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Dipeptide alcohol-based inhibitors of eukaryotic DNA polymerase α

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Abstract—We reported previously that a novel dipeptide alcohol, L-homoserylaminoethanol (Hse-Gly-ol), is a selective inhibitor of eukaryotic DNA polymerase ε (pol ε) [*Bioorg. Med. Chem.* **2004**, *12*, 957–962]. The discovery suggests that the dipeptide structure could be a chemical frame for a DNA polymerase inhibitor. Therefore, we chemically synthesized 27 different species of dipeptide alcohols, and tested this inhibitory capability. Compound **6** (L-aspartylaminoethanol, Asp-Gly-ol) was found to be the strongest pol α inhibitor. Compound **6** did not influence the activities of other replicative DNA polymerases such as δ and ε, and had no effect on the activities of prokaryotic DNA polymerases, nor DNA metabolic enzymes such as human immunodeficiency virus type 1 reverse transcriptase, T7 RNA polymerase and bovine deoxyribonuclease I. The inhibitory effect of compound **6** on pol α was dose-dependent, and 50% inhibition was observed at a concentration of 33.5 μM. Compound **6**-induced inhibition of pol α activity was non-competitive with both the DNA template-primer and the dNTP substrate. This is the first report on a water-soluble pol α-specific inhibitor, sought for precise biochemical studies of pol α. The relationships between the structures of dipeptide alcohols and the inhibition of eukaryotic DNA polymerases are discussed.

1. Introduction

DNA polymerase catalyzes the addition of deoxyribonucleotides to the 3′-hydroxyl terminus of a primed double-stranded DNA molecule. Eukaryotic cells contain at least 3 replicative DNA polymerases (pol α, δ and ε), mitochondrial DNA polymerase (pol γ) and at least 12 repair types of DNA polymerase (pol β, δ, ε, ζ, η, θ, ι, κ, λ, μ, σ and REV1).[1] Selective inhibitors of eukaryotic DNA polymerases, which are reportedly separated into 16 classes,[1] are useful as tools and molecular probes to distinguish DNA polymerases and to clarify their biological and in vivo functions.[2] We have found many DNA polymerase inhibitors including long chain fatty acids,[3–5] triterpenoids,[6–8] steroids,[9,10] cerebrosides,[11] glycolipids,[12–16] flavonoids[17] and vitamin A such as retinal.[18] Most of the new inhibitors directly bound to the polymerase protein, and subsequently inhibited its activities.[5,10] In the process, we found an interesting compound that selectively inhibits pol ε activity.[19] This natural compound belongs to a class of compounds known as dipeptide alcohols, L-homoserylaminoethanol, produced by cultured cells of a higher plant, tobacco (*Nicotina tabacum* L.). It was of great interest that the compound is water-soluble. It is generally very difficult to find inhibitor compounds with such properties. If such inhibitors are found, it is extremely useful to study pol ε since the pol ε molecule is mostly unknown. Although we have reported on more than 30 natural compounds as described above,[20] all were water-insoluble. Moreover, the fact that water-soluble dipeptide compound could be a polymerase inhibitor is quite interesting. The in vivo control system for DNA polymerase activities may involve peptide hormone.

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We newly designed dipeptide alcohols of 27 compounds similar to L-homoserylaminoethanol, and tested their inhibitory activities. We succeeded in chemically synthesizing them. Some of them inhibited the activities of DNA polymerases. In this report, on a series of peptide inhibitors, which may reflect the in vivo system, we describe the inhibitory effect of these novel dipeptide alcohols and the structure–function relationships of the dipeptides and DNA polymerases. We also discuss the inhibitory mechanisms.

2. Results

2.1. Chemistry

Dipeptide alcohol analogues were synthesized according to the method reported by Kuriyama et al.[19] At first, we randomly synthesized various dipeptide alcohols and screened for DNA polymerase inhibitors. In the compounds, aminoethanol was found to be essential for DNA polymerase inhibitory activity (data not shown). Therefore, the 27 compounds of dipeptide alcohols, which were based aminoethanol, were chemically synthesized (Table 1). The structures of the products were confirmed by spectroscopy including FABMS and ^{13}C and ^{1}H NMR analyses. The novel compounds were **1** (L-homoserylaminoethanol),[19] **14** (glutaryl-aminoethanol), **15** (L-2-aminobutanoyl-aminoethanol), **18** (L-homoseryl-3-amino-1-propanol), **20** (L-homoseryl-glycine) and **22** (L-homoseryl-2-aminopropanol) (Table 1).

2.2. Effect of synthesized dipeptide alcohols on the activities of mammalian DNA polymerases

As shown in Table 1, we prepared 27 dipeptide alcohols by chemical synthesis. The amino acids used were randomly chosen to obtain information on the inhibition-related amino acids. The relative activities of pol α, β, δ and ε at 100 μM of the compounds are shown in Figure 1. We reported previously that compound **1** (L-homoserylaminoethanol) was a specific inhibitor of pol ε with an IC_{50} value of 270 μM.[19] Compounds **5**, **6**, **7** and **20** inhibited the activity of calf pol α, the IC_{50} values being less than 100 μM. In these compounds, the inhibitory effect of compound **6** (L-aspartylaminoethanol, Asp-Gly-ol) was the strongest. However, none of the compounds effective at 100 μM influenced the activities of rat pol β, human pol δ and human pol ε (Fig. 1). It is of interest that these compounds including **6** are inhibitors of replicative polymerases such as pol α. We concentrate on the characterization of compound **6** in the rest of this report.

2.3. Effects of compound 6 on the activities of DNA polymerases and on other DNA metabolic enzymes

Figure 2 shows the inhibition dose–response curves of compound **6** against five mammalian DNA polymerases. The inhibition by compound **6** was dose-dependent. This compound effectively inhibited pol α, with 50% inhibition observed at a dose of 33.5 μM (Fig. 2). Aphidicolin, a potent inhibitor of mammalian pol α, shows complete inhibition at 40 μM,[21] thus the effect

