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L-Homoserylaminoethanol, a novel dipeptide alcohol inhibitor of eukaryotic DNA polymerase ε from a plant cultured cells, *Nicotina tabacum* L.

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Abstract—We found a novel inhibitor specific to eukaryotic DNA polymerase ε (pol ε) from plant cultured cells, *Nicotina tabacum* L. The compound (compound **1**) was a dipeptide alcohol, L-homoserylaminoethanol. The 50% inhibition of pol ε activity by the compound was 43.6 μg/mL, and it had almost no effect on the activities of the other eukaryotic DNA polymerases such as α, β, γ and δ, prokaryotic DNA polymerases, nor DNA metabolic enzymes such as human telomerase, human immunodeficiency virus type 1 reverse transcriptase, T7 RNA polymerase, human DNA topoisomerase I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I. Kinetic studies showed that inhibition of pol ε by the compound was non-competitive with respect to both template-primer DNA and nucleotide substrate. We succeeded in chemically synthesizing the stereoisomers, L-homoserylaminoethanol and D-homoserylaminoethanol, and found both were effective to the same extent. The IC_{50} values of L- and D-homoserylaminoethanols for pol ε were 42.0 and 41.5 μg/mL, respectively. This represents the second discovery of a pol ε-specific inhibitor, and the first report on a water-soluble peptide-like compound as the inhibitor, which is required in biochemical studies of pol ε.

1. Introduction

Eukaryotic cells contain at least three 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] The purpose of these studies was to use them as tools and molecular probes to distinguish DNA polymerases and to clarify their biological and in vivo functions. In the process, we have found many DNA polymerase inhibitors including long chain fatty acids,[2–4] triterpenoids,[5–7] steroids,[8,9] cerebrosides,[10] glycolipids,[11–15] flavonoids,[16] and vitamin A such as retinal,[17] and characterized many new inhibitors of eukaryotic DNA polymerases which directly bind to the polymerase protein.[4,9]

Keywords: DNA polymerase ε; Dipeptide alcohol; L-Homoserylaminoethanol; D-Homoserylaminoethanol; Enzyme inhibitor.
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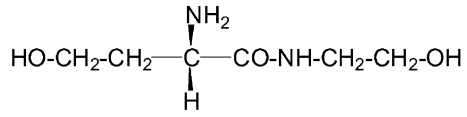
Although we have reported on more than 30 natural compounds that inhibit only the activities of eukaryotic DNA polymerases,[2–18] all the compounds were water-insoluble and not yet sufficient for extensive investigation of the processes, and subsequently we have continued screening for new agents. In this study, we described a novel compound that selectively inhibits only pol ε activity. The natural compound is a dipeptide alcohol, L-homoserylaminoethanol, produced by a higher plant, tobacco (*Nicotina tabacum* L.) cultured cells, and this compound exhibited characteristic inhibition spectra for DNA polymerases and DNA metabolic enzymes (Chart 1).

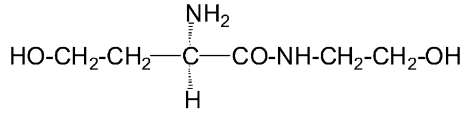
2. Results

2.1. Effects of compound 1 on the activities of DNA polymerases and other DNA metabolic enzymes

We found a compound that selectively inhibited eukaryotic pol ε from plant cultured cells, *Nicotina tabacum* L. The compound belongs to the class of compounds known as dipeptide alcohol (i.e., compound **1**). Compound **1** was tested for inhibition of eukaryotic DNA polymerases such as α, β, γ, δ and ε, prokaryotic DNA polymerases and DNA metabolic enzymes. Figure 1 shows the inhibition dose–response curves of compound **1** against five mammalian DNA polymerases. Human pol ε activity was selectively inhibited by compound **1**, and the IC_{50} value was 43.6 μg/mL. On the other hand, compound **1** had no significant influence on the activities of mammalian pol α, β, γ and δ, plant pol α and β, prokaryotic DNA polymerases such as the Klenow fragment of *E. coli* pol I, Taq pol and T4 pol. The three-dimensional structures of pol ε would be greatly different from those of other eukaryotic and 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 inhibition modes of compound **1** did not change (data not shown). Compound **1** 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 pol, human DNA topoisomerase I and II, T4 polynucleotide kinase and bovine deoxyribonuclease I (Fig. 2).



L-Homoserylaminoethanol (Compound **1**)



D-Homoserylaminoethanol

Chart 1. Structures of L-homoserylaminoethanol (compound **1**) and D-homoserylaminoethanol.

