Structural Relationship of Lithocholic Acid Derivatives Binding to the N-Terminal 8-kDa Domain of DNA Polymerase β†

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Received April 7, 2004; Revised Manuscript Received June 4, 2004

ABSTRACT: We reported previously that lithocholic acid (LCA, 3-α-hydroxy-5-β-cholan-24-oic acid), one of the major compounds in the secondary bile acids, selectively inhibited the activity of mammalian DNA polymerase β (pol β) [Mizushina, Y., Ohkubo, T., Sugawara, F., and Sakaguchi, K. (2000) *Biochemistry 39*, 12606−12613]. The purpose of this study was to investigate the molecular structural relationship of LCA and its 10 chemically synthesized derivatives. The inhibitory activities of pol β by some derivative compounds were stronger than that by LCA, and these compounds bound tightly to the 8-kDa domain fragment but not to the 31-kDa domain fragment of pol β. Biacore analysis demonstrated that the 8-kDa domain bound selectively to compound **9** (3-α-*O*-lauroyl-5-β-cholan-24-oic acid), which was the strongest pol β inhibitor tested, as a 1:1 complex with a dissociation constant (K_d) of 1.73 nM. From computer modeling analysis (i.e., molecular dynamics analysis), the 8-kDa domain had two inhibitor binding areas. Three amino acid residues (Lys60, Leu77, and Thr79) of the 8-kDa domain bound to LCA and compound **2** (3-α-methoxy-5-β-cholan-24-oic acid), and four amino acid residues (Leu11, Lys35, His51, and Thr79) of the 8-kDa domain bound to compound **9**. From these results, the structure−function relationship among pol β and its selective inhibitors was discussed.

We have screened eukaryotic DNA polymerase inhibitors as molecular probes to distinguish DNA polymerases and to clarify their biological and in vivo functions. As reported previously (*1*), one of them was the secondary bile acid, lithocholic acid (LCA,[1] 3-α-hydroxy-5-β-cholan-24-oic acid), which is known to promote tumorigenesis in rats induced by the monoalkylating agent, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (*2*, *3*). LCA exerts its activity without further modification by intestinal bacteria, but the molecular mechanism of the cancer-promoting activity by this bile acid has long remained obscure (*4*). LCA was an inhibitor of mammalian DNA polymerase β (pol β) (*1*). As to why LCA is a pol β inhibitor, at present, we are analyzing the structure and function of pol β using the inhibitor from two different viewpoints to understand the role of each of the polymerases in vivo, and to develop a drug design strategy for cancer chemotherapy agents.

pol β has a modular two-domain structure, with apparent flexibility within a protease-sensitive region between residues 82−86, which separates the two domains (*5*, *6*). Treatment with trypsin yields an N-terminal domain fragment (8 kDa), which retains binding affinity for single-stranded DNA (ssDNA) and a C-terminal domain fragment (31 kDa) with reduced DNA polymerase activity (*5*, *6*). We reported previously the mode of biochemical inhibition by LCA and unsaturated long-chain fatty acids such as nervonic acid (NA) using two of the pol β fragments, which were separated proteolytically (*1*, *7*, *8*). Both LCA and NA were found to bind to the 8-kDa DNA-binding domain fragment and to suppress binding to the template-primer DNA. The biochemical mode of inhibition of pol β by LCA was the same as those by the fatty acids, although the chemical structure of LCA greatly differed from those of the fatty acids. On ^{1}H-^{15}N HMQC NMR analysis of the 8-kDa domain of pol β with LCA, the chemical shifts were observed only in residues, mainly helix 3, helix 4, and the 79−87 turn of the same face (*1*). This region was comprised mainly of three amino acid residues (Lys60, Leu77, and Thr79) of pol β on the LCA-interaction interface (*1*). On the other hand, the chemical shifts by binding NA to pol β were mainly of four amino acid residues (Leu11, Lys35, His51, and Thr79) (*8*).

† This work was partly supported by a grant-in-aid for Kobe-Gakuin University Joint Research (B) (to Y.M. and H.Y.). Y.M. acknowledges grants-in-aid from the Hyogo Science and Technology Association, The Japan Food Chemical Research Foundation, and Grant-in-aid 16710161 for Scientific Research, The Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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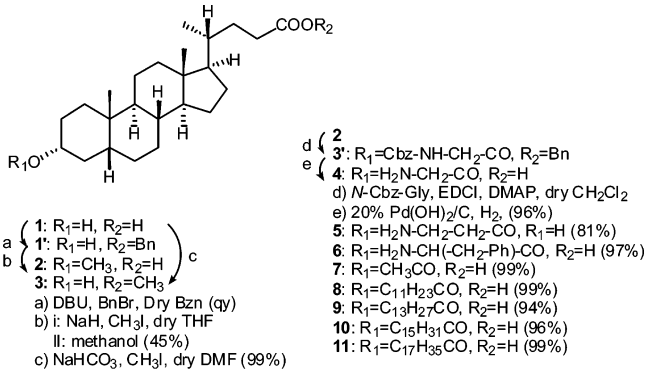
[1] Abbreviations: LCA, lithocholic acid; NA, nervonic acid; pol, DNA-directed DNA polymerase (E. C. 2.7.7.7); poly(dA), polydeoxyriboadenylic acid; oligo(dT)$_{12-18}$, oligo(12−18) deoxyribothymidylic acid; dTTP, 2′-deoxythymidine 5′-triphosphate; HIV-1, human immunodeficiency virus type-1; ssDNA, single-stranded DNA.

10.1021/bi049307r CCC: $27.50
Published on Web 07/27/2004

Scheme 1: Structure of LCA and Its Derivatives[a]



[a] Chemical yields (%) were expressed in parentheses. Abbreviations: qy, quantitive yield; Bn, benzyl; DBU, 1,8-diazabicyclo[4,3,0]undec-7-ene; BnBr, benzyl bromide; NaH, sodium hydride; THF, tetrahydrofuran; DMF, *N*,*N*-dimethylformamide; Cbz, carbobenzyloxy; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DMAP, (dimethylamino)pyridine.

These results indicated that the binding region of LCA and NA on the 8-kDa domain of pol β was different, and this domain fragment had two inhibitor binding areas. In this study, we modeled the structural interactions of LCA derivatives with the 8-kDa domain of pol β based on the NMR mapping results for the 8-kDa domain with or without LCA or NA. Some of the structurally modified LCAs did not bind to the LCA-binding site but to the NA-binding site. These studies may help to further clarify the structure and function of pol β three-dimensionally and to synthesize a theoretically selective DNA polymerase inhibitor.

