Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 287–290

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Biotinylated Lithocholic Acids for Affinity Chromatography of Mammalian DNA Polymerases α and β

Madoka Watanabe,[a] Shinya Hanashima,[a] Yoshiyuki Mizushina,[b] Hiromi Yoshida,[b] Masahiko Oshige,[a] Kengo Sakaguchi[a] and Fumio Sugawara[a,*]

[a]*Department of Applied Biological Science, Frontier Research Center for Genomic Drug Discovery, Tokyo University of Science, Noda, Chiba 278-8510, Japan*

[b]*Laboratory of Food & Nutritional Sciences, Department of Nutritional Science, High Technology Research Center, Kobe-Gakuin University, Nishi-ku, Kobe, Hyogo 651-2180, Japan*

Received 6 July 2001; accepted 26 October 2001

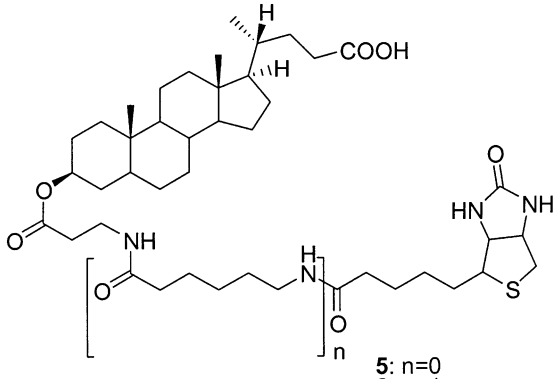
Abstract—Biotinylated lithocholic acids have been synthesized. The compounds inhibited mammalian DNA polymerases α and β with dose-dependent manner. The streptavidine columns conjugated with the synthetic biotinylated compounds chromatographed both two enzymes eluted by KCl solution at the different concentrations.

Lithocholic acid (LCA, **1**), one of secondary bile acids, is known to promote tumorigenesis in rats induced by the monoalkylating agent, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine.[1,2] LCA inhibits the activity of eukaryotic DNA polymerase (pol) β, which is known to be involved in DNA excision repair, and DNA pol α, which roles DNA replication process in cell cycle.[3] The IC_{50} value of DNA pol α is less than that of DNA pol β (Fig. 1). These observations suggest the possible mechanisms for tumorigenesis by LCA. Recently, Mizushina et al. indicated that LCA binds the template-primer binding domain (8 kDa) but not to the catalytic domain (31 kDa) of DNA pol β. On ^{1}H–^{15}N HMQC NMR experiment of DNA pol β associated with LCA, the 8 kDa domain bound to LCA as a 1:1 complex.[4] Three amino acid residues (Lys60, Leu77 and Thr79) in this domain were mainly interacted with LCA. It is important to isolate and use the specific inhibitors of DNA pol as molecular probes to understand not only in vivo functions but also precise biological cross talks in cell cycle. It is also mentioned that the DNA pol inhibitors can be useful for cancer chemotherapy with DNA targeting drugs such as bleomycin.[5]

The isolations of DNA pols by using many types of column chromatography (e.g., phoshocellulose, human serum conjugated sepharose, rabbit IgG conjugated sepharose and then monoclonal antibody conjugated sepharose column chromatography for DNA pol β) are still laborious and time-consuming works, because recent researches have revealed that eukaryotic cells contain at least twelve types of DNA pol. From this reason, we aimed to synthesize biotinylated LCAs and develop affinity column chromatography for further biochemical research of DNA pol.