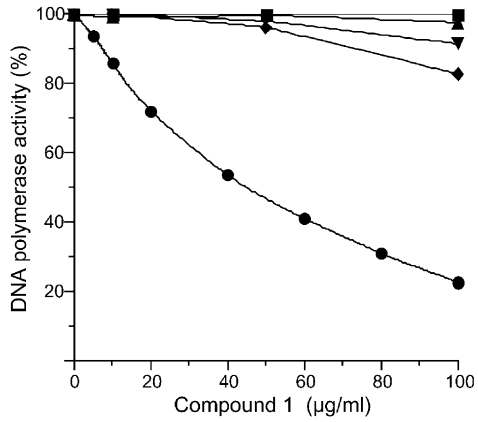


Figure 1. Mammalian DNA polymerase inhibition dose–response curves of compound **1**. The enzymes used (0.05 units of each) were calf pol α (square), rat pol β (reverse triangle), human pol γ (diamond), human pol δ (triangle) and human pol ε (circle). The DNA polymerase activities were measured as described in Experimental section. DNA polymerase activity in the absence of the compound was taken as 100%.

We determined the chemical structure of compound **1** by spectrophotometries including mass-spectrometry and NMR analysis. Compound **1** was a novel dipeptide alcohol, L-homoserylaminoethanol. Therefore, this is the first report of a novel compound that is a water-soluble eukaryotic pol ε inhibitor. In the biochemical studies, whether the inhibitor is water-soluble or not is crucial.

2.2. Effects of reaction conditions on DNA polymerase ε inhibition

To determine the effects of a non-ionic detergent on the binding of compound **1** to pol ε, a neutral detergent, Nonidet P-40 (NP-40), was added to the reaction mixture at a concentration of 0.05 or 0.1%. In the absence of compound **1**, DNA polymerase activity was taken as 100%. The pol ε inhibitory effect of compound **1** at 10 and 100 μg/mL was not affected by the addition of NP-40 to the reaction mixture, suggesting that the binding interaction to the enzyme by compound **1** is hydrophilic (Table 1). We also tested whether an excess amount of a substrate analogue, poly(rC) (100 μg/mL), or a protein, BSA (100 μg/mL), could prevent the inhibitory effects of compound **1**, to determine whether the effects of the compound were due to their non-specific adhesion to the enzymes, or to selective binding to specific sites. Poly(rC) and BSA showed little or no influence on the effects of compound **1**, suggesting that the binding to the DNA polymerase occurs selectively (Table 1).

2.3. Mode of inhibition of DNA polymerase ε by compound 1

To elucidate the inhibitory mechanism, the extent of inhibition was measured as a function of the concentration of either DNA template-primer or deoxyribonucleoside triphosphates in the absence or presence of compound **1** (Fig. 3). In the kinetic analyses, poly(dA)/oligo$(dT)_{12–18}$ and dTTP were used as the DNA tem-

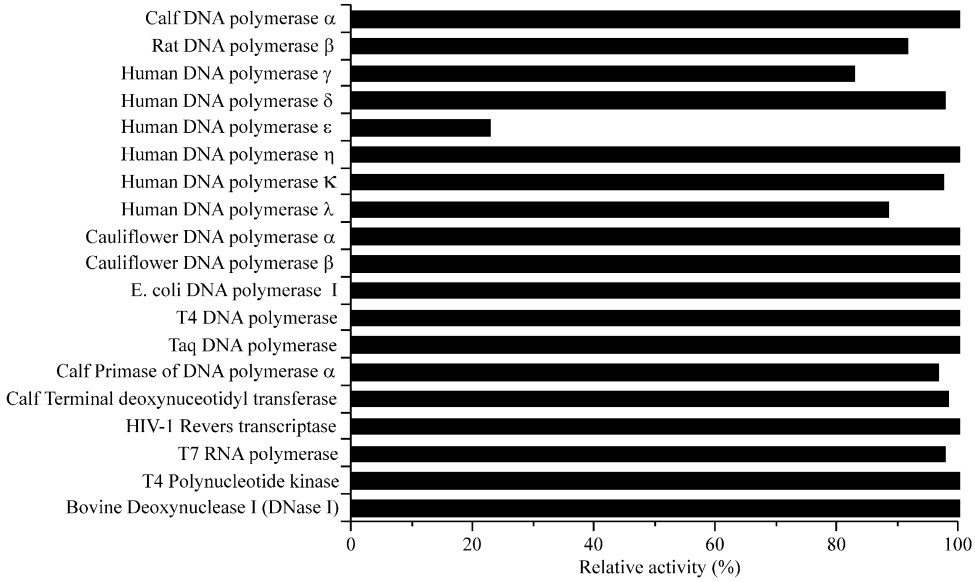


Figure 2. Effect of compound **1** on the activities of various DNA polymerases and other DNA metabolic enzymes % of relative activity. Compound **1** (100 μg/mL) was incubated with each enzyme (0.05 units). Enzyme activity in the absence of compound was taken as 100%.