MATERIALS AND METHODS

Materials. Nucleotides such as [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP, 43 Ci/mmol) and chemically synthesized template-primers such as poly(dA) and oligo(dT)$_{12-18}$ were purchased from Amersham Biosciences Inc. (Buckinghamshire, U.K.). LCA, NA, and all other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Syntheses of LCA Derivatives. As shown in Scheme 1, benzyl ester (**1′**) of LCA (compound **1**) was obtained from the reaction with benzyl bromide and 1,8-diazabicyclo[5,4,0]-undec-7-ene (DBU) as a catalyst in quantitative yield. The reaction of **1′** and CH_3I with NaH in dry tetrahydrofuran (THF) yielded 3-*O*-methyl ether, and addition of methanol afforded 3-α-methoxy-5-β-cholan-24-oic acid (compound **2**) in 45% yield from **1′**. The reaction of compound **1** with CH_3I and $NaHCO_3$ in dry *N*,*N*-dimethylformamide (DMF) afforded 3-α-hydroxy-5-β-cholan-24-oic acid methyl ester (compound **3**) in 99% yield. The compound **1′** in dry CH_2Cl_2 was reacted with the mixture of (dimethylamino)pyridine (DMAP), 1-ethyl-3-(dimethylaminopropyl)carbodiimide (EDCI), and *N*-carbobenzoxyglycine (*N*-Cbz-Gly) to yield **3′**, which was treated with 20% $Pd(OH)_2$ on carbon under a hydrogen atmosphere to give 3-α-*O*-glycyl-5-β-cholan-24-oic acid (compound **4**) in 96% yield from **1′**. In a similar manner, 3-α-*O*-β-alanyl-5-β-cholan-24-oic acid (compound **5**) was obtained in 81% yield from **1′**, 3-α-*O*-L-phenylalanyl-5-β-cholan-24-oic acid (compound **6**) in 97% yield from **1′**, 3-α-*O*-acetoyl-5-β-cholan-24-oic acid (compound **7**) in 99% yield from **1′**, 3-α-*O*-lauroyl-5-β-cholan-24-oic acid (compound **8**) in 99% yield from **1′**, 3-α-*O*-myristoyl-5-β-chloan-24-oic acid (compound **9**) in 94% yield from **1′**, 3-α-*O*-palmitoyl-5-β-chloan-24-oic acid (compound **10**) in 96% yield from **1′**, and 3-α-*O*-stearoyl-5-β-chloan-24-oic acid (compound **11**) in 99% yield from **1′**. The structures of synthetic products were determined by spectroscopic means including Fourier transform (FT)-NMR and FT-IR.

2. 3-α-Methoxy-5-β-cholan-24-oic acid. $[\alpha]_D$ +26.1 (*c* 1.88, $CHCl_3$). IR: 2939, 2868, 1709, 1379, and 1093 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.36 (s, 3H), 3.17 (m, 1H), 2,39 (m, 1H), 2.24 (m, 1H), 1.96−0.96 (m, 26H), 0.92 (s, 3H), 0.9l (s, 3H), 0.64 (s, 3H). ^{13}C NMR(100 MHz, $CDCl_3$) δ: 179.9, 80.5, 56.4, 55.9, 55.5, 42.7, 42.0, 40.3, 40.1, 35.8, 35.8, 35.2, 34.8, 32.6, 31.1, 30.8, 28.1, 27.3, 26.7, 26.3, 24.2, 23.4, 20.8, 18.2, 12.0.

3. 3-α-Hydroxy-5-β-cholan-24-oic acid methyl ester. $[\alpha]_D$ +34.4 (*c* 0.72, $CHCl_3$). IR: 2939, 2866, 2360, 2341, 1732, and 1377 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.3.66 (s, 3H), 3.62 (m, lH), 2.35 (m, lH), 2.22 (m, lH), 1.99−0.97 (m, 26H), 0.92 (s, 3H), 0.90 (s, 8H), 0.64 (s, 8H). ^{13}C NMR (l00 MHz, $CDCl_3$) δ: 174.8, 71.8, 56.5, 55.9, 51.5, 42.7, 42.1, 40.4, 40.1, 36.4, 35.8, 35.4, 35.3, 34.6, 31.0.

4. 3-α-*O*-Glycyl-5-β-cholan-24-oic acid. $[\alpha]_D$ +34.70 (*c* 0.6, $CHCl_3$). IR: 1751 and 1681 cm^{-1}. ^{1}H NMR (400 MHz, MeOD + TFA-d_1) δ: 4.8l (m, 1H), 3.74 (s, 2H), 2.29 (m, 1H), 2.17 (m, 1H), 1.98−0.99 (m, 26H), 0.92 (s, 3H), 0.89 (s, 3H), 0.64 (s, 3H). ^{13}C NMR (100 MHz, MeOD + TFA-d_1) δ: 178.6, 168.0, 78.3, 57.8, 57.5, 43.9, 43.8, 41.8, 41.8, 37.2, 36.7, 35.9, 35.7, 33.2, 32.8, 32.2, 32.0, 31.9, 29.1, 28.1, 27.5, 25.2, 23.7, 21.9, 18.7, 12.4.

5. 3-α-*O*-β-Alanyl-5-β-cholan-24-oic acid. $[\alpha]_D$ +24.9 (*c* l.14, MeOH). IR: 1712 and 1223 cm^{-1}. ^{1}H NMR (400 MHz, MeOD + TFA-d_1) δ: 4.78 (m, lH), 3.19 (t, 2H, J = 6.8 Hz), 2.71 (t, 2H, J = 6.8 Hz), 2.88 (m, lH), 2.20 (m, lH), 2.04−2.02 (m, 26H), 0.97 (s, 8H), 0.97 (s, 8H), 0.70 (s, 3H). ^{13}C NMR (l00 MHz, MeOD + TFA-d_1) δ: 178.2, 171.7, 76.8, 57.9, 57.5, 43.9, 43.3, 41.8, 41.5, 37.2, 36.7, 36.4, 36.0, 35.7, 33.8, 32.5, 32.3, 32.0, 29.2, 28.2, 27.6, 25.2, 23.8, 21.9, 18.7, 12.5.

6. 3-α-*O*-L-Phenylalanyl-5-β-cholan-24-oic acid. $[\alpha]_D$ +95.2 (*c* 0.59, MeOH + CH_3COOH). IR: 1743 and 1710 cm^{-1}. ^{1}H NMR (400 MHz, MeOD + TFA-d_1) δ: 7.29 (s, 5H), 4.69 (m, lH), 4.16 (t, lH, J = 6.8 Hz), 3.14 (m, 2H), 2.34−2.06 (m, 2H), 1.99−0.96 (m, 26H), 0.89 (s, 3H), 0.89 (s, 3H), 0.62 (s, 3H). ^{13}C NMR (l00 MHz, MeOD + TFA-d_1) δ: 176.2, 170.1, 135.8, 130.6, 130.0, 128.7, 77.9, 67.1, 57.8, 57.4, 43.9, 43.1, 41.7, 41.4, 38.0, 37.1, 36.6, 35.9, 35.6, 33.1, 32.2, 31.8, 29.2, 28.1, 27.5, 27.3, 25.2, 23.8, 21.9, 18.8, 12.5.

7. 3-α-*O*-Acetoyl-5-β-cholan-24-oic acid. The data were described in ref 9.

