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Bioorganic & Medicinal Chemistry 12 (2004) 2597–2601
Bioorganic & Medicinal Chemistry

Taxol derivatives are selective inhibitors of DNA polymerase α

Masahiko Oshige,[a,†] Mika Takenouchi,[d] Yasutaro Kato,[b] Sinji Kamisuki,[b] Toshifumi Takeuchi,[b] Kouji Kuramochi,[a] Isamu Shiina,[e] Yoshihito Suenaga,[e] Yo-ichi Kawakita,[e] Kazufumi Kuroda,[f] Noriyuki Sato,[d] Susumu Kobayashi,[a,c] Fumio Sugawara[a,b] and Kengo Sakaguchi[a,b,*]

[a]*Frontier Research Center for Genome & Drug Discovery, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba 278-8510, Japan*
[b]*Department of Applied Biological Science, Faculty of Science and Technology, Tokyo University of Science, Noda-shi, Chiba-ken 278-8510, Japan*
[c]*Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamazaki, Noda-shi, Chiba-ken 278-8510, Japan*
[d]*Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, South 1, West 17, Chuo-ku, Sapporo 060-8556, Japan*
[e]*Department of Applied Chemistry, Faculty of Science, Tokyo University of Science, Kagurazaka, Shinjuku-ku, Tokyo 162-8601, Japan*
[f]*Mitsubishi Chemical Institute of Life Science, Minami-ohtani 11, Machida-shi, Tokyo 194-8511, Japan*

Received 11 December 2003; revised 10 March 2004; accepted 11 March 2004

Available online 2 April 2004

Abstract—During screening for mammalian DNA polymerase inhibitors, we found and succeeded in isolating a potent inhibitor from a higher plant, *Taxus cuspidate*. The compound was unexpectedly determined to be taxinine, an intermediate of paclitaxel (taxol) metabolism. Taxinine was found to selectively inhibit DNA polymerase α (pol.α) and β (pol.β). We therefore, tested taxol and other derivatives and found that taxol itself had no such inhibitory effect, and only taxinine could inhibit both pol.α and β. The other compounds used, one derivative, cephalomannine, and five intermediates synthesized chemically inhibited only the pol.α activity in vitro. None of the compounds, including taxinine, influenced the activities of the other DNA polymerases, which are reportedly targeted by many pol.β inhibitors. With both pol.α and β, all of the compounds tested noncompetitively inhibited with respect to both the DNA template-primer and the dNTP substrate.

1. Introduction

We have screened natural compounds for selective inhibition of mammalian DNA polymerases and found many effective examples,[1] some of which have been developed for clinical use as immunosuppressive or antitumor agents.[2] Before our trial, novel DNA polymerase inhibitors had not attracted particular notice because it was thought that eukaryotic cells contain three replicative DNA polymerases (pol.α, δ, and ε), mitochondrial DNA polymerase (pol.γ), and one repair type of DNA polymerase (pol.β) and because inhibitors to replicative pol.α and β were already well established, aphidicolin and dideoxyTTP (ddTTP), respectively. However, many new forms of DNA polymerases from eukaryotes were found and as of 2003, at least 16 species (pol.α, β, γ, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ, ϕ, pol.I-like 1, and pol.I-like 2) are known.[3] Aphidicolin is not necessarily a specific inhibitor of pol.α, and ddTTP cannot be employed as a pol.β-specific inhibitor. We have, therefore, continued our search for new natural compounds that selectively inhibit each of these eukaryotic DNA polymerases for use as tools and molecular probes to clarify their biological and in vivo functions.[1]

In the process, we recently identified taxinine as an inhibitor from a higher plant, the Japanese yew (*Taxus cuspidata*). The compound is an intermediate of

Keywords: Paclitaxel (taxol); Taxinine; Taxol derivatives; Enzyme inhibition; DNA polymerase α; DNA polymerase β.
* Corresponding author. Tel.: +81-471-24-1501x3409; fax: +81-471-23-9767; e-mail: kengo@rs.noda.tus.ac.jp
† Present address of first author (M.O.): Indiana University School of Medicine, Cancer Research Institute, 1044W. Walnut St., R4/151, Indianapolis, IN 46202, USA.