Table 1. Effects of poly (rC), bovine serum albumin (BSA) or Nonidet P-40 (NP-40) on the inhibition of human pol ε activiy by compound **1**

Compound **1** added to the reaction mixture	Relative pol ε activity (%)	
Without the compound		
None (control)	100	
+ 100 μg/mL poly (rC)	100	
+ 100 μg/mL BSA	100	
+ 0.05% NP-40	100	
+ 0.1% NP-40	100	
20 μg/mL Compound **1**		Ratio
20 μg/mL Compound **1**	72.0	(1.00)
20 μg/mL Compound **1** + 100 μg/mL poly (rC)	70.7	(0.98)
20 μg/mL Compound **1** + 100 μg/mL BSA	68.5	(0.95)
20 μg/mL Compound **1** + 0.05% NP-40	72.4	(1.01)
20 μg/mL Compound **1** + 0.1% NP-40	73.0	(1.01)
100 μg/mL Compound **1**		Ratio
100 μg/mL Compound **1**	22.5	(1.00)
100 μg/mL Compound **1** + 100 μg/mL poly (rC)	20.2	(0.90)
100 μg/mL Compound **1** + 100 μg/mL BSA	21.0	(0.93)
100 μg/mL Compound **1** + 0.05% NP-40	22.6	(1.00)
100 μg/mL Compound **1** + 0.1% NP-40	21.8	(0.97)

100 μg/mL poly (rC), 100 μg/mL BSA and 0.05% or 0.1% NP-40 was added to the reaction mixture.

In the absence of compound **1**, human pol ε activity (0.05 units) was taken as 100%.

plate-primer and nucleotide substrate, respectively. Double reciprocal plots of the results indicated that the compound **1**-mediated inhibition of the pol ε activity was non-competitive with both the DNA template-primer and nucleotide substrate. In the case of the DNA template-primer, 52.3 and 33.4% decreases in maximum velocity (V_{max}) were observed in the presence of 15 and 30 μg/mL compound **1**, respectively, whereas the apparent Michaelis constant (K_m) was unchanged at 15.6 μM (Fig. 3A). The K_m for the nucleotide substrate was unchanged at 1.82 μM, and the V_{max} for the nucleotide substrate increased from 58.8 to 31.3 pmol/h in the presence of 30 μg/mL compound **1** (Fig. 3B). When activated DNA and four deoxyribonucleoside triphosphates were used as the DNA template-primer and nucleotide substrate, respectively, the inhibition of pol ε by compound **1** was the same as when using poly(dA)/oligo(dT)$_{12-18}$ and dTTP (data not shown). These findings suggested that the binding site of compound **1** to pol ε is different from the DNA template-primer and nucleotide substrate binding sites of pol ε.

The inhibition constant (K_i) values for pol ε, obtained from Dixon plots from Figure 3C and D, were found to be 15.6 μg/mL and 24.8 μg/mL for the DNA template-primer and nucleotide substrate, respectively. The affinity of compound **1** may be stronger at the DNA template-primer binding than at the nucleotide substrate-binding of pol ε.

2.4. Inhibition of DNA polymerase ε activity by synthesized homoserylaminoethanol

We succeeded in the chemical synthesis of compound **1** (i.e., native type, L-homoserylaminoethanol) and its unnatural enantiomer (i.e., D-homoserylaminoethanol). These synthesized compounds inhibited human pol ε activity identical to the natural compound, and the IC_{50} values of L-homoserylaminoethanol from nature and those of the synthetic L-homoserylaminoethanol and D-homoserylaminoethanol were similar (Table 2). This suggested that natural and unnatural stereoisomers of homoserylaminoethanol exhibited the same inhibitory activities toward pol ε. The inhibitory activities of pol ε from fruit fly (*D. melanogaster*) and budding yeast (*S. cerevisiae*) by these compounds were as strong as that of pol ε from human (Table 2). Therefore, both enantiomers must be selective inhibitors of pol ε.

