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Structural analysis of isosteviol and related compounds as DNA polymerase and DNA topoisomerase inhibitors

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Abstract

Isosteviol (*ent*-16-ketobeyeran-19-oic acid) is a hydrolysis product of stevioside, which is a natural sweetener produced in the leaves of *Stevia rebaudiana* (Bertoni) Bertoni. In this report, we prepared isosteviol and related compounds from stevioside by microbial transformation and chemical conversion and assayed the inhibitory activities toward DNA metabolic enzymes and human cancer cell growth. Among twelve compounds obtained, only isosteviol (compound **3**) potently inhibited both mammalian DNA polymerases (pols) and human DNA topoisomerase II (topo II), and IC_{50} value for pol α was 64.0 μM. This compound had no inhibitory effect on higher plant (cauliflower) pols, prokaryotic pols, human topo I, and DNA metabolic

Abbreviations: topo, DNA topoisomerase; pol, DNA polymerase; dTTP, 2′-deoxythymidine 5′-triphosphate; dNTP, deoxyribonucleoside triphosphates; DMSO, dimethylsulfoxide; dsDNA, double stranded DNA; EtBr, ethidium bromide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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enzymes such as human telomerase, T7 RNA polymerase, and bovine deoxyribonuclease I. With pol α, isosteviol acted non-competitively with the DNA template-primer and nucleotide substrate. Isosteviol prevented the growth of human cancer cells, with LD_{50} values of 84–167 μM, and 500 μg of the compound caused a marked reduction in TPA (12-*O*-tetradecanoylphorbol-13-acetate)-induced inflammation (inhibitory effect, 53.0%). The relationship between the structure of stevioside-based compounds and these activities were discussed.

Keywords: Isosteviol; DNA polymerase; DNA topoisomerase; Enzyme inhibitor; Cytotoxicity; Anti-inflammation

Introduction

Stevioside (compound **1**) is a glycoside formed by three glucose molecules and steviol (compound **2**), a diterpenic carboxylic alcohol (Fig. 1). Stevioside is a high-intensity sweetener that tastes about 300 times sweeter than sucrose (0.4% solution). In many countries, it is used as a low-calorie sweetener in a wide range of food products and beverages. Stevioside has been used for several years as a sweetener in South America, Asia, Japan, China, and different countries of the European Union, while in the United States, it has been used as a dietary supplement since 1995. *Stevia rebaudiana* (Bertoni) Bertoni products are approved for sweetening purposes in Brazil, Korea, and Japan (Kinghorn, 2002). The advantages of stevioside as a dietary supplement for human subjects are manifold: it is stable, it is non-calorific, it maintains dental health by reducing the intake of sugar, and it has possibilities for use by diabetic and phenylketonuria patients and obese persons.

We have reported that novel anti-TPA-induced inflammatory compounds, a novel terpeno benzoic acid (i.e., myrsinoic acid A (MAA)), novel triterpenoids (i.e., tormentic acid (TA) and euscaphic acid (EA)) and some phenolic compounds such as curcumin were inhibitors of mammalian DNA polymerase α, β and λ (pol α, β and λ) (Mizushina et al., 2000, 2003; Murakami et al., 2002). Although tumor promoters are compounds that promote tumor formation (Hecker, 1978), these compounds also cause inflammation and are generally used as artificial inflammation inducers for the screening of anti-inflammatory agents (Fujiki and Sugimura, 1987). Tumor promoter-induced inflammation can be distinguished from acute inflammation, which is exudative and accompanied by fibroblast proliferation and granulation. TPA not only causes inflammation but influences mammalian cell growth (Nakamura et al., 1995), suggesting that the molecular basis of the inflammation is the pol reaction related to cell proliferation. Here, we investigated the mode of action of stevioside, isosteviol and related compounds against pols and DNA metabolic enzymes, which are responsible for DNA replication leading to cell proliferation and DNA repair/recombination and, then, the relation between the degree of the cell growth inhibitory effect and the anti-inflammatory activity.

