(1*E*)-3-hydroxy-1-propenyl]-7-methyl-oxo-1*H*-2-benzopyran-7-yl 2-hydroxy-4-methoxy-6-methylbenzoate, and was named kasanosin B.

The structures of kasanosin A (**1**) and kasanosin B (**2**) were similar to that of rubiginosin A and rubiginosin B, respectively.[16,17] Rubiginosins A and B were isolated from the inedible mushroom *Hypoxylon rubiginosum* by Asakawa et al. These compounds were reported to inhibit nitric oxide production in RAW 264.7 cells.[18]

2.4. Inhibition by isolated compounds of the activities of DNA polymerases and other DNA metabolic enzymes

First, the isolated compounds **1** (kasanosin A) and **2** (kasanosin B) were investigated as to whether they inhibited the activities of the ten mammalian pols, such as families A (i.e., pol γ), B (i.e., pols α, δ, and ε), X (i.e., pols β and λ, and TdT), and Y (i.e., pols η, ι, and κ). As shown in Figure 3A, these compounds at 100 μM were found to significantly inhibit the activities of pols β and λ in family X. The inhibition of compounds **1** and **2** on the activities of pols β and λ was dose-dependent, and 50% inhibition of rat pol β was observed at concentrations of 27.3 and 60.1 μM, respectively (Fig. 4A), and the IC_{50} values for human pol λ were 35.0 and 72.9 μM, respectively (Fig. 4B). These results suggested that compound **1** was an approximately 2.2-fold stronger inhibitor than compound **2**, and the inhibitory effect of these compounds on pol β was stronger than on pol λ. Although TdT also belongs to the pol X family, these compounds did not inhibit the activity of calf TdT (Fig. 3A). On the other hand, families A, B, and Y of mammalian pols (Fig. 3A), a higher plant (cauliflower) pol α, a fish (cherry salmon) pol δ, and prokaryotic pols such as Klenow fragment of *Escherichia coli* pol I, T4 pol, and *Taq* pol (Fig. 3B) did not influence the activities by compounds **1** and **2**.

The same concentration (i.e., 100 μM) of these compounds also did not suppress the activities of DNA metabolic enzymes, such as calf primase 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. These results suggested that compounds **1** and **2** could selectively inhibit the activities of pols β and λ, but could not influence the other DNA metabolic enzymes in vitro. Since these compounds are azaphilone derivatives, the backbone structure of the azaphilone might be important for selective inhibition.

3. Discussion

As described in this report, we found novel potent inhibitors specific to eukaryotic pols β and λ of family X from cultures of *Talaromyces* sp. derived from seaweed. The natural compounds were found to be azaphilone derivatives, kasanosin A (**1**) and kasanosin B (**2**).

Based on sequence homology, eukaryotic pols can be further subdivided into four different families: A, B, X, and Y.[5] Family A pols contain both replicative and repair polymerases, such as mitochondrial pol γ. Family B pols mostly contain replicative polymerases and include the major eukaryotic pols α, δ, and ε. Y-family pols differ from others in having low fidelity on undamaged templates and in their ability to replicate through damaged DNA. Family X contains the well-known pol β as well as others, such as pols λ and μ, and TdT.[3,5] Pol β is required for short-patch base excision repair,