8. 3-α-*O*-Lauroyl-5-β-cholan-24-oic acid. $[\alpha]_D$ +31.5 (*c* 0.74, $CHCl_3$). IR: 2929, 2858, 2360, 2341, and 1716 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.73 (m, lH), 2.62 (t, 2H, J = 7.6 Hz), 2.42−2.22 (m, 2H), 1.98−1.00 (m, 44H) 0.93 (s, 3H), 0.92 (s, 3H), 0.88 (t, 3H, J = 6.8 Hz), 0.64 (s, 3H). ^{13}C NMR (l00 MHz, $CDCl_3$) δ: 179.8, 173.5, 74.1, 56.5, 56.0, 42.7, 41.9, 40.4, 40.1, 35.8, 35.3, 35.0, 34.8, 34.6, 32.8, 31.9, 30.9, 30.7, 29.6, 29.4, 29.8, 29.2, 29.1, 28.2, 27.0, 26.7, 26.3, 25.0, 24.2, 23.3, 22.7, 20.8, 18.2, 14.1, 12.0.

9. 3-α-*O*-Myristoyl-5-β-chloan-24-oic acid. $[\alpha]_D$ +26.6 (*c* 0.88, $CHCl_3$). IR: 2929, 2856, and 1712 cm^{-1}. ^{1}H NMR (400

MHz, $CDCl_3$) δ: 4.73 (m, lH), 2.44−2.22 (m, 2H), 2.26 (t, 2H, $J = 7.2$ Hz), 1.98−1.00 (m, 48H), 0.97 (s,3H), 0.92 (s, 3H), 0.88 (t, 3H, $J = 6.8$ Hz), 0.65 (s, 3H). ^{13}C NMR (l00 MHz, $CDCl_3$) δ: 179.3, 173.5, 74.1, 56.5, 56.0, 42.7, 41.9, 40.4, 40.1, 35.8, 35.3, 35.0, 34.8, 34.6, 32.3, 31.9, 30.8, 30.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 29.1, 28.2, 27.0, 26.7, 26.8, 25.1, 24.2, 23.3, 22.7, 20.8, 18.2, 14.1, 12.0.

10. 3-α-*O*-Palmitoyl-5-β-chloan-24-oic acid. $[\alpha]_D$ +29.4 (*c* 0.71, $CHCl_3$). IR: 2929, 2856, and 1712 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.73 (m, lH), 2.43−2.2l (m, 2H), 2.26 (t, 2H, $J = 7.6$ Hz), 1.98−1.00 (m, 52H), 0.93 (s, 3H), 0.91 (s, 3H), 0.88 (t, 8H, $J = 6.8$ Hz) 0.65 (s, 8H). ^{13}C NMR (l00 MHz, $CDCl_3$) δ: 180.1, 173.5, 74.1, 56.5, 56.0, 42.7, 41.9, 40.4, 40.1, 35.8, 35.3, 35.0, 34.8, 34.6, 32.3, 31.9, 31.1, 30.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.2, 29.1, 28.2, 27.0, 26.7, 26.3, 25.1, 24.2, 23.3, 22.7, 20.8, 18.2, 14.1, 12.0.

11. 3-α-*O*-Stearoyl-5-β-chloan-24-oic acid. $[\alpha]_D$ +27.4 (*c* 0.77, $CHCl_3$). IR: 2927, 2854, and 1708 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 4.73 (m, lH), 2.42−2.19 (m, 2H), 2.26 (t, 2H, $J = 7.2$ Hz), 1.98−1.00 (m, 56H), 0.93 (s, 3H), 0.91 (s, 3H), 0.88 (t, 3H, $J = 7.2$ Hz), 0.65 (s, 3H). ^{13}C NMR (l00 MHz, $CDCl_3$) δ: 179.8, 173.5, 74.1, 56.5, 56.0, 42.7, 41.9, 40.4, 40.1, 35.8, 35.4, 35.0, 34.8, 34.6, 32.8, 31.9, 31.2, 30.9, 29.6, 29.5, 29.4, 29.3, 29.1, 28.2, 27.0, 26.7, 26.3, 25.1, 24.2, 23.3, 22.7, 20.8, 18.3, 14.1, 12.0.

Enzymes. We purified pol α from calf thymus by immunoaffinity-column chromatography as described previously (*10*). Recombinant rat DNA polymerase β (pol β) was purified from *Escherichia coli* JMpβ5 as described by Date et al. (*11*). The C-terminal 31-kDa domain fragment was prepared by controlled proteolysis at 25 °C with trypsin (1500:1, w/w) for 90 min. This fragment was purified as previously described by Kumar et al. (*5*, *6*). The N-terminal 8-kDa domain of rat pol β (residues 2−87) was overexpressed in *E. coli* strain BL21 harboring the expression plasmid "Lys-87" constructed in our laboratory. Overproduction of the N-terminal 8-kDa domain and the purification procedure were performed essentially as described in our previous report (*8*). pol I (α-like) and II (β-like) from a higher plant, cauliflower inflorescence, were purified according to the methods outlined by Sakaguchi et al. (*12*). *E. coli* Klenow fragment of pol I and human immunodeficiency virus type 1 (HIV-1) reverse transcriptase were purchased from Worthington Biochemical Corp. (Freehold, NJ). T4 DNA polymerase, *Taq* DNA polymerase, and calf-terminal deoxynucleotidyl transferase were purchased from Takara (Tokyo, Japan). Bovine pancreas deoxyribonuclease I was purchased from Stratagene Cloning Systems (La Jolla, CA).

DNA Polymerase Assay. The activities of DNA polymerases (0.05 units each) were measured as described previously (*7*, *13*). For DNA polymerases, poly(dA)/oligo(dT)$_{12-18}$ and dTTP were used as the template-primer DNA and nucleotide substrate, respectively. The substrates of terminal deoxynucleotidyl transferase used were oligo(dT)$_{12-18}$ (3′-OH) and dTTP as the template primer and nucleotide substrate, respectively. For HIV reverse transcriptase, poly(rA)/oligo(dT)$_{12-18}$ and dTTP were used as the template primer and nucleotide substrate, respectively. LCA and its derivatives were dissolved in dimethyl sulfoxide (DMSO) at various concentrations, sonicated for 30 s, and then added to the reaction mixture. The activity without the inhibitor was considered to be 100%, and the remaining activity at each inhibitor concentration was determined as a percentage of this value. A total of 1 unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleoside triphosphates (i.e., dTTP) into synthetic template primers (i.e., poly(dA)/oligo(dT)$_{12-18}$, A/T = 2:1) in 60 min at 37 °C under the normal reaction conditions for the enzyme (*7*, *13*).

Other Enzyme Assays. Bovine deoxyribonuclease I was measured in each of the standard assays according to the specifications of the manufacturer as described by Lu and Sakaguchi (*14*), respectively.