Table 1. Structures of synthesized dipeptide alcohols

Compound no.	Abbreviation	Chemical structure
1[a]	Hse-Gly-ol	HO–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
2	Ser-Gly-ol	HO–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
3	Thr-Gly-ol	H_3C–CH(OH)–CH(NH_2)–CO–NH–CH_2–CH_2–OH
4	Met-Gly-ol	H_3C–S–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
5	Cys-Gly-ol	HS–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
6	Asp-Gly-ol	HOOC–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
7	Asn-Gly-ol	H_2NOC–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
8	Gly-Gly-ol	H–CH(NH_2)–CO–NH–CH_2–CH_2–OH
9	Ala-Gly-ol	H_3C–CH(NH_2)–CO–NH–CH_2–CH_2–OH
10	Propionyl-Gly-ol	H_3C–CH_2–CO–NH–CH_2–CH_2–OH
11	Butyryl-Gly-ol	H_3C–CH_2–CH_2–CO–NH–CH_2–CH_2–OH
12	Isobutyryl-Gly-ol	H_3C–CH(CH_3)–CO–NH–CH_2–CH_2–OH
13	Succinyl-Gly-ol	HOOC–CH_2–CH_2–CO–NH–CH_2–CH_2–OH
14[a]	Glutaryl-Gly-ol	HOOC–CH_2–CH_2–CH_2–CO–NH–CH_2–CH_2–OH
15[a]	2-Aminobutanoyl-Gly-ol	H_3C–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
16	Phe-Gly-ol	Ph–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
17	His-Gly-ol	Imidazole–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
18[a]	Hse-amino-1-propanol	HO–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–CH_2–OH
19	Tyr-Gly-ol	HO–Ph–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
20[a]	Hse-Gly-OH	HO–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–COOH
21	Hse-Gly-NH_2	HO–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–$CONH_2$
22[a]	Hse-Ala-ol	HO–CH_2–CH(NH_2)–CO–NH–CH(CH_3)–CH_2–OH
23	Val-Gly-ol	H_3C–CH(CH_3)–CH(NH_2)–CO–NH–CH_2–CH_2–OH
24	Leu-Gly-ol	H_3C–CH(CH_3)–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
25	Glu-Gly-ol	HOOC–CH_2–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–OH
26	Asp-Gly-OH	HOOC–CH_2–CH(NH_2)–CO–NH–CH_2–COOH
27	Asp-aminoethylamine	HOOC–CH_2–CH(NH_2)–CO–NH–CH_2–CH_2–NH_2

[a] Novel compound.

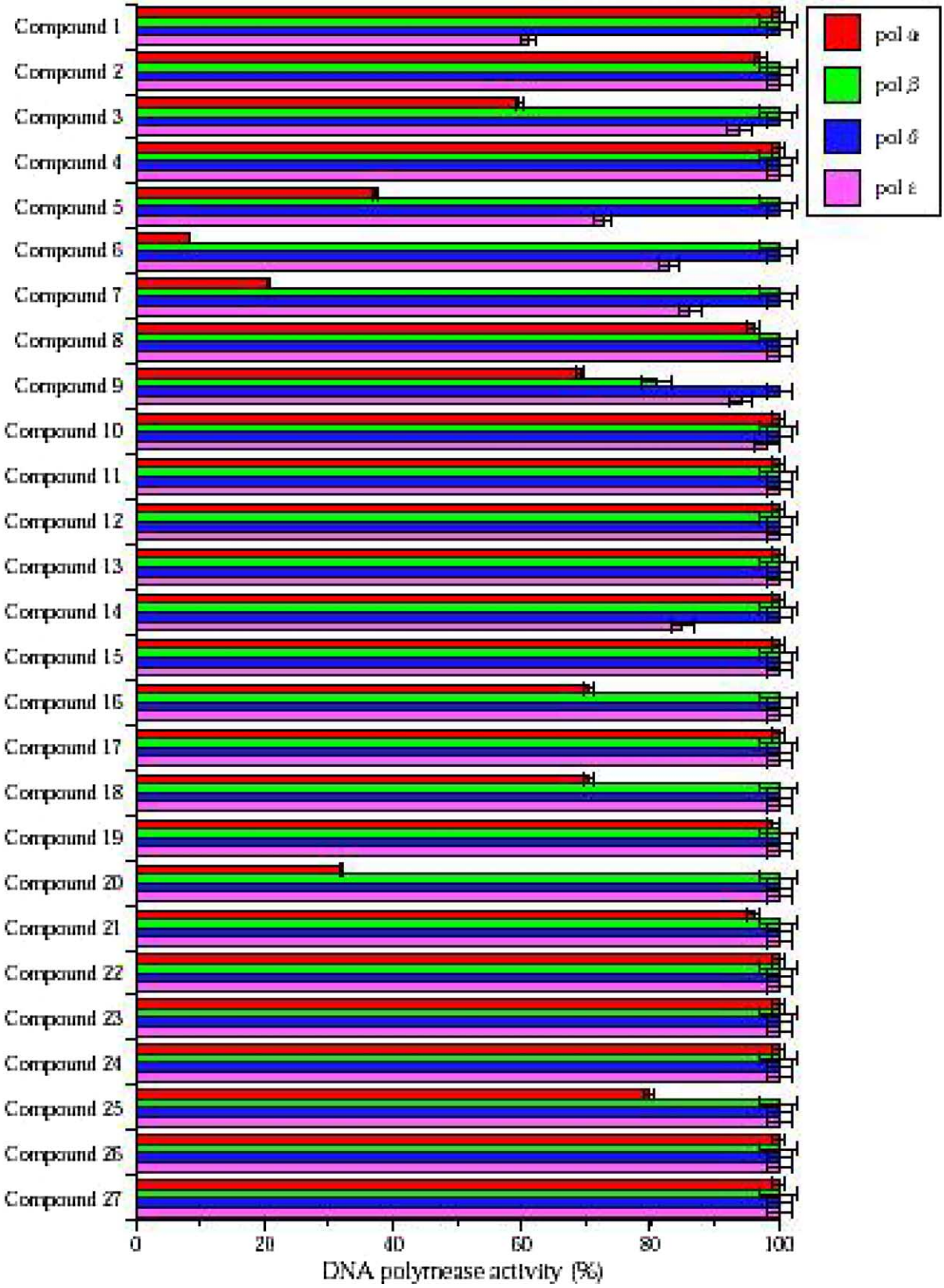

Figure 1. Effect of chemically synthesized dipeptide alcohols on the activities of mammalian DNA polymerases. % Of relative activity. Dipeptide alcohols (100 μM each) were incubated with each DNA polymerase (0.05 units). Enzyme activity in the absence of compound was taken as 100%. Data are shown as means ± SEM of three independent experiments.

of compound **6** on this enzyme was almost the same as that of aphidicolin. As shown in Table 2, compound **6** also inhibited the activity of pol α from fish (cherry salmon), insect (*Drosophila melanogaster*) and plant