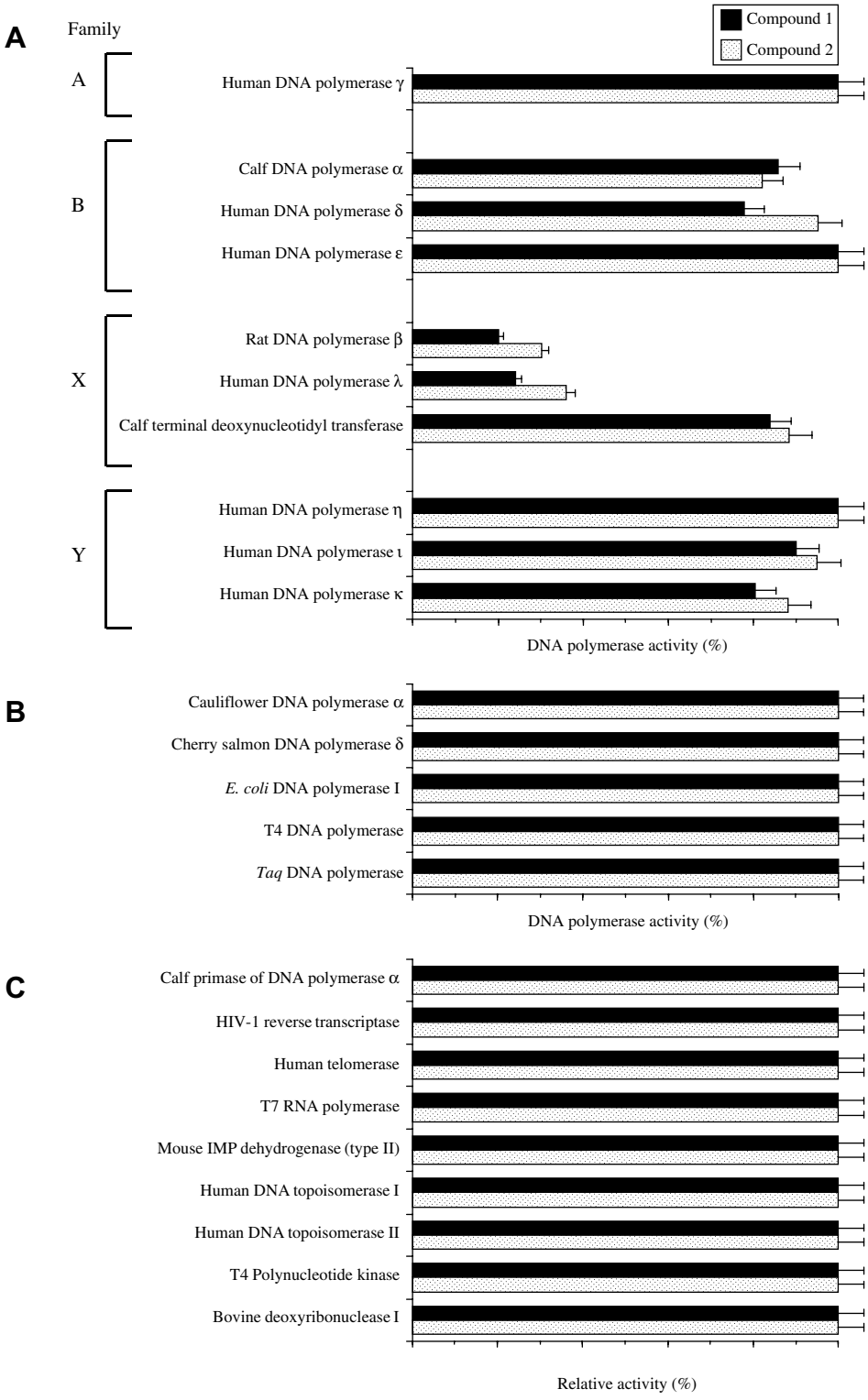

Figure 3. Effect of compounds **1** (kasanosin A) and **2** (kasanosin B) on the activities of various DNA polymerases and other DNA metabolic enzymes. (A) Mammalian pols, (B) plant, fish, and prokaryotic pols, and (C) other DNA metabolic enzymes. Compound **1** (black bars) and compound **2** (gray bars) (100 μM each) were incubated with each enzyme (0.05 U). % of relative activity. Enzymatic activity was measured as described previously.[6,7,11] Enzyme activity in the absence of the compounds was taken as 100%. Data are shown as means ± SEM of four independent experiments.

a DNA repair pathway that is essential for repairing abasic sites.[3] Pols λ and μ are involved in non-homologous end joining, a mechanism for rejoining DNA double-strand breaks. TdT is only expressed in lymphoid tissue and adds 'n nucleotides' to double-strand breaks formed during V(D)J recombination to promote immunological diversity. The yeast *Saccharomyces cerevisiae* has only one pol of family X, pol 4, which is involved