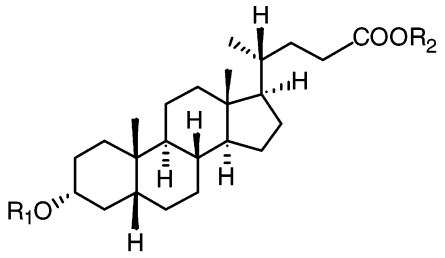
Gel Mobility Shift Assay. The gel mobility shift assay was carried out as described by Casas-Finet et al. (*15*). The binding mixture (a final volume of 20 μL) contained 20 mM Tris-HCl at pH 7.5, 40 mM KCl, 50 μg/mL bovine serum albumin (BSA), 10% dimethyl sulfoxide, 2 mM EDTA, 2.2 nmol of M13 plasmid DNA (single stranded and singly primed), and the 8-kDa domain fragment of purified pol β. Various concentrations of LCA and its derivatives were added to the binding mixture. The mixture was incubated at 25 °C for 30 min. Samples were run on 1.2% agarose gel in 0.1 M Tris-acetate at pH 8.3, containing 5 mM EDTA at 50 V for 2 h. The agarose gel was stained and detected with ethidium bromide.

Surface Plasmon Resonance Analysis. pol β and LCA derivatives binding analyses were performed using a Biosensor Biacore instrument (BIACORE X) (Biacore, Sweden). CM5 research-grade sensor chips (Biacore, Sweden) were used. All buffers were filtered before use. The 8-kDa domain fragment of pol β (1.87 nmol) in coupling buffer (10 μM sodium acetate at pH 4.7) was injected over a CM5 sensor chip at 20 μL/min to capture the protein in the carboxymethyl dextran matrix of the chip by the NHS/EDC coupling reaction (60 μL of mix) as described (*16*). Unreacted *N*-hydroxysuccinimide ester groups were inactivated using 1 M ethanolamine-HCl (pH 8.0). This reaction immobilized about 5000 response units (RU) of both proteins. Binding analysis of LCA derivatives was performed in a running buffer including the compounds (5 mM potassium phosphate buffer (pH 7.0) and 10% DMSO) at a flow rate of 20 μL/min at 25 °C. Kinetic parameters were determined using BIA evaluation 3.1 software.

LCA Derivatives Docking Modeling. The molecular docking of LCA derivatives and the 8-kDa domain of pol β were done using a fixed docking procedure in the Affinity program within Insight II modeling software (Accerlys Inc., San Diego, CA). The calculations used a CVFF force field in the Discovery program and a Monte Carlo strategy in the Affinity programs (*17*). Each energy-minimized final-docking position of LCA derivatives was evaluated using the interactive score function in the Ludi module. The Ludi score includes the contribution of the loss of translational and rotational entropy of the fragment, number and quality of hydrogen bonds, and contributions from ionic and lipophilic interactions to the binding energy.

RESULTS AND DISCUSSION

Effects of LCA Derivatives on the Activity of Rat DNA Polymerase β. As briefly described in the Introduction, we found and reported that LCA is an inhibitor of eukaryotic DNA polymerases, and among the DNA polymerases, the β



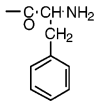
Compound	R_1	R_2
1	H	H
2	CH_3	H
3	H	CH_3
4	-CO-CH_2-NH_2	H
5	-CO-$(CH_2)_2$-NH_2	H
6		H
7	CO-CH_3	H
8	CO-$(CH_2)_{10}$-CH_3	H
9	CO-$(CH_2)_{12}$-CH_3	H
10	CO-$(CH_2)_{14}$-CH_3	H
11	CO-$(CH_2)_{16}$-CH_3	H

FIGURE 1: Structure of LCA and its derivatives: compound **1**, LCA (3-α-hydroxy-5-*β*-cholan-24-oic acid); compound **2**, 3-α-methoxy-5-*β*-cholan-24-oic acid; compound **3**, 3-α-hydroxy-5-*β*-cholan-24-oic acid methyl ester; compound **4**, 3-α-*O*-glycyl-5-*β*-cholan-24-oic acid; compound **5**, 3-α-*O*-*β*-alanyl-5-*β*-cholan-24-oic acid; compound **6**, 3-α-*O*-L-phenylalanyl-5-*β*-cholan-24-oic acid; compound **7**, 3-α-*O*-acetoyl-5-*β*-cholan-24-oic acid; compound **8**, 3-α-*O*-lauroyl-5-*β*-cholan-24-oic acid; compound **9**, 3-α-*O*-myristoyl-5-*β*-chloan-24-oic acid; compound **10**, 3-α-*O*-palmitoyl-5-*β*-chloan-24-oic acid; and compound **11**, 3-α-*O*-stearoyl-5-*β*-chloan-24-oic acid.

type was most sensitive to inhibition by LCA (*1*). The purpose of this study was to investigate the inhibitory mechanism more precisely by using 10 chemically synthesized derivatives of LCA prepared in our laboratory. The chemical structure of LCA and 10 derivatives are shown in Figure 1.

LCA (compound **1**) dose-dependently inhibited the activities of pol α and *β*, and the IC_{50} value of compound **1** were 52.1 and 11.0 *μ*M, respectively (parts A and B of Figure 2 and Table 1). The inhibitory effects of pol α and *β* by 3-α-methoxy-LCA (compound **2**) were stronger than those by LCA, and the inhibition of pol *β* activity by compound **2** was approximately 10-fold stronger than that of pol α activity. The carboxyl group at carbon position 21 in LCA (i.e., R_2 of LCA in Figure 1) is thought to be important for the inhibition of pol α and *β*, because the compound that was modified from the carboxyl group to a methyl ester (compound **3**) could not inhibit the activity (Table 1). Compounds **1** and **2** inhibited the activities of pol α and *β* more potently than the compounds that were modified at carbon position 3 in LCA (i.e., R_1 of LCA in Figure 1) from the hydrogen group to amino acid groups containing the hydrophilic group such as $-NH_2$ (i.e., compounds **4**−**6**), which were weaker than those of compounds **1** and **2** (Table 1).

Compounds **7**−**11** are the esters of acetic acid (C2), lauric acid (C12), myristic acid (C14), palmitic acid (C16), and stearic acid (C18) at position C3 of α-OH of LCA (i.e., R_1 of LCA in Figure 1), respectively. The inhibition of pol *β* activity by these compounds was stronger than that of pol α activity (parts C and D of Figure 2 and Table 1). In the chemically synthesized compounds, the inhibition of pol *β* by compound **9** was the strongest with an IC_{50} value of 0.35 *μ*M. The inhibitory effect of pol *β* activity by compound **9** was stronger than those of compounds **8** and **10**; therefore, the molecular length of compound **9** may be the best fit to bind to pol *β*. The length of C16 fatty-acid-conjugated LCA (i.e., compound **9**) is 25.65 Å (Figure 5K), and the three-dimensional molecular structure of compound **9** is suggested to be similar to that of NA, which is a C24-long chain monounsaturated fatty acid (parts G and J of Figure 5). Compounds **1**, **2**, **9**, and NA were used in the later experiments.