We report here that one of the compounds, isosteviol (compound **3**), is a potent inhibitor of mammalian pols and human DNA topoisomerase II (topo II). Pols and topos were suggested to be important cellular targets for chemical intervention in the development of anti-cancer agents. Isosteviol inhibited both the growth of human cancer cells and the TPA-induced inflammation. Therefore,

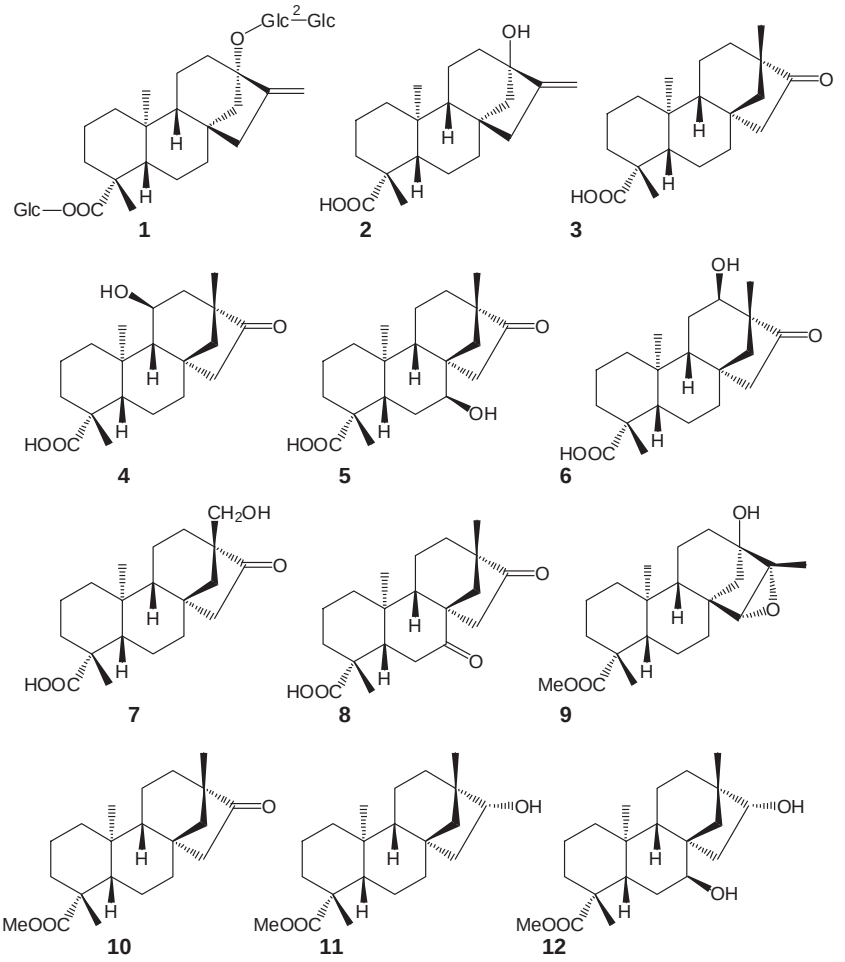

Fig. 1. Chemical structure of isosteviol and related compounds. Compound **1**: stevioside, compound **2**: steviol, compound **3**: isosteviol, compound **4**: 11β-hydroxyisosteviol, compound **5**: 7β-hydroxyisosteviol, compound **6**: 12β-hydroxyisosteviol, compound **7**: 17-hydroxyisosteviol, compound **8**: 7-oxoisosteviol, compound **9**: methyl *ent*-15β,16β-epoxy-13-hydroxykauran-19-oate, compound **10**: isosteviol methyl ester, compound **11**: methyl *ent*-16β-hydroxybeyeran-19-oate, and compound **12**: methyl *ent*-7α, 16β-dihydroxybeyeran-19-oate.

information concerning the structural characteristics of stevioside-based compounds may provide valuable insight for the design of new anti-cancer agents.