Synthesis of Biotinylated LCAs

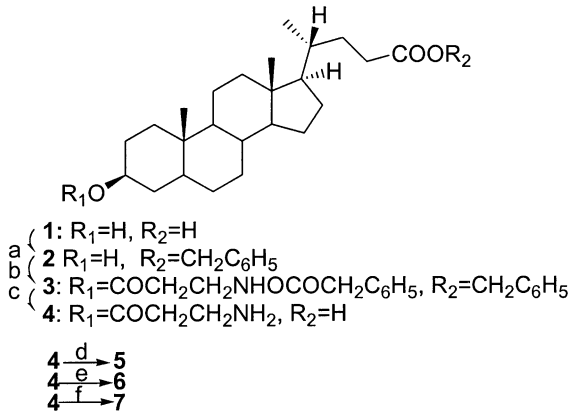
Benzyl ester (**2**) of LCA (**1**) was prepared from the reaction of benzyl bromide and DBU in benzene, then 3-hydroxyl was protected with the treatment of CBZ β-alanine with EDCI/DMAP in CH_2Cl_2 to give **3**. After debenzylation with $Pd(OH)_2$ under hydrogen atmosphere, the resulting amine (**4**) was treated with *N*-hydroxysuccinimide-biotin, biotinamidocaproate *N*-hydroxysuccinimide ester and 6-(biotinamidecaproylamide)caproic acid *N*-succinimide ester in DMF in the presence of Et_3N to give **5**, **6** and **7**, respectively (Scheme 1).



5: n=0
6: n=1
7: n=2

*Corresponding author. Tel.: +81-471-24-1501; fax: +81-471-23-9767; e-mail: sugawara@rs.noda.sut.ac.jp

0960-894X/02/$ - see front matter
PII: S0960-894X(01)00725-9



Scheme 1. Reagents and conditions: (a) benzyl bromide, DBU, benzene reflux, 16 h (75.2%); (b) CBZ-β-alanine, EDCI, DMAP, dry CH_2Cl_2, 16 h (99.0%); (c) $Pd(OH)_2$, H_2, EtOAc/EtOH/H_2O (10:1:1), 5 h (82.0%); (d) *N*-hydroxysuccinimide-biotin, DMF, Et_3N, 5 h (81.8%); (e) biotinamidocapronate *N*-hydroxysuccinimide ester, DMF, Et_3N, 5 h (69.2%); (f) 6-(biotinamidecaproylamide)caproic acid *N*-succinimide ester, DMF, Et_3N, 5 h (77.5%).

Effects of biotinylated lithocholic acids on the activities of mammalian DNA polymerases α and β

Figure 1 shows the inhibitory dose–response curves of LCA (**1**) and the biotinylated compounds (**5**–**7**) against the mammalian DNA pol α and β. All of these compounds dose-dependently inhibited the activities of calf DNA pol α and rat DNA pol β. LCA strongly inhibited the activity of DNA pol β rather than that of DNA pol α. On the other hand, the inhibitory dose curves for DNA pols α and β by **5** and **6** were almost similar, and **7** inhibited DNA pol α more effectively. The IC_{50} values of DNA pol α activity by LCA (**1**), compound **5**, **6**, and **7** were 40, 29, 42 and 10 μM, respectively (Fig. 1A). The IC_{50} values of pol β activity by **1**, **5**, **6** and **7** were 11, 31, 46 and 32 μM, respectively (Fig. 1B).