The thermal transition profiles of double- to single-stranded DNA with or without LCA derivatives showed that these compounds cannot bind to the DNA molecule (data not shown). These results suggested that LCA derivatives must inhibit the enzyme activities by interacting with the enzymes directly.

Effects of LCA Derivatives on the Activities of DNA Metabolic Enzymes. LCA and its derivatives such as compounds **1**, **2**, and **9** had no significant influence the activities of higher plant, cauliflower, pol α and *β*, prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* pol I, *Taq* DNA polymerase, and T4 DNA polymerase. The three-dimensional structures of mammalian DNA polymerases are greatly different from those of plant and prokaryotic DNA polymerases. Compounds **1** and **2** also did not inhibit any of the activities of other DNA-metabolic enzymes such as calf DNA primase of pol α, calf-terminal deoxynucleotidyl transferase, HIV-1 reverse transcriptase, and bovine deoxyribonuclease I. On the other hand, compound **9** inhibited the activities of terminal deoxynucleotidyl transferase and reverse transcriptase, but it did not influence the activities of primase of pol α and deoxyribonuclease I. Because this manner of inhibition by compound **9** was the same tendency as that by NA, compound **9** might be of the same inhibitory mode as NA. When activated DNA was used as the template-primer DNA instead of poly(dA)/oligo-$(dT)_{12-18}$, the inhibition modes of these compounds did not change. Therefore, the derivatives should be classified as inhibitors of mammalian DNA polymerases, especially pol *β*.

Analysis of Binding between LCA Derivatives and DNA Polymerase β. We prepared the whole enzyme of pol *β* with a molecular weight of 39 kDa, as well as the two domain fragments of 8 kDa, which is the template-primer DNA-binding domain, and 31 kDa, which is the catalytic part involved in DNA polymerization (see the Materials and Methods). Both fragments were obtained by controlled proteolysis and purified by FPLC Superose 12 chromatography to near homogeneity (see Figure 4 in ref 4).

The template-primer DNA-binding activity of the 8-kDa domain was analyzed by gel mobility shift assay. Figure 3 shows the results of the gel shift assay of single-stranded (ss) M13 plasmid DNA, the 8-kDa domain fragment of the

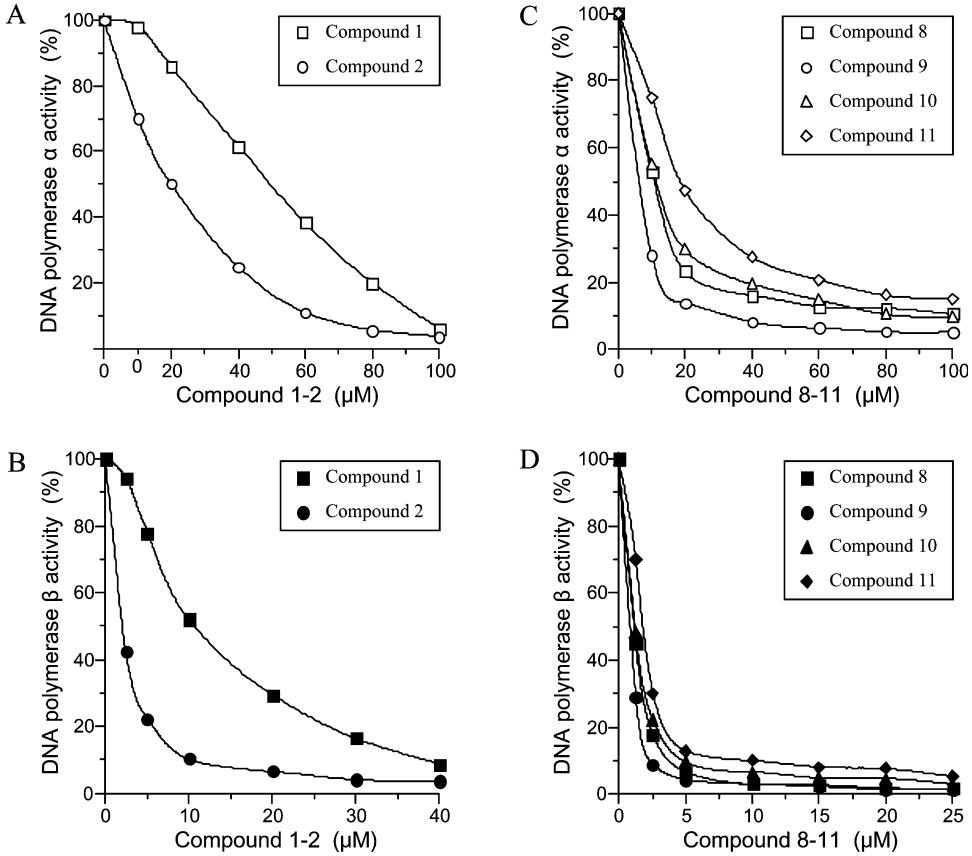


FIGURE 2: Mammalian DNA polymerase inhibition dose−response curves of LCA derivatives. The enzymes used (0.05 units of each) were calf pol α (A and C) and rat pol β (B and D). Compounds **1**, **2**, and **8**−**11** were measured at indicated concentrations. DNA polymerase activity in the absence of these compounds was taken as 100%.

Table 1: IC_{50} Values of LCA Derivatives on the Activities of Mammalian pol α and β

	IC_{50} value (μM)			IC_{50} value (μM)	
compound	pol α	pol β	compound	pol α	pol β
1 (LCA)	52.1	11.0	**7**	48.5	9.8
2	20.0	2.1	**8**	10.6	1.1
3	>500	>500	**9**	7.7	0.35
4	114.5	60.5	**10**	11.4	1.2
5	99.5	91.4	**11**	18.5	2.0
6	50.3	21.0	nervonic acid	1.1	2.8

pol-β-binding complex (lanes 2−10). In the binding assay, 0.2 nmol of the domain was added to M13 DNA at a concentration of 2.2 nmol (nucleotide). The 8-kDa domain fragment bound to M13 DNA and shifted in the gel (lane 2), but not the 31-kDa fragment, the catalytic domain without a DNA-binding site (see Figure 5A in ref 4). The molecular ratios of compounds and the 8-kDa domain are shown as inhibitor and enzyme (I/E) ratios in Figure 3. Compounds **1** (LCA) and **2** interfered with the complex formation M13 DNA and the 8-kDa domain to the same extent. At the I/E ratio of 10, interference by all of the compounds was complete (lanes 3, 5, 7, and 9). At a ratio of 1, interference by compounds **1** and **2** disappeared (lanes 4 and 6) and interference by compounds **9** and NA was nearly complete (lanes 8 and 10). These results suggested that one or a few molecules of compounds **1** and **2** or only one molecule of compounds **9** and NA competes with each molecule of M13 ssDNA and subsequently interferes with the binding of the 8-kDa domain fragment to DNA. The inhibitory activities of both of the 8-kDa domain binding to ssDNA and DNA