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doi:10.1016/j.bmc.2004.03.018

paclitaxel (taxol), an antitumor compound isolated from *Taxus brevifolia*,[4] which can halt mitosis and induce apoptosis. It is known that taxol directly affects tubulin polymerization, alters microtubule dynamics, and binds to Bcl-2, antiapoptotic human protein.[5] We here focused on whether this and other compounds belonging to the paclitaxel (taxol) group might directly influence the DNA polymerization system.

2. Experimental

2.1. Materials

Deoxynucleoside triphosphates and synthetic polynucleotides such as poly(dA), poly(rC), and oligo(dT)$_{12-18}$, and [^{3}H]2′-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) were purchased from Amersham Pharmacia Biotech (NJ, USA). All other reagents were of analytical grade and purchased from Wako (Osaka, Japan). Paclitaxel (taxol), Cephalomannine (compound **2**), Baccatin III, 10-Deacetylbaccatin III, 10-Deacetyltaxol, 7-epi-10-Deacetyltaxol were obtained from Wako (Osaka, Japan). Synthetic intermediates of taxol (eight compounds) were synthesized in our laboratory based on the methods described by Shiina et al.[6–10]

The names of eight compounds:

(1*S*,2*S*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-1-Acetoxymethyl-11,12-(carbonyldioxy)-1,2,4,9-tetrahydroxy-4a,8,13,13-tetramethyl-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydro-7,11-methanobenzocyclodecen-6-yl acetate (compound **3**).

1,2-*O*,*O*-Carbonyl-2-debenzoylbaccatin III (compound **4**).

(4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-12-Benzoyloxy-4,9,11-trihydroxy-4a,8,13,13-tetramethyl-1-methylene-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydro-7,11-methanobenzocyclodecen-6-yl 2,2,2-trichloroethyl carbonate (compound **5**).

(1*S*,2*S*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-12-Benzoyloxy-4,9,11-trihydroxy-4a,8,13,13-tetramethyl-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydrospiro[7,11-methanobenzocyclodecene-1,2′-oxiran]-2,6-yl diacetate (compound **6**).

(1*R*,2*S*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-11,12-Carbonyldioxy)-1-ethyl-1,2,4-trihydroxy-4a,8,13,13-tetramethyl-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydro-7,11-methano-benzocyclodecen-6,9-yl diacetate (compound **7**).

(4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-11,12-(Carbonyldioxy)-4,9-dihydroxy-4a,8,13,13-tetramethyl-1-methylene-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydro-7,11-methanobenzocyclodecen-6-yl acetate (not shown).

(1*S*,2*S*,2a*S*,3*R*,6*S*,6a*S*,8*R*,11*S*)-1,2-(Carbonyldioxy)-3,6,11-trihydroxy-6a,10,13,13-tetramethyl-7-oxo-1,2,2a,3,4,5,6,6a,7,8,11,12-dodecahydro-1,9-methanobenzocyclodecen-8-yl acetate (not shown).

(1*S*,2*S*,4*S*,4a*S*,6*R*,9*S*,11*S*,12*S*,12a*R*)-11,12-Carbonyldioxy)-4,9-dihydroxy-4a,8,13,13-tetramethyl-5-oxo-1,2,3,4,4a,5,6,9,10,11,12,12a-dodecahydrospiro[7,11-methanobenzocyclodecene-1,2′-oxiran]-2,6-yl diacetate (not shown).

2.2. Enzymes

Human DNA polymerase α, δ, and ε (pol.α, δ, and ε) and *Drosophila* pol.δ and ε were purified from Molt4 cells and embryos by protein-affinity column chromatography as described previously.[11] Purification of pol.α-primase complex from embryos was carried out by 5E12 antibody column chromatography followed by Heparin Sepharose 6 Fast Flow (Amersham Pharmacia Biotech) column chromatography.[12] Recombinant rat DNA polymerase β (pol.β), mouse DNA polymerase λ (pol.λ) and human Terminal deoxynucleotidyl transferase (TdT) were purified from *Escherichia coli* BL21 (DE3).[13] Calf DNA topoisomerase I (topo I) and human DNA topoisomerase II (topo II) were purchased from Takara (Tokyo, Japan) and TopoGen (OH, USA). *E. coli* DNA polymerase I, Taq DNA polymerase and T4 DNA polymerase from Takara (Tokyo, Japan).