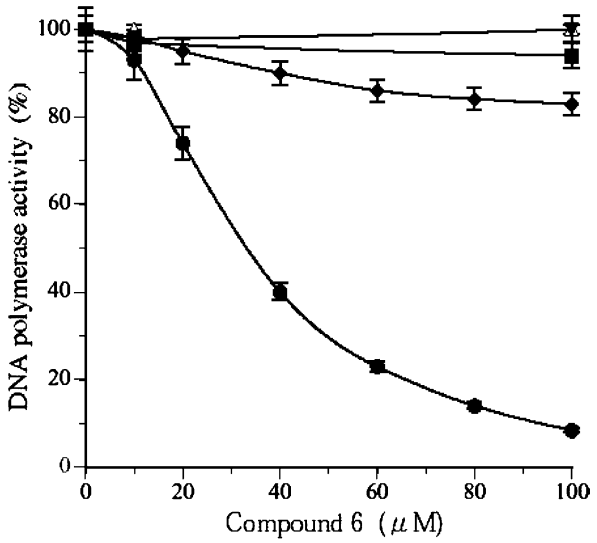

Figure 2. Mammalian DNA polymerase inhibition dose–response curves of compound **6**. The enzymes used (0.05 units of each) were calf pol α (circle), rat pol β (open-triangle), human pol γ (square), human pol δ (reverse-triangle) and human pol ε (diamond). The DNA polymerase activities were measured as described in Section 5. DNA polymerase activity in the absence of the compound was taken as 100%. Data are shown as means ± SEM of three independent experiments.

(cauliflower) to the same extent as mammalian (calf and mouse) pol α. On the other hand, compound **6** had no significant influence on the activities of mammalian pol β, γ, δ and ε, fish pol δ, insect pol δ and ε, plant pol β, prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* pol I, Taq pol and T4 pol. Hence, in three-dimensional structure, eukaryotic pol α would greatly differ from prokaryotic DNA polymerases. When activated DNA (i.e., a gapped DNA, which is digested by bovine deoxyribonuclease I) was used as the DNA template-primer, the mode of inhibition by compound **6** did not change (data not shown). Compound **6** also did not inhibit the activities of other DNA metabolic enzymes such as calf DNA primase of pol α, calf terminal deoxynucleotidyl transferase, human telomerase, human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, T7 RNA polymerase, human DNA topoisomerases I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I (Table 2).

Consequently, compound **6** is a novel water-soluble pol α inhibitor, which has never before been reported. Whether the inhibitor is water-soluble or not is crucial to the study of polymerases.

2.4. Mode of DNA polymerase α inhibition by compound 6

Next, to elucidate the mechanism of pol α inhibition, the extent of inhibition as a function of DNA template-primer or dNTP (2′-deoxyribonucleotide 5′-triphosphate) substrate concentrations was studied (Fig. 3). In the kinetic analysis, poly(dA)/oligo(dT)$_{12–18}$ and dTTP were used as the DNA template-primer and dNTP substrate,

Table 2. IC_{50} values of compound **6** for the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	IC_{50} values of compound **6** (μM)
Mammalian DNA polymerases	
Calf DNA polymerase α	33.5 ± 1.4
Mouse the largest subunit of DNA polymerase α	35.0 ± 1.0
Rat DNA polymerase β	>200
Human DNA polymerase γ	>200
Human DNA polymerase δ	>200
Human DNA polymerase ε	>200
Human DNA polymerase η	>200
Human DNA polymerase ι	>200
Human DNA polymerase κ	>200
Human DNA polymerase λ	>200
Fish DNA polymerases	
Cherry salmon DNA polymerase α	38.4 ± 1.5
Cherry salmon DNA polymerase δ	>200
Insect DNA polymerases	
D. melanogaster DNA polymerase α	34.6 ± 1.3
D. melanogaster DNA polymerase δ	>200
D. melanogaster DNA polymerase ε	>200
Plant DNA polymerases	
Cauliflower DNA polymerase I (α-like)	34.1 ± 1.1
Cauliflower DNA polymerase II (β-like)	>200
Procaryotic DNA polymerases	
E. coli DNA polymerase I (Klenow fragment)	>200
Taq DNA polymerase	>200
T4 DNA polymerase	>200
Other DNA metabolic enzymes	
Calf primase of DNA polymerase α	>200
Calf terminal deoxynucleotidyl transferase	>200
Human telomerase	>200
HIV-1 reverse transcriptase	>200
T7 RNA polymerase	>200
Human DNA topoisomerase I	>200
Human DNA topoisomerase II	>200
T4 polynucleotide kinase	>200
Bovine deoxyribonuclease I	>200

Compound **6** was incubated with each enzyme. The enzymatic activity was measured as described under Section 5. Enzyme activity in the absence of the compounds was taken as 100%. Data are shown as means ± SEM of three independent experiments.

respectively. Double-reciprocal plots of the results showed that compound **6**-induced inhibition of calf pol α activity was non-competitive with both the DNA template-primer and the dNTP substrate (Fig. 3A and B). In the case of the DNA template-primer, the apparent Michaelis constant (K_m) was unchanged at 13.0 μM, whereas 50% and 28.1% decreases in maximum velocity (V_{max}) were observed in the presence of 15 and 30 μM of compound **6**, respectively (Fig. 3A). The K_m for the dNTP substrate was 1.65 μM, and the V_{max} for the dNTP substrate decreased from 29.2 to 14.3 pmol/h in the presence of 30 μM of compound **6** (Fig. 3B). The inhibition constant (K_i) value, obtained from Dixon plots, was found to be 14 and 29 μM for the DNA template-primer and substrate dTTP, respectively (Fig. 3C and D). Because the K_i value for the template-primer was half that for the dNTP substrate, the affinity of compound **6** is higher for the enzyme–DNA template-