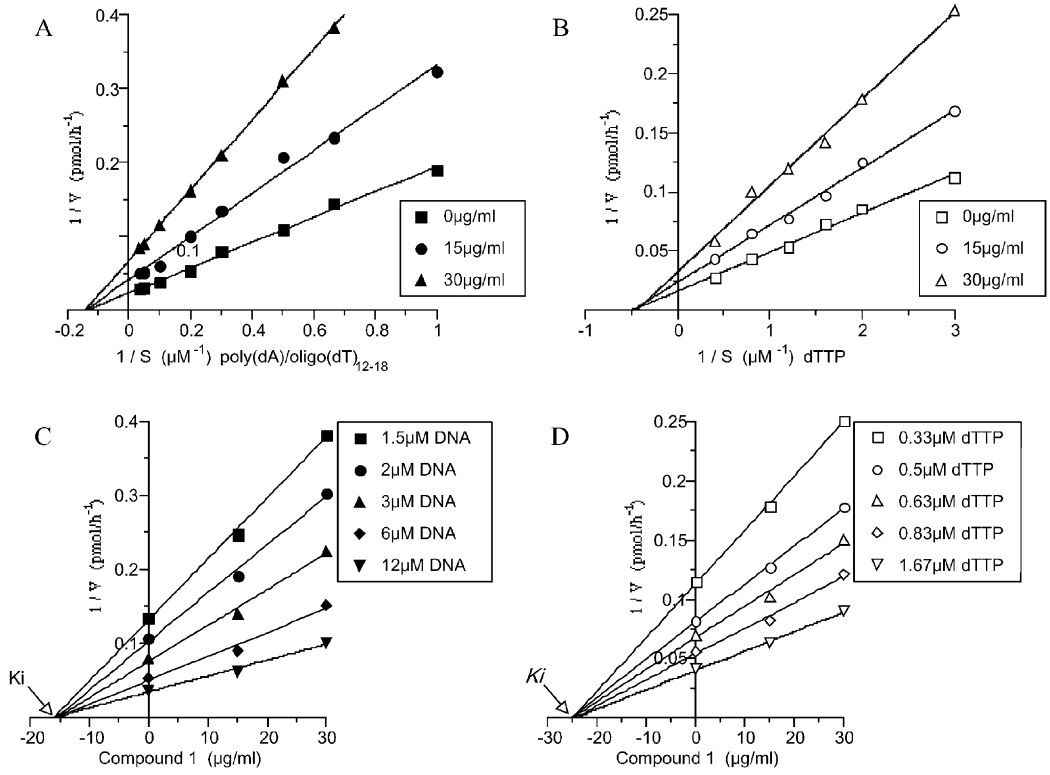


Figure 3. Kinetic analyses of pol ε inhibition by compound **1**. (A and B) Lineweaver-Burk double-reciprocal plots obtained by varying DNA template-primer concentrations (A), and nucleotide substrate concentrations (B). Activity of human pol ε was assayed in the absence (square) or presence of 15 (circle), and 30 (triangle) μg/mL compound **1**. (C and D) The inhibition constants (K_i) were determined as 15.6 and 24.8 mg/mL from a Dixon plot made on the basis of the same data for A and B, respectively. The amount of human pol ε in the assay mixture was 0.05 units.

Table 2. Concentration of the natural and synthesized homoserylaminoethanol (μg/mL) giving 50% inhibition of eukaryotic pol ε

Compound	Eukaryotic pol ε		
	Human[a]	Fruit fly[b]	Budding yeast[c]
Natural compound **1** (L-type)	43.6	48.1	32.8
Synthesized L-homoserylaminoethanol	42.0	46.5	34.2
Synthesized D-homoserylaminoethanol	41.5	49.3	34.0

The compounds were incubated with each enzyme (0.05 units). The enzymatic activity was measured as described in Materials and Methods. DNA polymerase activity in the absence of the compound was taken as 100%.
[a] *Homo sapiens*.
[b] *Drosophila melanogaster*.
[c] *Saccharomyces cerevisiae*.

3. Discussion

In this report, we described a novel inhibitor specific to eukaryotic pol ε. The compound was a dipeptide alcohol, L-homoserylaminoethanol. This is the second pol ε-specific inhibitor following sulfoquinovosyl-diacylglycerol (SQDG) also reported by us.[19] Compared with SQDG, the inhibitory effect of L-homoserylaminoethanol on pol ε was not as strong, but the compound showed marked differences that are crucial in the biochemical study of pol ε. As described in the Introduction, the purpose of screening was to use the inhibitors as tools and molecular probes to distinguish pol ε and other polymerases and to clarify the biological and in vivo biological functions. Although more than 30 DNA polymerase inhibitors including pol ε have been found in our laboratory so far, all of them were water-insoluble or unuseful for agents and drugs. In particular, as L-homoserylaminoethanol is water-soluble, it may be valuable as a tool or probe.