2.3. DNA polymerase assays

The activities of DNA polymerases were measured by previously described methods[13] with poly(dA)/oligo(dT)$_{12-18}$ and dTTP as the template-primer and nucleotide substrate, respectively. Compounds were dissolved in 10% dimethyl sulfoxide (DMSO) at various concentrations and aliquots were added to the reaction mixture for each assay. The activity without the inhibitor was considered to be 100% and the remaining activity at each concentration of inhibitor was determined as a percentage of this value. One unit of each DNA polymerase activity was defined as the amount of each enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleotide triphosphates (i.e., dTTP) into the synthetic template-primer (i.e., poly(dA)/oligo(dT)$_{12-18}$, A/T=2/1) in 60 min at 37 °C under the normal reaction conditions.[14] For kinetic analyses, the concentrations of template-primer or [^{3}H]dTTPs were varied. The inhibition mode was analyzed by Lineweaver–Burk plots, K_m were obtained from Double reciprocal plots, and K_i were from Dixon plots.

2.4. DNA topoisomerase assays

DNA relaxation activities of DNA topoisomerases were described previously.[15] Activity of topo I was analyzed in the same manner as described above except that reaction mixtures contained 10 mM Tris–HCl (pH 7.9), pUC19 plasmid DNA (200 ng), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol, and 1 unit of topo I. One unit was defined as the amount of enzyme capable of relaxing 0.25 μg of DNA in 15 min at 37 °C.

Topo II reactions were performed in 20 μL reaction mixtures containing 50 mM Tris–HCl buffer (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol (DTT), pBR322 plasmid DNA (200 ng), 2 μL of inhibitor solution (10% DMSO), and 1 unit of topo II. The mixtures were incubated at 37 °C for 30 min and reactions terminated by adding 2 μL of loading buffer consisting of 5% sarkosyl, 0.0025% bromophenol blue, and 25% glycerol. The mixtures were subjected to 1% agarose gel electrophoresis in TAE (Tris–acetate–EDTA) running buffer. The agarose gels were stained with ethidium bromide, and DNA was visualized on a UV transilluminator.

2.5. Cell culture and measurement of cell growth inhibition

A549 cells, a human lung carcinoma cell line, were cultured in Eagle's minimal essential medium with nonessential amino acids and MKN28 cells, a human gastric cancer cell line, in 1640 medium, both supplemented with 10% fetal calf serum. MCF-7 cells, a human breast cancer cell line, were cultured in Eagle's minimal essential medium with nonessential amino acids, 1.5 g/L $NaHCO_3$, 1 mM Na-pyruvate, and 10 μg/mL insulin. All taxol analogues were first dissolved in DMSO at 10 mM, and then diluted with standard medium before testing for effects on growth rates, determined by MTT assay.[16]

2.6. Isolation of taxinine

In a series of screenings for mammalian DNA polymerase inhibitors, we found a natural compound from a higher plant, the Japanese yew (*Taxus cuspidata*), which inhibits the activities of pol.α and β, used as the target. The compound was extracted with methanol from vegetable bodies (500 g dry wt) and evaporation of the solvent yielded a 5 g sample. This was partitioned between dichloromethane (1 L) and water (1 L), adjusted to pH 7.0, and the organic layer was evaporated. The fraction was then subjected to silica gel column chromatography (Wakogel C-100, 100 mesh, 5.0 × 50 cm) and eluted with dichloromethane–methanol (1:1 v/v). Active fractions (750 mg) were purified by a second silica gel column chromatography (Wakogel C-200, 200 mesh, 2.5 × 40 cm) using dichloromethane–methanol (1:1 v/v) and an aliquot (150 mg) was subjected to a third silica gel column chromatography (Wakogel C-300, 300 mesh, 1.5 × 20 cm) and then eluted with dichloromethane–methanol (1:1 v/v). Finally, the active compound (20 mg) was purified by Sephadex LH-20 column chromatography (1.5 × 20 cm) eluted with dichloromethane–methanol (1:1 v/v). Negative FABHR (fast atom bombardment high resolution) mass and ^{1}H, ^{13}C, and DEPT (distortion less enhancement by polarization transfer) NMR spectroscopic analyses indicated the inhibitor to be taxinine (see compound **1** in Fig. 1), previously reported as a bio-antimutagen isolated from the same plant.[4]