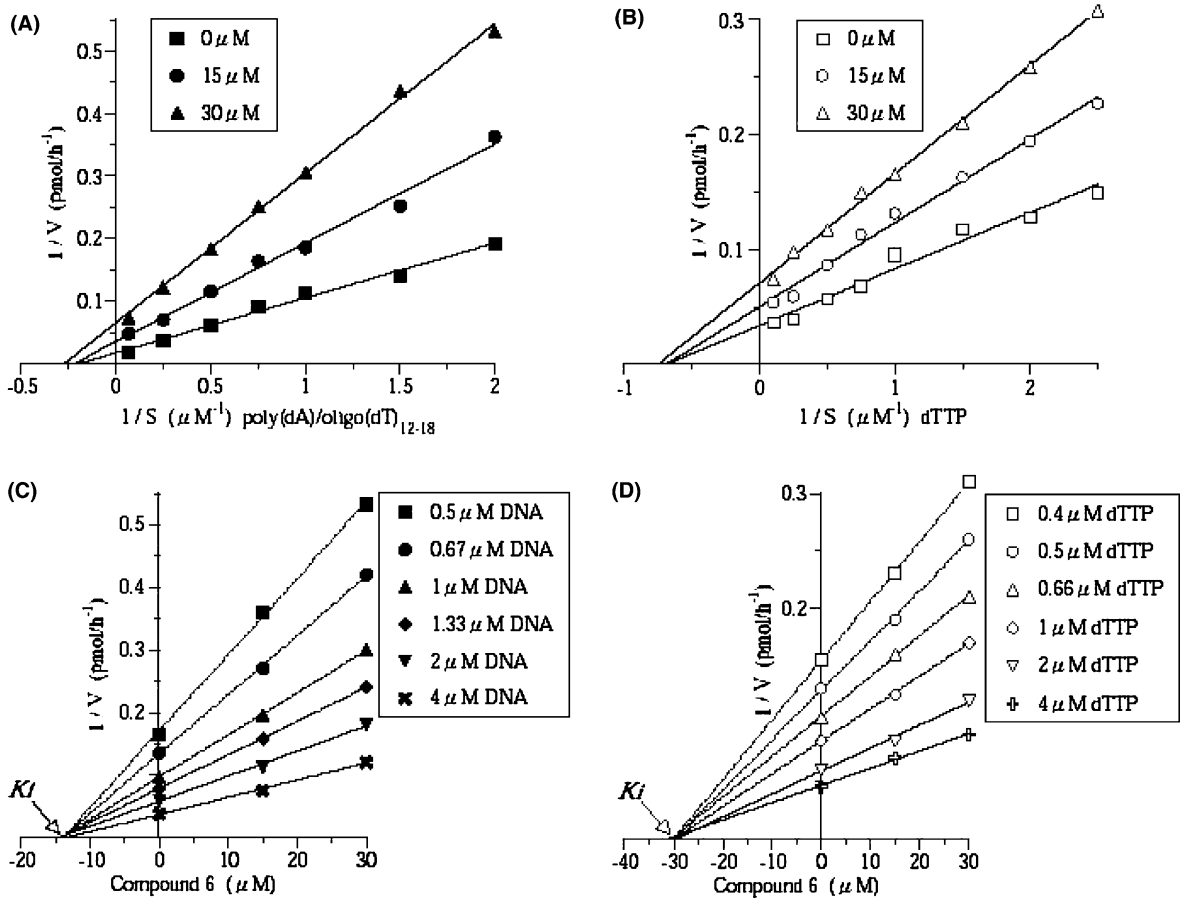

Figure 3. Kinetic analysis of the inhibition of calf thymus DNA polymerase α by compound **6**. (A and B) Lineweaver–Burk double-reciprocal plots obtained by varying DNA template-primer (i.e., poly(dA)/oligo(dT)$_{12–18}$) concentrations (A), and dNTP substrate (i.e., dTTP) concentrations (B). Activity of calf pol α was assayed in the absence (square) or presence of 15 (circle), and 30 (triangle) μM of compound **6**. (C and D) The inhibition constant (K_i) were determined as 14 and 29 μM from a Dixon plot made on the basis of the same data for A and B, respectively. The amount of calf pol α in the assay mixture was 0.05 units.

primer binary complex than for the enzyme–DNA–dNTP ternary complex. When activated DNA and four deoxynucleoside triphosphates were used as the template-primer and dNTP substrate, respectively, the inhibition of calf pol α by compound **6** was non-competitive with the DNA template-primer and the dNTP substrate (data not shown). The core domain (p110) of the largest subunit of mouse pol α, which has the catalytic activity, was similarly inhibited (data not shown). From a biochemical viewpoint, this mode of action is unusual.

On the other hand, the inhibition of pol α by aphidicolin was competitive with each of the four dNTPs substrate for binding to a pol α–DNA binary complex, but its K_i depended on the sequence context, varying from 0.2 to 2 μM. Kinetic evidence showed that inhibition proceeds through the formation of a pol α–DNA–aphidicolin ternary complex.[21] In contrast, inhibition of pol α by compound **6** was non-competitive with the four deoxynucleoside triphosphates (data not shown). The mode of the inhibitory effect of compound **6** on pol α was quite different from that of aphidicolin. Because the K_i value for compound **6** was higher than that for aphidicolin, the complex formation affinity of pol α and compound **6** might be lower than that of pol α and aphidicolin. The effect of aphidicolin on the inhibitory effect of compound **6** was additive (data not shown), therefore, the pol α binding site of compound **6** might be different from that of aphidicolin.

3. Discussion

We previously reported a novel inhibitor specific to eukaryotic DNA polymerase ε (pol ε) from cultured plant, *N. tabacum* L. cells.[19] This compound (**1**) was a dipeptide alcohol, L-homoserylaminoethanol (Hse-Gly-ol), and had a weak inhibitory effect on pol ε activity, with an IC_{50} value of 270 μM (43.6 μg/mL). As previously reported, the synthetic D-enantiomer (D-homoserylaminoethanol) also shows pol ε inhibitory activity identical to the natural L-enantiomer. To know the universality of the dipeptide inhibition, then, based on the structure of Hse-Gly-ol, we designed and chemically synthesized 26 different dipeptide alcohols.

As shown in Figure 1, there was no better inhibitor of pol ε than the key compound **1** among the synthetic compounds. However, the pol α inhibitory activity absent in compound **1** was generated in many synthetic compounds. Notably, compounds **5**, **6**, **7** and **20** selectively inhibited pol α activity with IC_{50} values of less

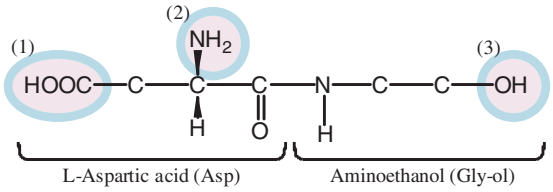

Figure 4. Chemical structure of dipeptide alcohol-based inhibitor of eukaryotic DNA polymerase α. Compound **6** (L-aspartylaminoethanol, Asp-Gly-ol). The essential groups ((1)–(3)) for eukaryotic pol α inhibitory activity in dipeptide alcohol are shown.