SQDG, a sulfo-glycolipid, has been developed as a clinically promising immunosuppressant, and another sulfo-glycolipid, sulfoquinovosyl-monoacylglycerol, has strong anti-tumor activity as described previously.[11–15] Inhibitors of the replicative DNA polymerases, such as pol ε, could be potential candidates for new immunosuppressants and anti-cancer drugs. Therefore, L-homoserylaminoethanol should also be tested as an immunosuppressive or an anti-tumor agent. If it was effective, L-homoserylaminoethanol could be a more promising agent in the clinical usage, because of water-soluble. Since L-homoserylaminoethanol selectively and non-competitively inhibits pol ε activity with the DNA template-primer and nucleotide 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.

To obtain more information about the molecular basis for differential inhibition spectra exhibited by dipeptide alcohols and development of anti-cancer agents, we are trying rational drug designs of other dipeptide alcohols that are based on the structure of L-homoserylaminoethanol.

4. Experimental

4.1. General methods

The purity of the dipeptide alcohol was checked by HPTLC on Silica Gel $60F_{254}$ (E. Merck) using the solvent system PrOH-AcOH-H_2O (4:1:1), and a chlorine-*o*-tolidine reagent was used for detection, and further determined by HPLC on a column of Develosil RPAQUEOUS-AR-5 (4.6×250 mm, Nomura Chemical, Ltd., Seto, Japan) using 0.005N HCl as eluant at a flow rate of 1 mL/min. The equipment for HPLC was the Waters Delta 600 system. Optical rotations were measured with a Jasco DIP-370 digital polarimeter. ^{1}H 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. The assignment of proton and carbon signals in NMR was determined from extensive homonuclear decoupling experiments, DEPT, ^{1}H-^{13}C COSY, and HMBC spectral data. FABMS were measured using glycerol as a matrix on a Jeol JMS-SX 102A spectrometer.

5. Materials

Deoxyribonucleoside triphosphates such as [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP) (43 Ci/mmol) and synthetic polynucleotides such as poly(dA), poly(rA), poly(rC) and oligo(dT)[12–18], were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries (Osaka, Japan). The cells of the tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) were cultured by the manufacturer's method as described by Nagata et al.[20]

5.1. Preparation and isolation of the peptide

The cultured cells of the higher plant (*Nicotina tabacum* L., 300 g, dry weight) were extracted with 50% methanol. The extract (1.6 g) was applied to an Amberlite IR-120B column (250 mL, H^+ from) and eluted with 0.5 M NH_4OH. The concentrated eluate (190 mg) was applied to a Dowex 1-X2 column (1.5×70 cm, OH^- form) and eluted with water, and then the elution was subjected to an Amberlite CG-50 column (1.0×45 cm, NH_4^+ form) and eluted with water. The elutuin was rechromatographed to Dowex 1-X2 column (1.0×40 cm, OH^- form) and eluted with water to give compound **1** (5.5 mg).

5.2. Structural determination of compound 1

Compound **1** was determined to have the molecular formula $C_6H_{14}N_2O_3$ by HRFABMS. The ^{13}C NMR spectroscopic data showed the presence of a single carbonyl (δ 180.3), a single methine (δ 55.2), and four methylene (δ 39.3, 44.2, 61.2, 62.8) carbon atoms. The methylene groups at δ 61.2 and 62.8 were attributed to the hydroxymethyl carbon. Decoupling experiments and HMBC spectrum of compound **1** elucidated a $HOCH_2$–CH_2–CH-CO– moiety and a –CH_2–CH_2OH moiety. These results suggest that compound **1** is a pseudodipeptide consisting of homoserine and aminoethanol. In comparative studies, the natural product and synthetic L-homoserylaminoethanol showed identical NMR data and the same retention time on HPLC. From the specific rotation values in H_2O for the natural product (+21.8°) and the synthetic sample (+18.3°), the structure of compound **1** was determined to be L-homoserylaminoethanol.