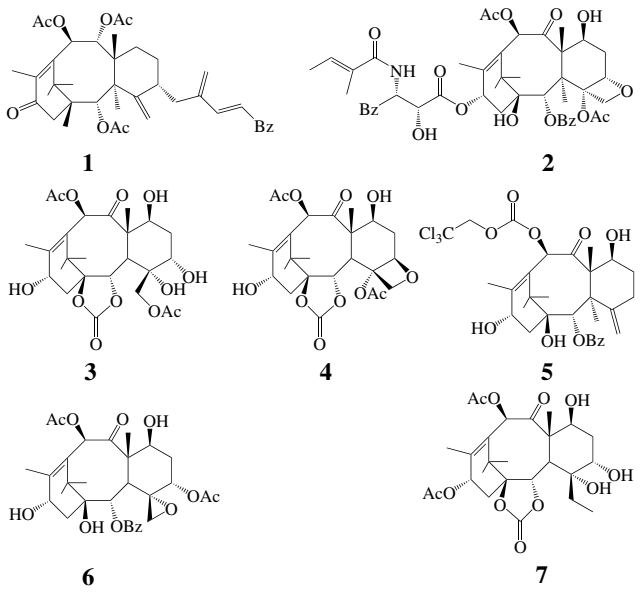

Figure 1. Taxol analogues as DNA polymerase inhibitors. (**1**) Taxinine; (**2**) Cephalomannine; (**3**–**7**) intermediates for taxol synthesis.[6–10]

3. Results and discussion

3.1. Effects of taxol and its derivatives on the activities of mammalian DNA polymerases and DNA topoisomerases

Table 1 shows the values of 50% inhibition (IC_{50}) of all the compounds tested against mammalian pol.α, β, δ, ε, λ, TdT, topo I, topoII, *Drosophila* pol.α, δ, and ε. Although 14 DNA polymerase species have been reported as of 2003, those available as mammalian enzyme species are still limited. However, all of the enzyme species used in this report could be purified to near homogeneity.

Compound **1** (Taxinine) was effective at inhibiting human pol.α and β activity (IC_{50} values of 27 and 36 μM, respectively), but had no effect on the other DNA polymerase species and the DNA topoisomerases. However, taxol itself did not exert any inhibition (data not shown). Some of the intermediates such as compounds **2** (Cephalomannine), **3**–**7** could influence only pol.α. The IC_{50} values of the compounds **1**–**7** with mammalian pol.α were 27, 31, 44, 41, 85, 83, and 97 μM, respectively. Same as mammalian pol.α, *Drosophila* pol.α was inhibited by the compounds **1**–**7**. The IC_{50} values of with *Drosophila* pol.α were 30, 37, 45, 38, 79, 90, and 85 μM. None of the compounds shown in Fig. 1 had any significant influence on the activities of mammalian DNA pol.δ, ε, and λ, TdT, or topo I and II and *Drosophila* pol.δ and ε (Table 1). Prokaryotic DNA polymerases such as *E. coli* DNA polymerase I, Taq DNA polymerase, and T4 DNA polymerase were not targeted (data not shown). Compounds **1**–**4** were especially strong inhibitors of mammalian and *Drosophila* pol.α, like aphidicolin, which is a pol.α, δ, and ε inhibitor. The data indicate that some taxol group compounds are DNA polymerase inhibitors specific to pol.α or pol.α and β, but taxol itself is not. Since aphidicolin is

Table 1. IC_{50} values (μM) of taxol and its analogues on the activity of various DNA polymerases

Compound	Pol.α	Pol.β	Pol.δ	Pol.ε	Pol.λ	TdT	Topo I	Topo II	Dpol.α	Dpol.δ	Dpol.ε
1	27	36	>500	>500	>500	>500	>500	>500	30	>500	>500
2	31	>500	>500	>500	>500	>500	>500	>500	37	>500	>500
3	44	>500	>500	>500	>500	>500	>500	>500	45	>500	>500
4	41	>500	>500	>500	>500	>500	>500	>500	38	>500	>500
5	85	>500	>500	>500	>500	>500	>500	>500	79	>500	>500
6	83	>500	>500	>500	>500	>500	>500	>500	90	>500	>500
7	97	>500	>500	>500	>500	>500	>500	>500	85	>500	>500