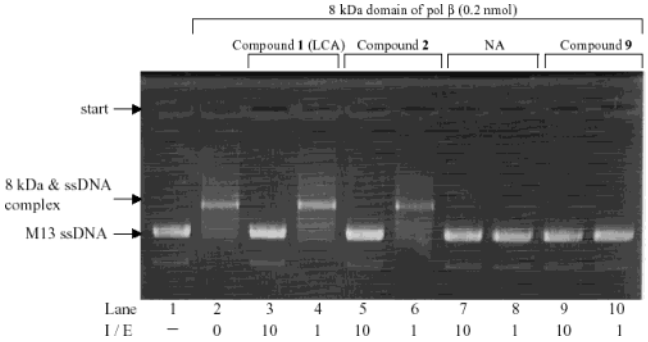


FIGURE 3: Gel mobility shift analysis. Gel shift analysis of binding between M13 single-stranded plasmid DNA and the 8-kDa domain of pol β. M13 DNA (2.2 nmol; nucleotide) was mixed with purified proteins and compounds. Lanes 2−10 contained the purified 8-kDa domain at a concentration of 0.2 nmol; lane 1 contained no enzyme. Lanes 3, 5, 7, and 9 were mixed with 2 nmol of compound **1**, compound **2**, NA, and compound **9**, respectively. Lanes 4, 6, 8, and 10 were mixed with 0.2 nmol of compound **1**, compound **2**, NA, and compound **9**, respectively. Samples were run on a 1.2% agarose gel in 0.1 M Tris-acetate (pH 8.3) containing 5 mM EDTA at 50 V for 2 h. A photograph of an ethidium-bromide-stained gel is shown.

polymerization of pol β by compounds **9** and NA were stronger than those by compounds **1** and **2**. The inhibitory mode of pol β by compounds **9** and NA might be different from that of compounds **1** and **2**. Like LCA (*1*), the derivatives must compete with the DNA template primer to bind to the 8-kDa domain.

The question thus arose as to whether the derivatives have a direct influence on the catalytic site. The catalytic 31-kDa domain fragment can bind to the DNA template primer,

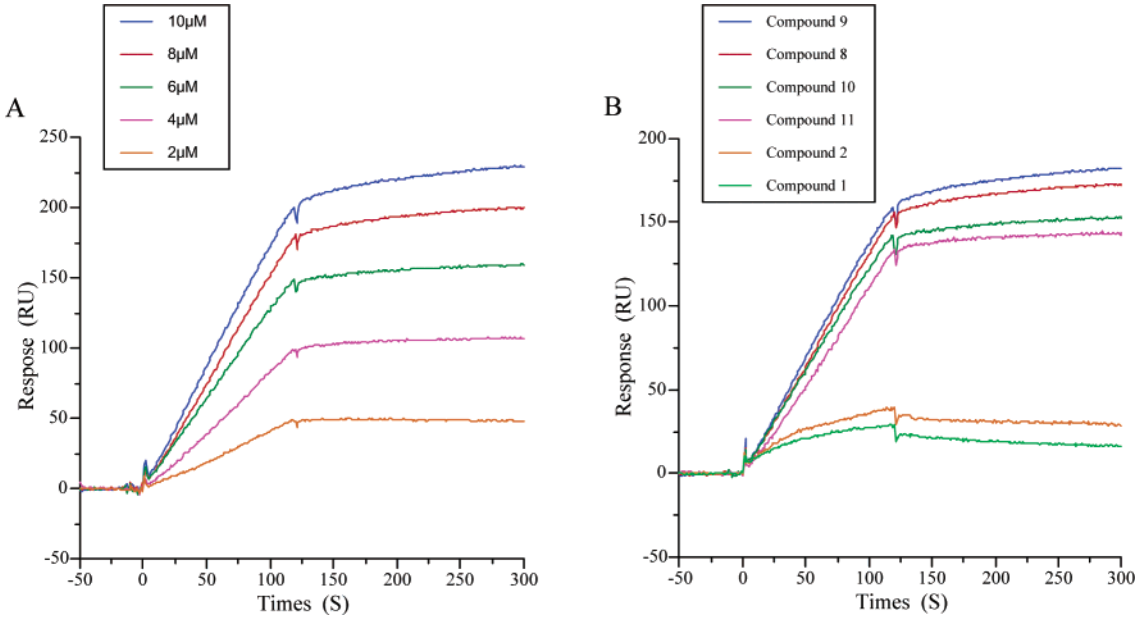


FIGURE 4: Biacore analysis of binding of LCA derivatives to immobilize the 8-kDa domain of pol β. (A) Five different indicated concentrations of compound **9** were injected over the 8-kDa domain for 120 s at 20 μL/min and dissociated for 180 s at 20 μL/min. (B) A total of 8 μM of compounds **1**, and **2**, and **8**−**11** were injected over the 8-kDa domain for 120 s at 20 μL/min and dissociated for 180 s at 20 μL/min. Binding to compounds was detected by the surface plasmon resonance signal (Biacore; see the Materials and Methods) and is indicated in response units. The background resulting from injection of the running buffer alone was subtracted from the data before plotting.

Table 2: Binding and Dissociation Constants between the 8-kDa Domain of pol β and LCA Derivatives[a]

compound	K_a (nM^{-1})	K_d (nM)	compound	K_a (nM^{-1})	K_d (nM)
1 (LCA)	1.29×10^{-4}	7710	**10**	1.25×10^{-1}	8.02
2	2.89×10^{-4}	3460	**11**	1.03×10^{-1}	9.68
8	3.19×10^{-1}	3.13	nervonic acid	5.88×10^{-2}	17.0
9	5.78×10^{-1}	1.73			

[a] K_a = binding constant, and K_d = dissociation constant.

although weakly. The 31-kDa domain fragment synthesized DNA without interference from any of the derivatives and NA, because DNA was synthesized even at an I/E ratio for the 31-kDa fragment of 1000 (data not shown). These results suggested that these compounds indirectly influence DNA polymerization on the 31-kDa catalytic site as a result of a lack of template primer and to compete with the substrate.

Binding between LCA Derivatives and the 8-kDa Domain of DNA Polymerase β. To confirm the biochemical data, the parameters for binding of LCA and its derivatives were determined using the 8-kDa domain of pol β immobilized to the sensor chip in a Biacore. Five different concentrations of compound **9**, which was the strongest pol β inhibitor in the synthesized LCA derivatives, were used for the binding analysis (Figure 4A). The 8-kDa domain (1.87 nmol each) was conjugated to the CM5 sensor chip, and then the compound (2, 4, 6, 8, and 10 μM) was added to the conjugated proteins. Compound **9** bound to the 8-kDa domain and dissociated from the protein (Figure 4A). Compound **9** also bound to the 39-kDa pol β at almost the same rate as the 8-kDa domain (data not shown). The dissociation constant (K_d) of binding of compound **9** to the 8-kDa domain was determined to be 1.73 nM from the data in Figure 4A (Table 2). Therefore, compound **9** directly interacts with the 8-kDa domain.