than 100 μM, and compound **6** was the strongest pol α inhibitor among the compounds tested, with an IC_{50} value of 33.5 μM (Fig. 2). So, based on the pol α inhibitory activity of compounds synthesized in the present study, we discuss the minimum structural feature for the inhibition. A generic formula for dipeptide alcohol-based eukaryotic pol α inhibitors is illustrated in Figure 4. First of all, note that the strongest inhibitor **6** resulted from the replacement of the CH_2OH group in the amino acid moiety of compound **1** with a COOH group ((1) of Fig. 4). The replacement of this COOH with a $CONH_2$ (compound **7**) or an SH (compound **5**) group slightly decreased the pol α inhibitory activity. The oxidation of either CH_2OH group in both terminal ends of compound **1** to give compound **6** or **20** lowered or abolished its inhibition towards pol ε but generated a potent inhibitory activity towards pol α. However, that of both the hydroxymethyl groups to give compound **26** nullified inhibitory activity towards not only pol ε but also pol α. The pol α inhibitory activity was nullified by the introduction of a methyl (compound **15**), an imidazole (compound **17**), a phenol (compound **19**) or an isopropyl (compound **24**) group in place of the COOH group in compound **6**. The importance of NH_2 to the inhibitory effect is apparent from the abolishment in activity resulting from the absence of NH_2 in compounds **10**–**14** ((2) of Fig. 4). The presence of the alcoholic OH group in the aminoalcohol moiety is also crucial for the inhibitory activity, as seen in compounds **26** and **27** ((3) of Fig. 4). Interestingly, replacement of the CH_2OH group in the aminoalcohol moiety of compound **1** with a COOH group to give compound **20** also generated fairly good inhibitory activity against pol α, lacking pol ε inhibitory activity. Compound **20** has the same molecular size as the strongest compound **6**, and has the COOH and alcoholic OH groups at both terminal ends and the NH_2 group essential for the inhibitory activity as well. However, there is a significant difference in the pol α inhibitory activity of the two compounds. This means that pol α has fairly strict requirements in terms of both the amino acid moiety and the aminoalcohol moiety at the dipeptide aminoalcohol binding site.

Aphidicolin is a selective inhibitor of both pol α and eukaryotic DNA replicative polymerases such as pol δ and ε, indicating that this polymerase is essential for DNA replication,[22] and this inhibitor has been very useful for studying the DNA replication system.[23] However, aphidicolin is not capable of distinguishing among pol α, δ and ε. Since compound **6** did not affect the activities of replicative DNA polymerases such as δ and ε, instead of aphidicolin, it could be useful as a tool to study the biochemical functions of pol α and as a molecular probe to distinguish the structure of pol α.

Inhibitors of the replicative DNA polymerases, such as pol α, could be potential candidates for anti-cancer drugs. Therefore, compound **6** should also be tested as an anti-tumour agent. If it was effective, this compound could be a promising agent for clinical usage, because it is water-soluble. Since these compounds selectively and non-competitively inhibit pol α activity with the DNA template-primer and dNTP substrates, the inhibitory effect may not be affected by dilution with an excess amount of nucleotide substrates. This may be another advantage as a clinical agent.

4. 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 (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Wako Ltd (Osaka, Japan).

4.1. Preparation and purification of dipeptide alcohol analogues

Dipeptide alcohol analogues were synthesized according to the method reported by Kuriyama et al.[19] Fmoc-amino acid derivatives were introduced to amino alcohol-Trt(2-Cl)-resin (Watanabe Chemical Ind. Ltd, Hiroshima, Japan) using *O*-(7-azabenzotriazol-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as a coupling reagent. The protected dipeptide alcohol–resin thus obtained was treated with 95% TFA in the presence of triisopropylsilane (TIPS) at room temperature for 120 min, followed by 20% piperidine in DMF. The crude product was purified by reverse phase HPLC on a Develosil ODS-HG-5 column (2 × 25 cm) (Nomura Chemical Co. Ltd, Seto, Japan) with a linear gradient of aqueous TFA/CH_3CN as a solvent system at a flow rate of 10 mL/min. Acylamino alcohol analogues were synthesized in the same manner as above using an appropriate fatty acid anhydride, as an acylating agent. Dipeptides and dipeptide amides were also synthesized in the same manner as above using Fmoc-amino acid-TrtA-PEG-resin and Fmoc-NH-SAL-PEG-resin (Watanabe Chemical Ind. Ltd, Hiroshima, Japan). The purities of all synthetic dipeptide alcohol analogues are over 98% by HPLC.

4.1.1. L-Homoserylaminoethanol (1). $[\alpha]_D$ +18.3 (*c* 0.44, H_2O); ^{1}H NMR (D_2O): δ 1.80 (1H, m), 1.91 (1H, m), 3.38 (2H, t, *J* = 5.5 Hz), 3.54 (1H, dd, *J* = 6.0, 7.3 Hz), 3.64–3.72 (4H, m); ^{13}C NMR (D_2O): δ 39.1, 44.2, 55.0, 61.1, 62.8, 179.9; HRMS (FAB) *m*/*z* 163.1084 $[M+H]^+$ ($C_6H_{15}N_2O_3$ requires 163.1083).

4.1.2. L-Serylaminoethanol (2). $[\alpha]_D$ +4.0 (*c* 0.47, H_2O); ^{1}H NMR (D_2O): δ 3.39 (2H, dt, *J* = 1.8, 5.5 Hz), 3.52

(1H, t, J = 5.5 Hz), 3.69 (2H, t, J = 5.5 Hz), 3.74 (2H, dd, J = 2.8, 5.5 Hz); ^{13}C NMR (D_2O): δ 44.3, 58.9, 62.8, 66.5, 178.2; HRMS (FAB) m/z 149.0928 $[M+H]^+$ ($C_5H_{13}N_2O_3$ requires 149.0926).

4.1.3. L-Threonylaminoethanol (3). $[\alpha]_D$ −5.9 (c 0.48, H_2O); ^{1}H NMR (D_2O): δ 1.21 (3H, d, J = 6.4 Hz), 3.29 (1H, d, J = 5.0 Hz), 3.39 (2H, t, J = 5.5 Hz), 3.69 (2H, t, J = 5.5 Hz), 4.01 (1H, dq, J = 5.0, 6.4 Hz); ^{13}C NMR (D_2O): δ 21.4, 44.2, 62.8, 63.1, 71.6, 178.3; HRMS (FAB) m/z 163.1084 $[M+H]^+$ ($C_6H_{15}N_2O_3$ requires 163.1083).

4.1.4. L-Methionylaminoethanol (4). $[\alpha]_D$ +16.7 (c 0.36, H_2O); ^{1}H NMR (D_2O): δ 1.87 (1H, m), 1.95 (1H, m), 2.11 (3H, s), 2.57 (2H, t, J = 7.3 Hz), 3.38 (2H, t, J = 5.5 Hz), 3.52 (1H, t, J = 7.3 Hz), 3.68 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 16.9, 32.1, 36.2, 44.2, 56.7, 62.7, 180.0; HRMS (FAB) m/z 193.1009 $[M+H]^+$ ($C_7H_{17}N_2O_2S$ requires 193.1011).