Mode of inhibition of DNA polymerase α and β by LCA and compound 7

To elucidate the inhibitory mechanism, the extent of inhibition was studied as a function of the DNA template-primer or nucleotide substrate concentrations (Table 1). In the kinetic analyses, poly(dA)/oligo(dT)$_{12–18}$ and dTTP were used as the DNA template-primer and nucleotide substrate, respectively. Double reciprocal plots of the results indicated that the LCA-mediated inhibition of the DNA pol α activity was non-competitive with both the DNA template-primer and the nucleotide substrate. In the case of the DNA template-primer, 56.3 and 31.8% decreases in maximum velocity (V_{max}) were observed in the presence of 25 and 50 pmol/h LCA, respectively, whereas the apparent Michaelis constant (K_m) was unchanged at 13.0 μM. The K_m value for the nucleotide substrate was 1.65 μM, and the V_{max} value for the nucleotide substrate decreased from 29.2 to 14.8 pmol/h in the presence of 50 μM LCA. The inhibition constant (K_i) value for DNA pol α, obtained from Dixon plots, was found to be 15.3 and 22.5 μM for the DNA template-primer and nucleotide substrate, respectively. The affinity of LCA might be higher at the DNA template-primer binding site than at the nucleotide substrate-binding site. On the other hand, the DNA pol β inhibition by LCA was competitive with both the DNA template-primer and the nucleotide substrate, because the V_{max} value was unchanged at a concentration of 111.0 and 62.5 pmol/h, respectively. The K_i values for DNA template-primer and nucleotide substrate were 4.70 and 6.62 μM, respectively. These data suggested that the binding modes of LCA to DNA pol α and DNA pol β are different from each other. As shown in Figure 1, the biotinylated LCA could inhibit the DNA pol α and β activity. The inhibition of DNA pol α activity by compound **7** was non-competitive for both the DNA template-primer (the K_m was unchanged at 13.0 μM) and the nucleotide substrate (the K_m was unchanged at 1.65 μM), and the inhibition of DNA pol β activity by compound **7** was competitive for both the DNA template-primer (the V_{max} was unchanged at 111 pmol/h) and the nucleotide substrate (the V_{max} was unchanged at 62.5 pmol/h). The inhibition mode for DNA pol α and β by biotinylated LCA was the same as that by LCA.

Interaction of LCA and DNA polymerases α and β

DNA pol α and β were charged to the biotinylated LCA-streptavidine columns and eluted by KCl solution.

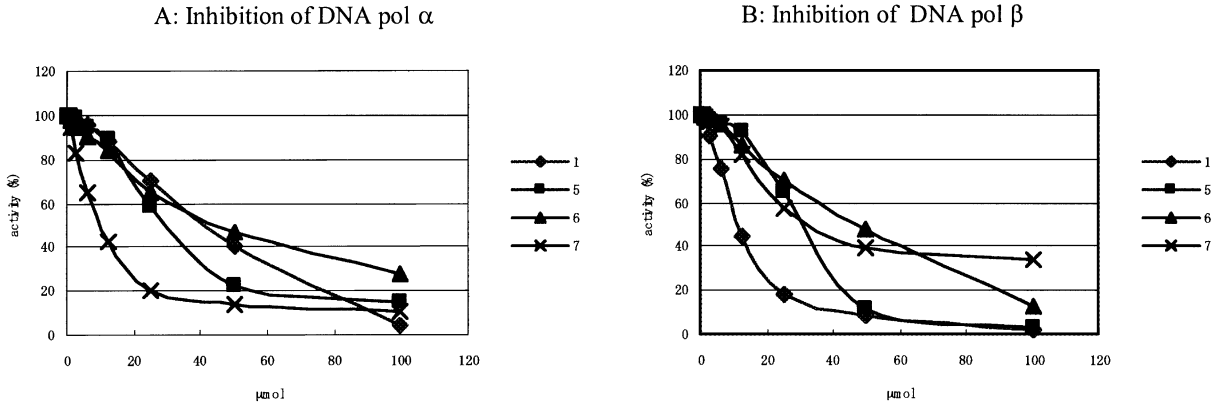


Figure 1. Dose–response curves of LCA (**1**) and the biotinylated compounds (**5**, **6**, **7**). The enzymes used (0.05 units each) were calf DNA pol α and rat DNA pol β. The DNA polymerase activities were measured as described in the text. DNA polymerase activity in the absence of compound was taken as 100%.

Table 1. Kinetic analysis of the inhibition by lithocholic acid **1** and the biotinylated compound **7** on the activities of DNA pol, as a function of the DNA template-primer dose and the nucleotide substrate concentration