A total of 8 μM of the LCA derivatives was analyzed to determine whether it would bind to the 8-kDa domain (Figure 4B). All of the compounds bound and dissociated from the protein. From the responses by the Biacore instrument, the 8-kDa domain binding of the derivatives can distinguish two groups; one is compounds **1** and **2**, and the other is compounds **8**−**11**. The response of compound **2** was higher than that of compound **1**. The response of compound **9** was the highest in compounds **8**−**11**. The K_d values of these compounds from Biacore analyses (Table 2) paralleled the observed IC_{50} values for pol β from the dose−response curves of parts B and D of Figure 2.

Modeling of the LCA Derivatives Interaction Interface on the 8-kDa Domain of pol β. Three-dimensional structural analysis using NMR indicated that the 8-kDa domain of pol β (residues 1−87) was formed by four α helices, packed as two antiparallel pairs (*18*, *19*). The pairs of α helices cross one another at 50°, giving them a V-like shape. The 8-kDa domain contains a "helix−hairpin−helix" motif (parts C, F, I, and L of Figure 5). The complex is in fast exchange on the NMR time scale, permitting us to follow the chemical-shift changes of the backbone NH and ^{15}N signals of the 8-kDa domain on complex formation by recording a series of ^{1}H-^{15}N HMQC spectra of the uniformly ^{15}N-labeled 8-kDa domain in the presence of LCA (compound **1**). Parts A and B of Figure 5 show the three-dimensional molecular structures of compound **1**, and the molecular length is 13.59 Å. From the NMR experiments and the docking simulation studies, the compound-**1**-binding interface of the 8-kDa domain consisted of one region mostly consisting of Lys60 in helix 3, Leu77 in helix 4, and Thr79 in the 79−87 unstructured linker segment (Figure 5C). Compound **1** must enter the pocket (i.e., the crevice between helix 3 and helix 4) formed by the three amino acids (i.e., Lys60, Leu77, and Thr79) in the 8-kDa domain.

Prasad et al. reported that template DNA [i.e., $p(dT)_8$] binding activity was impaired in site-directed mutants of Phe25, Lys35, Lys60, or Lys68 (*20*). The Helix-3−hairpin−

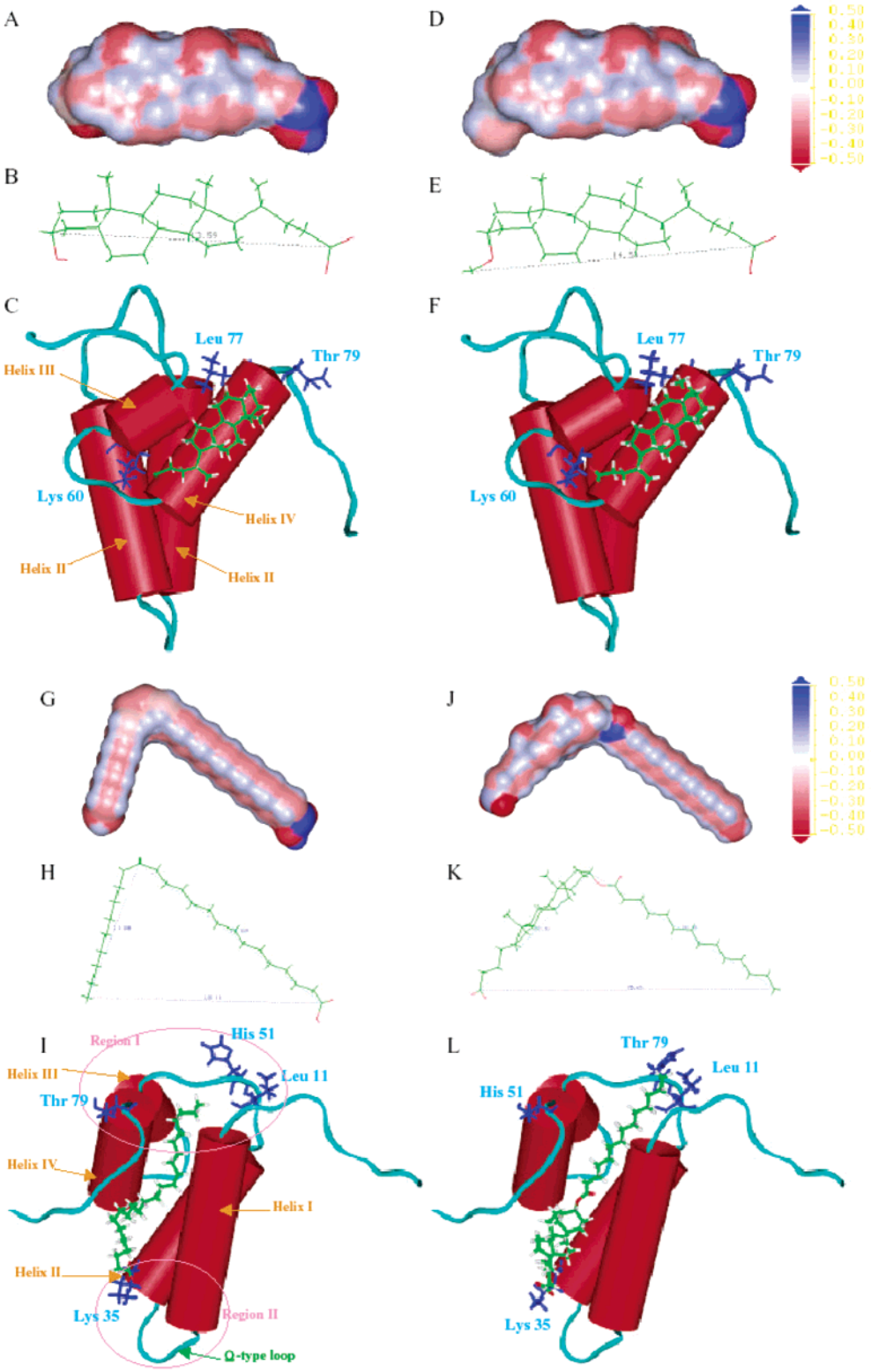


FIGURE 5: Docking simulation of LCA derivatives and the 8-kDa domain of pol β. (A, D, G, and J) Computer graphics of compound **1** (LCA) (A), compound **2** (D), NA (G), and compound **9** (J). Electrostatic potentials over molecular surfaces in the front view were analyzed using WebLab Viewer Lite (version 3.2, Accerlys Inc., San Diego, CA) software. Blue areas are hydrophilic, red areas are hydrophobic, and white areas are neutral. (B, E, H, and K) Stick models of compound **1** (B), compound **2** (E), NA (H), and compound **9** (K). (C, F, I, and L) Simulation of LCA derivatives interaction interface on the 8-kDa domain of pol β. Interactions of compound **1** (C), compound **2** (F), NA (I), and compound **9** (L). (C and F) Amino acid residues Lys60, Leu77, and Thr79, which were significantly shifted as the cross peaks from ^{1}H-^{15}N HMQC NMR experiments adding compound **1**, are depicted in blue (*1*). (I and L) Amino acid residues Leu11, Lys35, His51, and Thr79, which were significantly shifted as the cross peaks from ^{1}H-^{15}N HMQC NMR experiments adding NA, are depicted in blue (*8*). The Cα backbones of the X-ray crystal structures of the 8-kDa domain of pol β are shown in water blue. The carbons, oxygens, and hydrogens of the compounds were indicated as green, red, and white, respectively. The Protein Data Base codes of the 8-kDa domain of pol β is 1BNO. This figure was displayed using Insight II/Affinity (Accerlys Inc., San Diego, CA).