4.1.5. L-Cysteinylaminoethanol hydrochloride (5). $[\alpha]_D$ −10.9 (c 0.46, H_2O); ^{1}H NMR (D_2O): δ 3.06 (1H, dd, J = 6.4, 15.1 Hz), 3.11 (1H, dd, J = 5.0, 15.1 Hz), 3.43 (2H, t, J = 5.0 Hz), 3.70 (2H, t, J = 5.5 Hz), 4.20 (1H, dt, J = 1.4, 5.5 Hz); ^{13}C NMR (D_2O): δ 27.7, 44.5, 57.5, 62.1, 171.0; HRMS (FAB) m/z 165.0697 $[M+H]^+$ ($C_5H_{13}N_2O_2S$ requires 165.0698).

4.1.6. L-Aspartylaminoethanol hydrochloride (6). $[\alpha]_D$ +16.9 (c 0.43, H_2O); ^{1}H NMR (D_2O): δ 2.97 (1H, ddd, J = 1.4, 7.8, 17.8 Hz), 3.05 (1H, ddd, J = 1.4, 5.0, 17.8 Hz), 3.40 (2H, dt, J = 1.4, 5.0 Hz), 3.68 (2H, m), 4.32 (1H, dd, J = 5.0, 7.8 Hz); ^{13}C NMR (D_2O): δ 38.1, 44.5, 52.7, 62.6, 171.7, 176.2; HRMS (FAB) m/z 177.0874 $[M+H]^+$ ($C_6H_{13}N_2O_4$ requires 177.0875).

4.1.7. L-Asparaginylaminoethanol hydrochloride (7). $[\alpha]_D$ +19.0 (c 0.48, H_2O); ^{1}H NMR (D_2O): δ 2.92 (1H, dd, J = 7.3, 17.0 Hz), 2.98 (1H, dd, J = 5.5, 17.0 Hz), 3.41 (2H, dd, J = 4.6, 6.4 Hz), 3.68 (2H, m), 4.33 (1H, dd, J = 5.5, 7.3 Hz); ^{13}C NMR (D_2O): δ 37.9, 44.5, 52.9, 62.6, 171.7, 175.8; HRMS (FAB) m/z 176.1033 $[M+H]^+$ ($C_6H_{14}N_3O_3$ requires 176.1035).

4.1.8. Glycylaminoethanol hydrochloride (8). ^{1}H NMR (D_2O): δ 3.45 (2H, t, J = 5.5 Hz), 3.73 (2H, t, J = 5.5 Hz), 3.86 (2H, s); ^{13}C NMR (D_2O): δ 43.3, 44.3, 62.6, 170.1; HRMS (FAB) m/z 119.0819 $[M+H]^+$ ($C_4H_{11}N_2O_2$ requires 119.0821).

4.1.9. L-Alanylaminoethanol hydrochloride (9). $[\alpha]_D$ +12.0 (c 0.80, H_2O); ^{1}H NMR (D_2O): δ 1.57 (3H, d, J = 6.9 Hz), 3.44 (2H, m), 3.72 (2H, m), 4.10 (1H, q, J = 6.9 Hz); ^{13}C NMR (D_2O): δ 19.4, 44.3, 52.0, 62.6, 173.9; HRMS (FAB) m/z 133.0975 $[M+H]^+$ ($C_5H_{13}N_2O_2$ requires 133.0977).

4.1.10. Propionylaminoethanol (10). ^{1}H NMR (D_2O): δ 1.16 (3H, t, J = 7.8 Hz), 2.32 (2H, q, J = 7.8 Hz), 3.37 (2H, t, J = 5.5 Hz), 3.70 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 12.3, 31.9, 44.2, 62.8, 181.2; HRMS (FAB) m/z 118.0870 $[M+H]^+$ ($C_5H_{12}NO_2$ requires 118.0868).

4.1.11. Butyrylaminoethanol (11). ^{1}H NMR (D_2O): δ 0.95 (3H, t, J = 7.3 Hz), 1.65 (2H, m), 2.28 (2H, t, J = 7.3 Hz), 3.38 (2H, t, J = 5.5 Hz), 3.70 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 15.5, 21.7, 40.5, 44.1, 62.8, 180.3; HRMS (FAB) m/z 132.1026 $[M+H]^+$ ($C_6H_{14}NO_2$ requires 132.1025).

4.1.12. Isobutyrylaminoethanol (12). ^{1}H NMR (D_2O): δ 1.15 (6H, d, J = 6.9 Hz), 2.56 (1H, m), 3.37 (2H, t, J = 5.5 Hz), 3.70 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 21.4 (2 × C), 37.9, 44.1, 62.8, 184.3; HRMS (FAB) m/z 132.1026 $[M+H]^+$ ($C_6H_{14}NO_2$ requires 132.1025).

4.1.13. Succinylaminoethanol (13). ^{1}H NMR (D_2O): δ 2.60 (2H, t, J = 6.9 Hz), 2.70 (2H, t, J = 6.9 Hz), 3.38 (2H, t, J = 5.5 Hz), 3.69 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 32.3, 33.2, 44.2, 62.8, 178.0, 180.1; HRMS (FAB) m/z 162.0765 $[M+H]^+$ ($C_6H_{12}NO_4$ requires 162.0766).

4.1.14. Glutaryl-aminoethanol (14). ^{1}H NMR (D_2O): δ 1.93 (2H, m), 2.37 (2H, t, J = 7.3 Hz), 2.45 (2H, t, J = 7.3 Hz), 3.37 (2H, t, J = 5.5 Hz), 3.70 (2H, t, J = 5.5 Hz); ^{13}C NMR (D_2O): δ 23.5, 35.8, 37.6, 44.2, 62.7, 179.0, 181.0; HRMS (FAB) m/z 176.0924 $[M+H]^+$ ($C_7H_{14}NO_4$ requires 176.0923).

4.1.15. 2-Aminobutanoylaminoethanol hydrochloride (15). $[\alpha]_D$ +21.8 (c 1.10, H_2O); ^{1}H NMR (D_2O): δ 1.04 (3H, t, J = 7.6 Hz), 1.96 (2H, m), 3.46 (2H, t, J = 5.5 Hz), 3.73 (1H, m), 3.98 (1H, t, J = 6.7 Hz); ^{13}C NMR (D_2O): δ 11.1, 27.1, 44.3, 57.3, 62.6, 172.9; HRMS (FAB) m/z 147.1134 $[M+H]^+$ ($C_6H_{15}N_2O_2$ requires 147.1134).