Compd	Enzyme (0.05 units each)	Substrate	Compd concn (μM)	K_m (μM)	V_{max} (pmol/h)	K_i (μM)	Inhibition mode
1	Pol α	Template-primer[a]	0		55.6		
			25	13.00	31.3	15.3	Non-competitive
			50		17.7		
		Nucleotide substrate[b]	0		29.2		
			25	1.65	24.9	22.5	Non-competitive
			50		14.8		
	Pol β	Template-primer[a]					
			5	9.41	111.0	4.7	Competitive
			10	18.3			
		Nucleotide substrate[b]	0	3.05			
			5	6.28	62.5	6.6	Competitive
			10	7.86			
7	Pol α	Template-primer[a]	0		55.6		
			5	13.00	33.5	7.7	Non-competitive
			10		21.3		
		Nucleotide substrate[b]	0		29.2		
			5	1.65	24.3	5.5	Non-competitive
			10		17.8		
	Pol β	Template-primer[a]	0	6.47			
			10	9.98	111.0	12.8	Competitive
			20	21.70			
		Nucleotide substrate[b]	0	3.05			
			10	6.67	62.5	20.1	
			20	9.49			Competitive

[a]Poly(dA)/oligo(dT)$_{12-18}$ (=2/1).
[b]dTTP.

In the columns conjugated with the compounds **5**, **6** and **7**, the activities of DNA pol α were appeared at 430, 450 and 500 mM of KCl concentrations, respectively. The activities of DNA pol β were observed at 400, 370 and 340 mM KCl concentrations from the streptavidine sepharose columns conjugated with the compounds **5**, **6**, and **7**, respectively. Longer linker between LCA and biotin tended to elute by low concentration of KCl. Thus, compound **7** was the best candidate for DNA pol affinity chromatography in the tested compounds. The streptavidine column chromatography by using compound **7** is able to isolate DNA pol α and β from the mixture (Fig. 2). The high incorporation of dTTP for activated DNA indicated the enzymatic activity of polymerase β and the high incorporation of poly(dA)/oligo(dT) implied the activity of DNA pol α. Thus, the isolation of the mixture of DNA pols α and β was enabled on the affinity column chromatography used by the derivative of inhibitor. In addition to this, the column may be possible to isolate currently unknown enzymes regarding to DNA replication and/or repair process in the cell cycle.

Experimental

NMR and FABMS data

Synthetic compounds were characterized with both NMR and FABMS. FAB mass spectral data were collected on a JEOL JMS-700 with negative ion mode on glycerol matrix and NMR data were obtained from Bruker DRX-400 (400 MHz for ^{1}H in CD_3OD. Chemical shifts were expressed by δ ppm from TMS as internal standard. Optical rotation values were determined on JASCO polarimeter P1010.

Compound **5**: $C_{37}H_{59}N_2O_6S$. ^{1}H NMR (CD_3OD): LCA unit δ 0.69 (3H, s), 0.94 (3H, d, J=6.6 Hz), 0.96 (3H, s), 1.05–1.21 (6H, m), 1.26–1.34 (4H, m), 1.41–1.46 (6H, m), 1.50–1.76 (5H, m), 1.82–1.92 (3H, m), 2.03 (1H, d, J=11.6 Hz), 2.18 (2H, dd, J=6.4, 6.4 Hz), 2.19 (1H, m), 4.76 (1H, m); biotin unit: δ 1.42 (2H, m), 1.61 (2H, m), 1.70 (2H, m), 2.33 (2H, m), 2.71 (1H, d, J=12.7 Hz), 2.91 (1H, dd, J=5.0, 12.7 Hz), 3.19 (1H, m), 4.30 (1H, dd, J=4.4, 7.8 Hz), 4.48 (1H, dd, J=5.0, 7.8 Hz); β-Ala unit: δ 2.45 (2H, t, J=6.6 Hz), 3.42 (2H, J=6.6

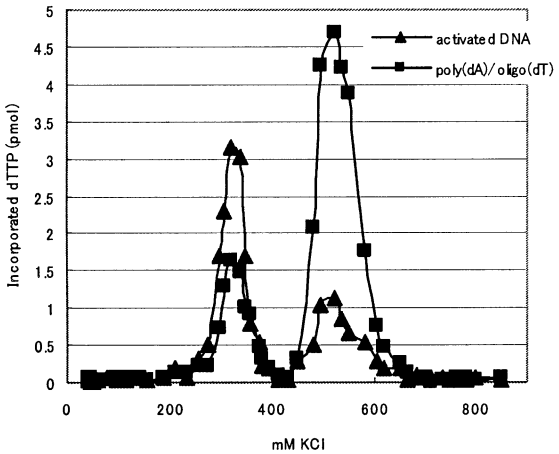


Figure 2. DNA pol α and β elution profiles from streptavidine sepharose column conjugated with **7**.