Table 3: Molecular Length of Three-Dimensional Structure of LCA Derivatives Calculated by Computer Simulation[a]

compound	molecular length (Å)	compound	molecular length (Å)
1 (LCA)	13.59	**10**	27.90
2	14.53	**11**	29.92
8	23.64	nervonic acid	18.14
9	25.65		

[a] These data were obtained by Insight II (Accerlys Inc.).

Helix-4 motif and residues in an adjacent Ω-type loop connected helix 1 and helix 2 from the ssDNA interaction surface. Because compound **1** bound to the ssDNA-binding region of the 8-kDa domain and competed for binding with template DNA as shown in Figure 3, two of the maps were compared. The only amino acid residue shifted by both compound **1** binding and ssDNA binding was Lys60 in helix 3. Leu77 and Thr79 are different from the other DNA-binding sites (Phe25, Lys35, and Lys68). Compound **1** probably competes with template DNA at residue Lys60 and binds to the site, which subsequently inhibits the ssDNA-binding activity on the 8-kDa domain of pol β.

Compound **2**, which is of the $-OCH_3$ group instead of the $-OH$ group, was located at carbon position 3 in compound **1** (parts A and D of Figure 5). The molecular length of compound **2** is 0.94 Å longer than that of compound **1** (parts B and E of Figure 5 and Table 3). The docking simulation of the 8-kDa domain of pol β and compound **2** was shown in Figure 5F. The binding interface of the 8-kDa domain of compound **2** was in the same position as that of compound **1**, and the fitting of compound **2** to the pocket (i.e., the crevice between helix 3 and helix 4) was better than that of compound **1**. These results suggested that the molecular length of the compound was important for both fitting to bind the pocket and the inhibitory activity of DNA polymerase and ssDNA binding.

As was also reported previously, unsaturated long-chain fatty acids such as NA were also potent inhibitors of pol β (*7*). The two-dimensional ^{1}H-^{15}N HMQC NMR spectra for the 8-kDa-domain−NA complex were analyzed (*8*). NA contains a free carboxyl group and a double bond in the cis configuration. The length of the three-dimensional structure of the NA molecule was shown to be 18.14 Å on a computer simulation (parts G and H of Figure 5). From the analyses of amino acid residues displaying chemical-shift changes on binding to NA in the solution structure of the 8-kDa domain with or without NA, the NA-binding interface of the 8-kDa domain consists of two regions: one consists of Leu11 in the 1−13 unstructured segment, His51 in the 45−55 turn, and Thr79 in the 79−87 unstructured linker segment ("region I" in Figure 5I), while the other consists of an Ω-type loop including helix 1 and helix 2 ("region II" in Figure 5I). The only site shifted not only by NA binding but also by ssDNA binding was Lys35 in the Ω-type loop including helix 1 and helix 2. NA probably competes with template DNA at residue Lys35 and binds to the site, which subsequently inhibits the ssDNA-binding activity on the 8-kDa domain.

Compounds **8**−**11** have almost the same three-dimensional molecular structures as NA, such as "V-shape" (parts G and J of Figure 5). The molecular lengths of these compounds were shown in Table 3. Because compound **9** was the strongest inhibitor of pol β in compounds **8**−**11** and NA, the molecular length of compound **9** (i.e., 25.65 Å) might be the best fit to the pocket of the 8-kDa domain. Therefore, a docking simulation of compound **9** and the 8-kDa domain of pol β was performed, with the compound-**9**-binding interface of the 8-kDa domain being the same pocket (i.e., the crevice between helix 1 and helix 2) as NA (Figure 5L). These results suggested that Lys35, which is a hydrophilic amino acid in "region II", bound to the carboxyl ends of compound **9** and that Leu11 and His51, which are hydrophilic amino acids in "region I", bound to the methyl end of compound **9**.

In conclusion, the inhibitory effects of LCA and its derivatives on pol β activity occur by the binding between the 8-kDa domain and the compounds as a 1:1 complex. On the pol β protein, one molecule of compounds **2** and **9** competed with one molecule of the template-primer DNA and subsequently interfered with the binding of the template primer to the 8-kDa domain (Figure 3). The binding of these compounds indirectly inhibited catalytic activity on the 31-kDa domain. These data indicated that there were at least two compound-binding pockets for sterol inhibitors such as LCA in the 8-kDa domain of pol β. The inhibitory action of pol β by compound **2** was similar to the effect by compound **9**, although compounds **2** and **9** are structurally unrelated. Both the hydrophilic end (carboxyl group) and hydrophobic sites in the compound played crucial roles in the inhibition. The hydrophilic carboxyl group at carbon position 21 in compounds **2** and **9** are thought to be important for the inhibition of pol β, because the compound that was modified from the carboxyl group to a carboxyl ester (i.e., compound **3**) could not inhibit the activity (Table 1). The terpenoid rings and alkyl chain (i.e., hydrophobic site) of the compound are also required for inhibition. The hydrophilic ssDNA-binding amino acid residues in the 8-kDa domain interact with compounds **2** and **9** (i.e., Lys60 for compound **2** and Lys35 for compound **9**), although the binding to the 8-kDa domain includes the DNA-binding amino acids residues.

Inhibitors of DNA polymerases such as pol α could be anticancer agents as reported previously (*21*), and the polymerases have emerged as important cellular targets for chemical intervention in the development of anticancer agents. The chemical LCA frames could be used for screening new anticancer chemotherapy agents. Compound **9**, which is the strongest pol α and β inhibitor in the synthesized LCA derivatives, could prevent the growth of human gastric cancer cells (NUGC-3) (data not shown). Moreover, these compounds may lead to the blockage of DNA synthesis of base excision repair by the inhibition of pol β activity to rescue the cells containing the damaged DNA under clinical radiation therapy or chemotherapy. LCA derivatives such as compound **9** could be useful in developing a drug design strategy for cancer chemotherapy agents, which help clinical radiation therapy or cancer chemotherapy.

The synthesized compounds based on LCA (compound **1**) inhibited the activities of pol α and β more potently than compound **1**. We would like to develop a theoretically ideal DNA polymerase inhibitor from structural relationship studies of previously reported fatty acids and LCA derivatives, which were investigated in this study.

ACKNOWLEDGMENT

We are grateful to Dr. A. Matsukage of Japan Women's University and Dr. T. Date of Kanazawa Medical University for preparing rat pol β and its 8-kDa domain fragment, respectively.

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BI049307R