4.1.16. L-Phenylalanylaminoethanol hydrochloride (16). $[\alpha]_D$ +41.7 (c 0.83, H_2O); ^{1}H NMR (D_2O): δ 3.25 (2H, m), 3.34 (2H, m), 3.55 (1H, m), 3.63 (1H, m), 4.25 (1H, t, J = 7.3 Hz), 7.35 (1H, d, J = 6.8 Hz), 7.42–7.49 (4H); ^{13}C NMR (D_2O): δ 39.7, 44.2, 57.4, 62.4, 130.8, 131.9 (2 × C), 132.2 (2 × C), 136.7, 172.0; HRMS (FAB) m/z 209.1289 $[M+H]^+$ ($C_{11}H_{17}N_2O_2$ requires 209.1290).

4.1.17. L-Histidylaminoethanol hydrochloride (17). $[\alpha]_D$ +35.9 (c 0.84, H_2O); ^{1}H NMR (D_2O): δ 3.41 (2H, m), 3.45 (2H, m), 3.64–3.71 (2H, m), 4.32 (1H, t, J = 7.1 Hz), 7.50 (1H, s), 8.76 (1H, s); ^{13}C NMR (D_2O): δ 28.9, 44.4, 55.1, 62.4, 121.2, 128.7, 137.2, 170.9; HRMS (FAB) m/z 199.1194 $[M+H]^+$ ($C_8H_{15}N_4O_2$ requires 199.1195).

4.1.18. L-Homoserylaminoethanol hydrochloride (18). $[\alpha]_D$ +15.6 (c 0.77, H_2O); ^{1}H NMR (D_2O): δ 1.83 (2H, m), 2.14 (2H, m), 3.38 (2H, m), 3.69 (2H, t, J = 6.4 Hz), 3.79 (2H, m), 4.13 (1H, t, J = 6.4 Hz); ^{13}C NMR (D_2O): δ 33.5, 35.5, 39.3, 54.6, 60.4, 61.9, 172.3; HRMS (FAB) m/z 177.1238 $[M+H]^+$ ($C_7H_{17}N_2O_3$ requires 177.1239).

4.1.19. L-Tyrosylaminoethanol hydrochloride (19). $[\alpha]_D$ +42.3 (c 1.10, H_2O); ^{1}H NMR (D_2O): δ 3.16 (2H, m),

3.33 (2H, m), 3.55 (1H, m), 3.63 (1H, m), 4.18 (1H, t, $J = 7.3$ Hz), 6.94 (2H, d, $J = 8.7$ Hz), 7.21 (2H, d, $J = 8.7$ Hz); ^{13}C NMR (D_2O): δ 38.9, 44.2, 57.5, 62.4, 118.6 (2 × C), 128.5, 133.6 (2 × C), 157.9, 172.1; HRMS (FAB) *m/z* 225.1238 $[M+H]^+$ ($C_{11}H_{17}N_2O_3$ requires 225.1239).

4.1.20. L-Homoserylglycine hydrochloride (20). $[\alpha]_D$ +27.3 (*c* 0.38, H_2O); ^{1}H NMR (D_2O): δ 2.20 (2H, m), 3.84 (2H, t, $J = 6.0$ Hz), 4.03 (1H, d, $J = 10.6$ Hz), 4.07 (1H, t, $J = 10.6$ Hz), 4.25 (1H, t, $J = 6.4$ Hz); ^{13}C NMR (D_2O): δ 35.4, 44.6, 54.5, 60.4, 172.7, 176.7; HRMS (FAB) *m/z* 177.0874 $[M+H]^+$ ($C_6H_{13}N_2O_4$ requires 177.0875).

4.1.21. L-Homoserylglycineamide hydrochloride (21). $[\alpha]_D$ +25.0 (*c* 0.70, H_2O); ^{1}H NMR (D_2O): δ 2.20 (2H, m), 3.84 (2H, t, $J = 6.0$ Hz), 4.03 (1H, d, $J = 10.6$ Hz), 4.07 (1H, t, $J = 10.6$ Hz), 4.26 (1H, t, $J = 6.4$ Hz); ^{13}C NMR (D_2O): δ 35.4, 44.8, 54.6, 60.4, 173.2, 176.4; HRMS (FAB) *m/z* 176.1033 $[M+H]^+$ ($C_6H_{14}N_3O_3$ requires 176.1035).

4.1.22. L-Homoseryl-2-aminopropanol hydrochloride (22). $[\alpha]_D$ +10.0 (*c* 0.72, H_2O); ^{1}H NMR (D_2O): δ 1.20 (3H, d, $J = 6.9$ Hz), 2.16 (2H, m), 3.56 (1H, dd, $J = 6.4$, 11.5 Hz), 3.65 (1H, dd, $J = 5.0$, 11.5 Hz), 3.81 (2H, m), 4.06 (1H, m), 4.13 (1H, t, $J = 6.4$ Hz); ^{13}C NMR (D_2O): δ 18.5, 35.6, 50.3, 54.7, 60.4, 67.0, 171.9; HRMS (FAB) *m/z* 177.1241 $[M+H]^+$ ($C_7H_{17}N_2O_3$ requires 177.1239).

4.1.23. L-Valylaminoethanol hydrochloride (23). $[\alpha]_D$ +24.5 (*c* 1.31, H_2O); ^{1}H NMR (D_2O): δ 1.08 (3H, d, $J = 6.9$ Hz), 1.09 (3H, d, $J = 6.9$ Hz), 2.25 (2H, m), 3.73 (1H, m), 3.46 (2H, m), 3.73 (2H, m), 3.81 (1H, d, $J = 6.0$ Hz); ^{13}C NMR (D_2O): δ 19.8, 20.4, 32.6, 44.3, 61.6, 62.6, 172.3; HRMS (FAB) *m/z* 161.1288 $[M+H]^+$ ($C_7H_{17}N_2O_2$ requires 161.1290).

4.1.24. L-Leucylaminoethanol hydrochloride (24). $[\alpha]_D$ +23.5 (*c* 1.48, H_2O); ^{1}H NMR (D_2O): δ 1.01 (6H, t, $J = 6.4$ Hz), 1.67–1.84 (3H), 3.44 (2H, t, $J = 6.4$ Hz), 3.69–3.76 (2H), 4.05 (1H, t, $J = 7.3$ Hz); ^{13}C NMR (D_2O): δ 23.9, 24.4, 26.7, 42.6, 44.4, 54.9, 62.5, 173.4; HRMS (FAB) *m/z* 175.1445 $[M+H]^+$ ($C_8H_{19}N_2O_2$ requires 175.1447).