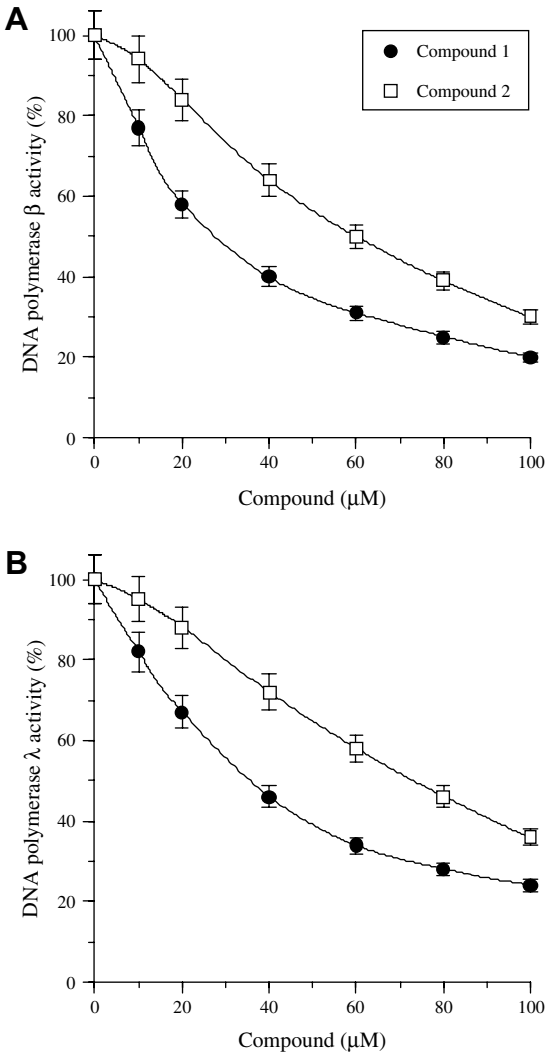

Figure 4. Mammalian DNA polymerase inhibition dose–response curves of compounds **1** (kasanosin A) and **2** (kasanosin B). Compounds **1** (closed circle) and **2** (open square) were incubated with rat pol β (A) and human pol λ (B) (0.05 units of each). Pol activities were measured as described in the Experimental section. Pol activity in the absence of the compounds was taken as 100%. Data are shown as means ± SEM of three independent experiments.

in non-homologous end joining.[3] Therefore, the inhibitors of family X pols could be immunosuppressive agents.

A pol is an enzyme that assists in DNA replication. Such enzymes catalyze the polymerization of deoxyribonucleotides alongside a DNA strand, which they read and use as a template.[19] The newly-polymerized molecule is complementary to the template strand and identical to the template's partner strand. On the other hand, TdT behaves like a pol in synthesizing a DNA chain by 5′- to 3′-polymerization of dNTPs (deoxythynucleoside 5′-triphosphates). Unlike a pol, TdT neither requires nor copies a template.[19] Because compounds **1** and **2** might be able to identify differences in the molecular catalytic mechanism between pols β, λ, and TdT among family X of pols, these compounds could selectively inhibit the activities of pols β and λ, but not influence TdT activity.

In conclusion, since compounds **1** and **2** have extremely high specificity for families of pols, these compounds could be useful molecular tools as pols β- and λ-specific inhibitors in studies to determine the precise roles of the pol family in vitro, and also might be useful to develop a drug design strategy for immunosuppressive and/or anti-cancer chemotherapy agents.

4. Experimental

4.1. Materials

Nucleotides and chemically synthesized DNA template-primers such as poly(dA), oligo(dT)$_{12-18}$, and [^{3}H]deoxythymidine 5′-triphosphate (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), and a plant (i.e., cauliflower) were purified, and prokaryotic pols and other DNA metabolic enzymes were purchased as described in our previous report.[6,7,11] The activities of all pols and other DNA metabolic enzymes were measured as described in previous reports.[6,7,11,20] The substrates of the pols were poly(dA)/oligo(dT)$_{12-18}$ and dTTP as the DNA template-primer and dNTP (2′-deoxyribonucleoside 5′-triphosphate) substrate, respectively. Compounds **1** and **2** were 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.[6,7]

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 ^{1}H and ^{13}C NMR spectra, respec-

tively. 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}).

Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (APIQSTAR pulsar i) under conditions of high resolution, using poly (ethylene glycol) as an internal standard.

4.4. Structure determination

4.4.1. Kasanosin A (1). Yellow powder: mp: 132–136 °C; $[\alpha]_D^{24}$ + 124.0 (*c* 0.05, MeOH); IR (film) ν_{max} 3376, 3024, 2927, 2855, 1722, 1643, 1531, 1463, 1411, 1336, 1258, 1215, 1191, 1163, 1092 cm^{-1}; HR-ESI–MS (*m*/*z*) calcd for $C_{22}H_{24}O_8Na$ ($[M+Na]^+$) 439.1363, found 439.1365.; ^{13}C and ^1H data, see Table 1.

4.4.2. Kasanosin B (2). Yellow powder: mp: 131–137 °C; $[\alpha]_D^{24}$ + 360.0 (*c* 0.15, MeOH); IR (film) ν_{max} 3367, 3019, 2928, 2856, 1722, 1643, 1606, 1529, 1462, 1407, 1260, 1216, 1163, 1092 cm^{-1}; HR-ESI–MS (*m*/*z*) calcd for $C_{22}H_{24}O_8Na$ ($[M+Na]^+$) 439.1363, found 439.1373.; ^{13}C and ^1H data, see Table 1.

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