Materials and methods

Materials

[^{3}H]-dTTP (2′-deoxythymidine 5′-triphosphate) (43 Ci/mmol), nucleotides, and chemically synthesized template-primers such as poly(dA) and oligo(dT)$_{12–18}$ were purchased from Amersham Biosciences (Buckinghamshire, UK). Supercoiled pUC19 plasmid DNA and pBR322 DNA were

obtained from Takara (Tokyo, Japan). All other reagents were of analytical grade and were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Chemicals

Sources of stevioside (compound **1**), isosteviol (*ent*-16-ketobeyeran-19-oic acid, compound **3**), 11β-hydroxyisosteviol (compound **4**), 7β-hydroxyisosteviol (compound **5**), 12β-hydroxyisosteviol (compound **6**), 17-hydroxyisosteviol (compound **7**), and 7-oxoisosteviol (compound **8**) were described in our recent paper (Akihisa et al., 2004). Thus, stevioside (compound **1**) was kindly donated by Horiuchi Foods, Co., Ltd. (Tokyo, Japan). Isosteviol (compound **3**) was obtained by hydrolysis of stevioside (compound **1**) with dilute hydrochloric acid. Incubation of compound **3** with *Aspergillus niger* IFO 4414 yielded 7β-hydroxyisosteviol (compound **5**), 11β-hydroxyisosteviol (compound **4**), and 12β-hydroxyisosteviol (compound **6**). Biotransformation of compound **3** by *Glomerella cingulata* IFO 9767 produced 17-hydroxyisosteviol (compound **7**). 7-Oxoisosteviol (compound **8**) was obtained from biotransformation of compound **3** by *Mortierella elongate* IFO 8570.

Steviol (compound **2**) was prepared from microbial transformation of stevioside (compound **1**) by using the fungus *A. niger* IFO 4414 (Subrahmanyam et al., 1999). Methyl *ent*-15β,16β-epoxy-13-hydroxykauran-19-oate (compound **9**) was obtained from compound **2** by epoxidation with *m*-chloroperbenzoic acid in dichloromethane followed by esterification with diazomethane. Esterification with diazomethane of isosteviol (compound **3**) afforded isosteviol methyl ester (compound **10**) (Avent et al., 1990a,b) and reduction of compound **10** with $NaBH_4$ in ethanol yielded methyl *ent*-16β-hydroxybeyeran-19-oate (compound **11**) (Ali et al., 1992). Reduction of 7β-hydroxyisosteviol (3) with $NaBH_4$ followed by esterification by diazomethane gave methyl *ent*-7α,16β-dihydroxybeyeran-19-oate (compound **12**) (Ali et al., 1992). Identification of compounds **2**, **9**, **10**, **11**, and **12** was performed by mass spectral and ^{1}H NMR spectral comparison with the corresponding compounds in the literature.

Enzymes

Pol α was purified from the calf thymus by immuno-affinity column chromatography as described previously (Tamai et al., 1988). Recombinant rat pol β was purified from *E. coli* JMpβ5 as described by Date et al. (1988). Recombinant His-pol λ was overexpressed and purified according to methods described previously (Shimazaki et al., 2002). 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. (1980). The Klenow fragment of pol I from *E. coli* and human immunodeficiency virus (HIV) type-1 reverse transcriptase (recombinant) was purchased from Worthington Biochemical Corp. (Freehold, NJ, USA). T7 RNA polymerase was purchased from Takara (Kyoto, Japan). Purified human placenta topo I and II (2 units/ml) were obtained from TopoGen, Inc. (Columbus, OH, USA). Bovine pancreas deoxyribonuclease I was purchased from Stratagene Cloning Systems (LaJolla, CA, USA).

DNA polymerase assays

Activities of pols were measured using methods described previously (Mizushina et al., 1996, 1997). The substrates of pols used were poly(dA)/oligo(dT)$_{12–18}$ and dTTP as template-primer DNA and nucleotide substrate, respectively. Isosteviol and related compounds were dissolved in dimethylsulfoxide

(DMSO) at various concentrations and sonicated for 30 s. Aliquots of 4 μl of sonicated samples were mixed with 16 μl of each enzyme (0.05 units) in 50 mM Tris–HCl (pH7.5) 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 activity at each concentration of inhibitor was determined relative to this value. One unit of pol 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 normal reaction conditions for each enzyme (Mizushina et al., 1996, 1997).