Hz). FABMS: *m*/*z* 672.62 ([M−H]$^-$). $[\alpha]_D$ +52.8 ° (*c* 0.1, MeOH).

Compound **6**: $C_{43}H_{70}N_4O_7S$. ^{1}H NMR: LCA unit δ 0.59 (3H, s), 0.85 (3H, d, *J* = 6.6 Hz), 0.86 (3H, s), 1.07–1.18 (6H, m), 1.28–1.34 (4H, m), 1.41–1.52 (6H, m), 1.57–1.70 (5H, m), 1.83–1.91 (3H, m), 2.03 (1H, d, *J* = 11.6 Hz), 2.18 (2H, t, *J* = 7.3 Hz), 2.20 (1H, m), 4.70 (1H, m); biotin unit: δ 1.43 (2H, m), 1.59 (2H, m), 1.66 (2H, m), 2.20 (2H, m), 2.72 (1H, d, *J* = 12.7 Hz), 2.91 (1H, dd, *J* = 5.0, 12.7 Hz), 3.18 (1H, m), 4.30 (1H, dd, *J* = 4.4, 7.8 Hz), 4.48 (1H, dd, *J* = 5.0, 7.8 Hz); β-Ala unit: δ 2.49 (2H, t, *J* = 6.6 Hz), 3.41 (2H, t, *J* = 6.6 Hz); aminocaproylamide unit δ 1.32 (2H, m), 1.43 (2H, m), 2.17 (2H, m), 2.25 (2H, m), 3.15 (2H, m). FABMS: *m*/*z* 785.55 ([M−H]$^-$). $[\alpha]_D$ +42.7 ° (*c* 0.1, MeOH).

Compound **7**: $C_{49}H_{81}N_5O_6S$. ^{1}H NMR (CD_3OD): LCA unit δ 0.69 (3H, s), 0.95 (3H, d, *J* = 6.4 Hz), 0.96 (3H, s), 1.03–1.18 (6H, m), 1.28–1.37 (4H, m), 1.41–1.54 (6H, m), 1.60–1.70 (5H, m), 1.83–1.90 (3H, m), 2.03 (1H, d, *J* = 11.6 Hz), 2.17 (3H, m), 4.70 (1H, m); biotin unit: δ 1.44 (2H, m), 1.63 (2H, m), 1.71 (2H, m), 2.30 (2H, m), 2.71 (1H, d, *J* = 12.7 Hz), 2.91 (1H, dd, *J* = 5.0, 12.7 Hz), 3.19 (1H, m), 4.29 (1H, dd, *J* = 4.4, 7.8 Hz), 4.48 (1H, dd, *J* = 5.0, 7.8 Hz); β-Ala unit: δ 2.49 (2H, t, *J* = 6.6 Hz), 3.41 (2H, t, *J* = 6.6 Hz); aminocaproylamide unit δ 1.28–1.32 (4H, m), 1.39–1.46 (4H, m), 2.13–2.18 (4H, m), 2.20–2.30 (4H, m), 3.10–3.20 (4H, m). FABMS: *m*/*z* 898.67 ([M−H]$^-$). $[\alpha]_D$ +5.7 ° (*c* 0.1, MeOH).