5.2.1. L-Homoserylaminoethanol (compound 1). Colorless syrup; $[\alpha]_D$ +21.8° (*c* 0.32, H_2O); ^{1}H NMR (500 Hz, D_2O) δ 1.80 (1H, m), 1.91 (1H, m), 3.37 (2H, t), 3.51 (1H, dd, J = 6.4, 8.7 Hz), 3.64–3.72 (4H); ^{13}C NMR (125 MHz, D_2O) δ 39,3 (t), 44.2 (t), 55.0 (d), 61.2 (t), 62.8 (t), 180.3 (s); HRFABMS m/z 163.1084 $[M+H]^+$ ($C_6H_{15}N_2O_3$ requires 163.1083).

5.3. Synthesis of L- and D-homoserylaminoethanols

N,N′-Diisopropylethylamine (104.5 μL, 0.6 mmol) was added to a suspension of aminoethanol (H-Gly-ol)-Trt(2-Cl)-resin (588 mg, 0.2 mmol, 200–400 mesh, 1% divinylbenzene polystyrene copolymer, Watanabe Chemical Industries, LTD., Hiroshima, Japan), Fmoc-L-Hse(Trt)-OH (233.5 mg, 0.4 mmol) and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (152.1 mg, 0.4 mmol) in DMF (10 mL). The reaction mixture was stirred for 2 h at room temperature, filtered and washed with DMF. The desired protected peptide-resin thus obtained was treated with trifluoroacetic acid (9.5 mL) in the presence of H_2O (0.25 mL) and triisopropylsilane (0.25 mL) at room temperature for 2 h, followed by treatment of 20% piperidine in DMF (10 mL) in the usual manner. The crude resulting H-L-Hse-Gly-ol (62.1 mg) was purified by reverse phase HPLC on a Develosil RPAQUEOUS-AR-5 column (20′ 250 mm) with an isocratic elution of 0.005 N HCl at a flow rate of 10 mL/min to give desired H-L-Hse-Gly-ol (19.2 mg).

5.3.1. L-Homoserylaminoethanol (H-L-Hse-Gly-ol). Colorless syrup; $[\alpha]_D$ +18.3° (*c* 0.44, H_2O); ^{13}C NMR (125 MHz, D_2O) δ 39,1 (t), 44.2 (t), 55.0 (d), 61.1 (t), 62.8 (t), 179.9 (s); FABMS m/z 163 $[M+H]^+$.

5.3.2. D-Homoserylaminoethanol (H-D-Hse-Gly-ol). Colorless syrup; $[\alpha]_D$ −22.8° (*c* 0.75, H_2O); ^{13}C NMR (125 MHz, D_2O) δ 39,1 (t), 44.2 (t), 55.0 (d), 61.1 (t), 62.8 (t), 179.9 (s); FABMS m/z 163 $[M+H]^+$.

6. Preparation of DNA polymerase and other DNA metabolic enzymes

DNA polymeases such as calf pol α, rat pol β, human pol δ, fruit fly (*D. melanogaster*) pol ε, human pol η, human pol κ and human pol λ were purified as described previously.[19] Human pol γ, cauliflower pol α and β, *E. coli* pol I, T4 pol, Taq pol, calf primase of pol α, calf terminal deoxynucleotidyl transferase, HIV-1 reverse

transcriptase, T7 RNA polymerase, T4 polynucleotide kinase and bovine deoxyribonuclease I were prepared as described previously.[21] Human telomerase and human DNA topoisomerase I and II were prepared as described previously.[16] Human pol ε was purified from the nuclear fraction of human peripheral blood cancer cells (Molt-4) by the second subunit of pol ε-conjugated affinity column chromatography (Oshige et al., in preparation). Yeast (*S. cerevisiae*) pol ε was purified by ion-exchange column chromatography as described by Hamatake et al.[22]

6.1. DNA polymerase assay

The activities of DNA polymerases were measured by the methods described previously.[2,3] The activity without the inhibitor was considered to be 100%, and the remaining activities at each concentration of inhibitor were determined as percentages of this value. One 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 the synthetic DNA 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] For kinetic analyses, the concentrations of DNA template-primer or [^{3}H]-dTTPs were varied. The inhibition mode was analyzed by Lineweaver–Burk plots, and Ki was obtained from Dixon plots.

6.2. Other DNA metabolic enzymes assays

The activities of primase of pol α, terminal deoxynucleotidyl transferase, reverse transcriptase, telomerase, RNA polymerase, DNA topoisomerases, polynucleotide kinase and deoxyribonuclease I were measured in each of the standard assays according to the manufacturer's specifications as described previously.[16,21]

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