DNA topoisomerase assays

Relaxation activity of topo II was determined by detecting the conversion of supercoiled plasmid DNA to its relaxed form (Spitzner et al., 1990). The topo II reaction was performed in 20 μl of a reaction mixture containing 50 mM Tris–HCl buffer (pH 8.0), 120 mM KCl, 10 mM $MgCl_2$, 0.5 mM ATP, 0.5 mM dithiothreitol, supercoiled pUC19 DNA (0.25 μg), 2 μl of inhibitor solution (10% DMSO) and 2 units of topo II. The reaction mixtures were incubated at 37 °C for 30 min and terminated by adding 2 μl of loading buffer consisting of 5% sarkosyl, 0.0025% bromophenol blue and 25% glycerol. The mixtures were subjected to 1% agarose gel electrophoresis in TAE (Tris–acetate–EDTA) running buffer. The agarose gel was stained with ethidium bromide (EtBr) and DNA was visualized on a UV transilluminator. The relaxation activity of topo I was analyzed in the same manner except that the reaction mixtures contained 10 mM Tris–HCl (pH 7.9), pBR322 DNA (0.25 μg), 1 mM EDTA, 150 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1 mM spermidine, 5% glycerol and 2 units of topo I. One unit was defined as the amount of enzyme capable of relaxing 0.25 μg of DNA in 15 min at 37 °C.

Other enzyme assays

Activities of T7 RNA polymerase and bovine deoxyribonuclease I were measured in standard assays according to the manufacturer's specifications as described by Nakayama and Saneyoshi (1985) and Lu and Sakaguchi (1991), respectively. Telomerase activity was determined using the polymerase chain reaction (PCR)-based telomeric repeat amplification protocol as described (Kim et al., 1994) with some modifications (Ueno et al., 2000).

Investigation of cytotoxicity in human cancer cells

To investigate the effects of isosteviol and related compounds in vivo, a human T cell acute lymphoblastic leukemia cell line, MOLT-4, a human B cell acute lymphoblastoid leukemia cell line, BALL-1, and a human gastric cancer cell line, NUGC-3, were obtained from Health Science Research Bank (Osaka, Japan). The cells were routinely cultured in RPMI1640 medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, 100 unit/ml penicillin, and 1.6 mg/ml $NaHCO_3$ at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The cytotoxicity of the compounds was investigated as follows. High concentrations (10 mM) of the compounds were dissolved in DMSO and stocked. Approximately 5×10^3 cells per well were inoculated in 96-well microplates, then the stock solution of

the compound was diluted to various concentrations and applied to each well. After incubation for 48 h, the survival rate was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983).

Anti-inflammatory assay

The mouse inflammatory test was performed according to Gschwendt's method (Gschwendt et al., 1984). This experiment complied with the regulations concerning animal experimentation and the care of experimental animals of Kobe-Gakuin University. Briefly, a methanol solution of the test compound (250 or 500 μg/20 μl) was applied to the inner part of the mouse ear. Thirty minutes after the test compound was applied, a TPA (12-*O*-tetradecanoylphorbol-13-acetate) solution (0.5 μg/20 μl of acetone) was applied to the same part of the ear. To the other ear of the same mouse, methanol and a TPA solution were applied as a control. After 7 h, a disk (6 mm diameter) was obtained from the ear and weighed. The inhibitory effect (IE) is presented as the ratio of the weight increase of the ear disks: IE: $\{[(\text{TPA only}) - (\text{tested compound plus TPA})]/[(\text{TPA only}) - (\text{vehicle})] \times 100\}$.

Computational analysis of isosteviol derivatives

A compound model was constructed and simple-minimized. Compound models were simulated with force field parameters based on the consistent valence force field (CVFF). Group-based cutoffs, 0.95 nm for van der Waals and 0.95 nm for Coulomb interactions, were introduced. The temperature was set at 298 K. Calculations based on simulation images were carried out using INSIGHT II (Accelrys Inc., San Diego, CA, USA). Electrostatic potentials on the surface of compounds were analyzed with WebLab ViewerLite (version 3.2, Accelrys Inc., San Diego, CA, USA) software.