DNA polymerase assay

DNA pol α was purified from calf thymus by immunoaffinity column chromatography as described previously.[6,7] Recombinant rat DNA pol β was purified from *Escherichia coli* JMpb5 as described by Date et al.[7]

The reaction mixtures for DNA pols α and β were described previously.[8,9] The substrates of DNA polymerases were used poly(dA)/oligo(dT)$_{12-18}$ and [^{3}H]-dTTP as template-primer DNA and nucleotide substrate, respectively. LCA and the biotinylated compounds (**5**, **6**, **7**) were dissolved in DMSO at various concentrations and sonicated for 30 s. Four μL of each of the sonicated sample was mixed with 16 μL of each enzyme (final 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 16 μL of each of the enzyme standard reaction mixtures, and incubation was carried out at 37 °C for 60 min. The activity without the inhibitor was considered 100%, and the remaining activities at each concentration of inhibitor were determined as percentages of this value. One unit of each DNA pol activity was defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of deoxyribonucleotide 5′-triphosphates (i.e., dTTP) into synthetic template-primers [i.e., poly(dA)/oligo(dT), A/T = 2/1] in 60 min at 37 °C under the normal reaction conditions for each enzyme.[8,9]

Affinity column chromatography conjugated with biotinylated lithocholic acids

Each biotinylated LCA (**5**–**7**) (5 mg each) was dissolved in 20% DMSO and mixed with 1.0 mL of streptavidine sepharose, respectively. The purified pol α (3 units) or pol β (2.0 mg) was loaded onto the columns equilibrated with 50 mM Tris–HCl (pH 7.5) and 50 mM KCl buffer. The column was washed with 8 mL of the same buffer and then the enzymes were eluted by a 24 mL linear gradient of KCl solutions from 50 to 950 mM in Tris–HCl (pH 7.5) buffer. DNA pols α and β activities and protein concentration of the fractions (0.4 mL each) were determined by standard DNA pol assay methods[8,9] and the method of Bradford,[10] respectively.

Acknowledgements

This work was partly supported by Inoue Research Award for Young Scientists from Inoue Foundation for Science (to Y.M.). This work was also supported partially supported by Grants-in-Aid 12780442 (to Y.M.) 12660103 (to F.S.) from the Ministry of Education, Science, Sports and Culture of Japan. F.S. also thanks Uehara Memorial Foundation and The Naitoh Foundation for financial support.

References and Notes

1. Narisawa, T.; Magadia, N. E.; Weisburger, J. H.; Wynder, E. L. *J. Natl. Cancer Inst.* **1974**, *53*, 1093.
2. Reddy, B. S.; Narisawa, T.; Weisburger, J. H.; Wyner, E. L. *J. Natl. Cancer Inst.* **1976**, *56*, 441.
3. Ogawa, A.; Murate, T.; Suzuki, M.; Nimura, Y.; Yoshida, S. *Jpn. J. Cancer Res.* **1998**, *89*, 1154.
4. Mizushina, Y.; Ohkubo, T.; Sugawra, F.; Sakaguchi, K. *Biochemistry* **2000**, *39*, 12606.
5. Deng, J.-Z.; Stark, S. R.; Sabat, M.; Hecht, S. M. *J. Nat. Prod.* **2000**, *63*, 1356.
6. Tamai, K.; Kojima, K.; Hanaichi, T.; Masaki, S.; Suzuki, M.; Umekawa, H.; Yoshida, S. *Biochim. Biophys. Acta* **1988**, *950*, 263.
7. Date, T.; Yamaguchi, M.; Hirose, F.; Nishimoto, Y.; Tanihara, K.; Matsukage, A. *Biochemistry* **1988**, *27*, 2983.
8. Mizushina, Y.; Tanaka, N.; Yagi, H.; Kurosawa, T.; Onoue, M.; Seto, H.; Horie, T.; Aoyagi, N.; Yamaoka, M.; Matsukage, A.; Yoshida, S.; Sakaguchi, K. *Biochim. Biophys. Acta* **1996**, *1308*, 256.
9. Mizushina, Y.; Yoshida, S.; Matsukage, A.; Sakaguchi, K. *Biochim. Biophys. Acta* **1997**, *1336*, 509.
10. Bradford, M. M. *Anal. Biochem.* **1976**, *2*, 248.