Taxol analogues were incubated with each enzymes (0.05 units). The enzyme activities were measured as described in Experimental. Enzyme activity in the absence of the compound was taken as 100%. Pol.α: human DNA polymerase α, pol.β: rat DNA polymerase β, pol.δ: human DNA polymerase δ, pol.ε: human DNA polymerase ε, pol.λ: mouse DNA polymerase λ, TdT: human Terminal deoxynucleotidyl transferase, topo I: calf DNA topoisomerase I, topo II: human DNA topoisomerase II, Dpol.α: *Drosohila* DNA polymeraseα, Dpol.δ: *Drosohila* DNA polymerase δ, Dpol.ε: *Drosohila* DNA polymerase ε.

at present the only commercially available inhibitor for pol.α. Compound **2** (Cephalomannine) in particular may have promise as a new pol.α-specific inhibitor for in vitro studies of DNA synthetic complexes. The correlation among the structures of these compounds and the inhibition effects to DNA polymerase was not completely clear in these results yet. However, if the compounds act as inhibitors in vivo, they would not only provide molecular tools for analyzing DNA polymerases, but might also be lead compounds for cancer chemotherapeutic agents. We therefore, also investigated their actions on the human lung carcinoma cell line A549, the human gastric cancer cell line MKN28, and the human breast cancer cell line MCF-7. However, none of the pol.α-specific inhibitors (compounds **1**–**7**) caused any suppression of cell growth. Presumably the pol.α-inhibitory effect may be too weak (data not shown).

3.2. Effects of reaction conditions on mammalian DNA polymerase inhibition

To determine the effects of a nonionic detergent on inhibition by compounds **1**–**7** of pol.α and β, a neutral detergent, Nonidet P-40 (NP-40), was added to the reaction mixture at a concentration of 0.1%. In the absence of any compound, pol.α activity was taken as 100%. The inhibitory effects of compounds **1**–**7** on pol.α and of compound **1** on pol.β at 100 μM were not affected by the addition of NP-40 to the reaction mixture, suggesting that the binding interactions to the enzymes are hydrophilic. We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μg/mL), or a protein, BSA (100 μg/mL), could prevent the inhibitory effects of compounds **1**–**7**. Poly(rC) and BSA showed little or no influence, suggesting that the binding to pol.α or β occur selectively (data not shown). The results were also similar on *Drosophila* pol.α (data not shown).

3.3. Mode of DNA polymerase inhibition by pol.α-inhibitory compounds

To elucidate the inhibition mechanisms of compounds **1**–**7** on mammalian pol.α, β, and *Drosophila* pol.α, the extent of inhibition as a function of DNA template-primer or dNTP substrate concentrations was studied (Table 2). For kinetic analyses, poly(dA)/oligo$(dT)_{12-18}$ and dTTP were used as the DNA template-primer and dNTP substrate, respectively. Double reciprocal plots showed that on mammalian and *Drosophila* pol.α, all the compounds were noncompetitive with both the DNA template and the dNTP substrate, and on pol.β, compound **1** were also noncompetitive (Table 2). On mammalian and *Drosophila* pol.α, the K_m values to the DNA template for compounds **1**–**7** were unchanged at 4.2, 3.3, 5.6, 4.0, 2.2, 3.6, 4.9 and 4.5, 3.9, 5.6, 4.0, 2.4, 3.3, 5.1 μM, respectively (Table 2). Similarly, on mammalian or *Drosophila* pol.α, the K_m values to the dNTP substrate were also unchanged at 0.93, 1.4, 1.1, 0.95, 1.2, 1.0, 0.94 and 1.0, 1.3, 1.2, 0.88, 1.2, 1.3, 1.1 μM, respectively (Table 2). On pol.β, compound **1** was noncompetitive with both the DNA template (K_m unchanged at 3.9 μM) and the dNTP substrate (K_m unchanged at 1.4 μM) (Table 2). For mammalian or *Drosophila* pol.α, and the DNA template, the inhibition constant (K_i) values obtained from Dixon plots were found to be 4.8, 6.0, 8.1, 7.3, 6.6, 5.4, 7.2 and 4.9, 5.9, 7.9, 7.0, 6.4, 4.9, 6.9 μM for compounds **1**–**7**, respectively, and for pol.β and the DNA template, to be 6.65 μM for compound **1**. On the other hand, the K_i values for mammalian or *Drosophila* pol.α, and dNTP were 4.1, 5.2, 7.0, 6.3, 7.5, 6.2, 5.1 and 4.0, 4.9, 7.1, 6.2, 7.1, 5.9, 4.1 μM for compounds **1**–**7**, respectively, and the K_i values for pol.β and dNTP was 7.1 μM for compound **1**. When activated DNA was used as the DNA template-primer, the inhibition of mammalian pol.α, β and *Drosophila* pol.α were the same as with the synthetic DNA template-primer (data not shown). These results suggested that none of all the compounds used here competitively binds to either the DNA template binding site or the dNTP substrate binding site on mammalian pol.α, β, and *Drosophila* pol.α, but that they may inhibit the DNA polymerases by binding somewhere on their surfaces.