4.1.25. L-Glutamylaminoethanol hydrochloride (25). $[\alpha]_D$ +32.4 (*c* 1.62, H_2O); ^{1}H NMR (D_2O): δ 2.20 (2H, q, $J = 7.3$ Hz), 2.56 (2H, t, $J = 7.3$ Hz), 3.37–3.46 (2H), 3.70 (2H, t, $J = 5.5$ Hz), 4.06 (1H, t, $J = 6.4$ Hz); ^{13}C NMR (D_2O): δ 28.7, 32.2, 44.4, 55.4, 62.5, 172.2, 179.3; HRMS (FAB) *m/z* 191.1033 $[M+H]^+$ ($C_7H_{15}N_2O_4$ requires 191.1032).

4.1.26. L-Aspartylglycine hydrochloride (26). $[\alpha]_D$ +21.6 (*c* 0.65, H_2O); ^{1}H NMR (D_2O): δ 3.05 (1H, dd, $J = 7.8$, 17.9 Hz), 3.14 (1H, dd, $J = 5.0$, 17.9 Hz), 4.07 (1H, d, $J = 10.2$ Hz), 4.11 (1H, d, $J = 10.2$ Hz), 4.46 (1H, dd, $J = 5.0$, 7.8 Hz); ^{13}C NMR (D_2O): δ 37.8, 44.2, 52.5, 171.9, 175.8, 175.9; HRMS (FAB) *m/z* 191.0669 $[M+H]^+$ ($C_6H_{11}N_2O_5$ requires 191.0668).

4.1.27. L-Aspartylaminoethylamine hydrochloride (27). $[\alpha]_D$ +7.6 (*c* 0.80, H_2O); ^{1}H NMR (D_2O): δ 3.04 (1H, dd, $J = 6.9$, 17.9 Hz), 3.10 (1H, dd, $J = 5.0$, 17.9 Hz), 3.23 (2H, t, $J = 6.4$ Hz), 3.63 (2H, t, $J = 6.4$ Hz), 4.38 (1H, dd, $J = 5.0$, 6.9 Hz); ^{13}C NMR (D_2O) δ: 38.0, 39.9, 41.7, 52.6, 172.5, 176.2; HRMS (FAB) *m/z* 176.1037 $[M+H]^+$ ($C_6H_{14}N_3O_3$ requires 176.1035).

4.2. Enzymes

DNA polymerase α (pol α) was purified from calf thymus by immuno-affinity column chromatography as described previously.[24] The amino-terminal (1–329) and the carboxyl-terminal (1280–1465) truncation mutant of the largest subunit of pol α, p110, was prepared as described previously.[25] Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date et al.[26] Human pol γ catalytic gene was cloned into pFastBac. Histidine-tagged enzyme was expressed using the BAC-TO-BAC HT Baculovirus Expression System according to the supplier's manual (Life Technologies, MD) and purified using ProBoundresin (Invitrogen Japan, Tokyo, Japan). Human pol δ and ε were purified by the nuclear fractionation of human peripheral blood cancer cells (Molt-4) using the second subunit of pol δ and ε-conjugated affinity column chromatography, respectively.[27] Recombinant human pol η and ι tagged with His_6 at their C-terminal were expressed in SF9 insect cells using the baculovirus expression system, and were purified by the cells as described previously.[28,29] A truncated form of pol κ (i.e., hDINB1DC) with a 6 × His-tag attached at the C-terminal was overproduced using the BAC-to-BAC Baculovirus Expression System kit (GIBCO BRL) and purified as described previously.[30] Recombinant His-pol λ was overexpressed and purified according to a method described previously.[31] Fish pol α and δ were purified from the testis of cherry salmon (*Oncorhynchus masou*).[32] *Drosophila* pol α, δ and ε were purified from the early embryos of *D. melanogaster* as described previously.[33,34] 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.[35] Human immunodeficiency virus (HIV) type-1 reverse transcriptase (recombinant) and the Klenow fragment of pol I from *E. coli* were purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). T4 DNA polymerase, Taq DNA polymerase, T7 RNA polymerase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan). Calf thymus terminal deoxynucleotidyl transferase and bovine pancreas deoxyribonuclease I were purchased from Stratagene Cloning Systems (LaJolla, CA, USA). Purified human placenta DNA topoisomerases I (2 units/μL) and IIα (2 units/μL) were purchased from TopoGen, Inc. (Columbus, OH).

5. Experimental

5.1. General experimental procedures

The purity of the synthetic dipeptides was determined by HPLC on a column of Develosil RPA-QUEOUS-AR-5 (4.6 × 250 mm, Nomura Chemical Ltd, Seto, Japan) with

a linear gradient of 0.01 N HCl/CH_3CN solvent system as eluent at a flow rate of 1 mL/min. The equipment used for HPLC was the Waters Delta 600 system. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on a Jeol ECP-500 spectrometer. Chemical shifts are expressed in ppm downfield from sodium 3-(trimethylsilyl)propionate (TSP) in D_2O as an internal standard. FABMS was measured using glycerol as a matrix on a Jeol JMS-700 spectrometer.

5.2. DNA polymerase assays

Activities of DNA polymerases were measured using methods described previously.[2,3] For DNA polymerases, poly(dA)/oligo$(dT)_{12-18}$ and dTTP were used as the template-primer DNA 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. For terminal deoxynucleotidyl transcriptase, oligo$(dT)_{12-18}$ (3′-OH) and dTTP were used as the template-primer and nucleotide substrate, respectively.

Synthesized dipeptide alcohols were dissolved in distilled water at various concentrations and sonicated for 30 s. Aliquots of 4 μL of sonicated samples were 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, except for Taq DNA polymerase, which was incubated at 74 °C for 60 min. The activity without the inhibitor was considered 100%, and the remaining activities at each concentration of inhibitor were determined relative to this value. One unit of each DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of deoxyribonucleotide 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 each enzyme.[2,3]

5.3. Other enzyme assays

Activities of DNA primase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were measured in each of the standard assays according to the manufacturer's specifications as described by Tamiya-Koizumi et al.,[36] Nakayama and Saneyoshi,[37] Soltis and Uhlenbeck[38] and Lu and Sakaguchi,[39] respectively. Telomerase activity was determined using the polymerase chain reaction (PCR)-based telomeric repeat amplification protocol as described[40] with some modifications.[41]

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