As described in Introduction, at least 16 species of DNA polymerases from eukaryotes (pol.α, β, γ, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ, ϕ, pol.I-like 1, and pol.I-like 2) are known, as of 2003. Selective inhibitors of DNA polymerases are useful tools and molecular probes and since pol.α is essential for DNA replication, its inhibition is very useful for studying the DNA replication systems.

Table 2. Inhibition modes, K_i, and K_m values (μM) of taxol analogues for mammalian DNA polymerase α, β, and *Drosophila* DNA polymerase α

	DNA			dTTP		
	Inhibition mode	K_i (μM)	K_m (μM)	Inhibition mode	K_i (μM)	K_m (μM)
Mammalian pol.α						
Compound **1**	Noncompetitive	4.8	4.2	Noncompetitive	4.1	0.93
Compound **2**	Noncompetitive	6.0	3.3	Noncompetitive	5.2	1.4
Compound **3**	Noncompetitive	8.1	5.6	Noncompetitive	7.0	1.1
Compound **4**	Noncompetitive	7.3	4.0	Noncompetitive	6.3	0.95
Compound **5**	Noncompetitive	6.6	2.2	Noncompetitive	7.5	1.2
Compound **6**	Noncompetitive	5.4	3.6	Noncompetitive	6.2	1.0
Compound **7**	Noncompetitive	7.2	4.9	Noncompetitive	5.1	0.94
Drosophila pol.α						
Compound **1**	Noncompetitive	4.9	4.5	Noncompetitive	4.0	1.0
Compound **2**	Noncompetitive	5.9	3.9	Noncompetitive	4.9	1.3
Compound **3**	Noncompetitive	7.9	5.6	Noncompetitive	7.1	1.2
Compound **4**	Noncompetitive	7.0	4.0	Noncompetitive	6.2	0.88
Compound **5**	Noncompetitive	6.4	2.4	Noncompetitive	7.1	1.2
Compound **6**	Noncompetitive	4.9	3.3	Noncompetitive	5.9	1.3
Compound **7**	Noncompetitive	6.9	5.1	Noncompetitive	4.1	1.1
Mammalian pol.β						
Compound **1**	Noncompetitive	6.7	3.9	Noncompetitive	7.1	1.4

The inhibition modes were analyzed by Lineweaver–Burk plots.
K_is were obtained from Dixon plots.
K_ms were from Double reciprocal plots.

Aphidicolin, once believed to be a pol.α-specific inhibitor, is now known to also inhibit the activities of pol.δ and ε.[17] However, there have been no previous reports of inhibitors capable of distinguishing among pol.α, δ, and ε, reported previously by us.[18] Unfortunately, however, Dehydroaltenusin could influence only the activity of mammalian pol.α. Surprisingly, it showed no effect on pol.α from any organisms except mammals.[13] The commonly applied agent dehydroaltenusin is not applicable for studies of pol.α from nonmammalian organisms but some of the taxol derives reported here might be suitable for this purpose.

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