Results and discussion

Effects of isosteviol and related compounds on DNA polymerases and other DNA metabolic enzymes

We, as a primary screening for inhibition of the DNA metabolic enzymes, tested the effects of isosteviol and related compounds (compounds **1**–**12**, Fig. 1) on mammalian pol α, β and λ and plant and prokaryotic pols. We decided that the compound, of which the IC_{50} values were lower than 200 μM, was an inhibitor of the enzyme activity. As shown in Table 1, compound **3** (isosteviol) inhibited the activity of mammalian pols (α, β and λ) but did not influence the activities of higher plant (cauliflower) pol I (α-like polymerase) and II (β-like polymerase) and prokaryotic pols such as the Klenow fragment of *E. coli* pol I. The other compounds, **1**–**2** and **4**–**12**, had no effect on the mammalian pols (Table 1). Human topo II activity was inhibited by compounds **1**, **3** and **7** (Table 1). On the other hand, human topo I activity was not influenced by any of the compounds at all. The compounds had little effect on the activities of higher plant (cauliflower) pol I (α-like polymerase) and II (β-like polymerase), prokaryotic pols such as the Klenow fragment of *E. coli* pol I, and DNA metabolic enzymes such as T7 RNA polymerase, human immunodeficiency virus (HIV) type-1 reverse transcriptase, human telomerase, and bovine deoxyribonuclease I (Table 1).

Table 1
IC_{50} values for isosteviol and related compounds on the activities of various DNA polymerases and other DNA metabolic enzymes

Enzyme	Compound (μM)											
	1	2	3	4	5	6	7	8	9	10	11	12
Calf pol α	>1000	>1000	64.0	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Rat pol β	>1000	>1000	177	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human pol λ	>1000	>1000	103	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Cauliflower pol I[a]	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Cauliflower pol II[b]	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
E. coli pol I	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
T7 RNA polymerase	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
HIV-RT[c]	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human telomerase	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human topo I	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Human topo II	90.0	>1000	190.0	>1000	>1000	>1000	82.5	>1000	>1000	>1000	>1000	>1000
Bovine DNase I[d]	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000

[a] α-like DNA polymerase.
[b] β-like DNA polymerase.
[c] Reverse transcriptase.
[d] Deoxyribonuclease I.

These observations suggested that some of the stevioside-based compounds could be inhibitors of mammalian pols and human topo II and, of these twelve compounds, isosteviol (compound **3**) was of greatest interest as an inhibitor of the mammalian pols and topo II. Structurally, all compounds consist of a tetracyclic diterpenoid with a beyerane skeleton and one carboxyl group (Fig. 1). Compound **1** is a glycoside formed by three glucose molecules, compounds **2**–**8** have one free carboxyl group and compounds **9**–**12** have one methyl ester of a carboxyl group. Compound **3** (isosteviol) has one ketone group and no hydroxyl groups. From the results in Table 1, no hydroxyl groups, one carboxyl group and one ketone group of diterpene acid might be considered to be important for the inhibition of mammalian pols.

To determine whether a tetracyclic diterpenoid such as compound **3** binds to DNA, the fluorescence emission spectra of ethidium bromide (EtBr) in the presence of calf thymus double-stranded DNA (dsDNA) and the compound were measured. When EtBr was intercalated with dsDNA, the fluorescence of the EtBr–dsDNA complex increased and the maximum emission wavelength was 595 nm. At high concentrations (i.e., 1000 μM) of compound **3**, no decrease in the fluorescence of EtBr was observed (data not shown). Thus, compound **3** did not bind and intercalate with the dsDNA, suggesting that it must inhibit the activity by interacting with the enzyme directly.

Therefore, we concentrated on the properties of compound **3** in the latter part of this study.

*Effects of compound **3** on the activities of mammalian DNA polymerases and human DNA topoisomerases*

Fig. 2 shows the inhibition dose–response curves of compound **3** against mammalian pols and human topos. Pol α is a replicative pol and pol β and λ are repair and recombination related pols.

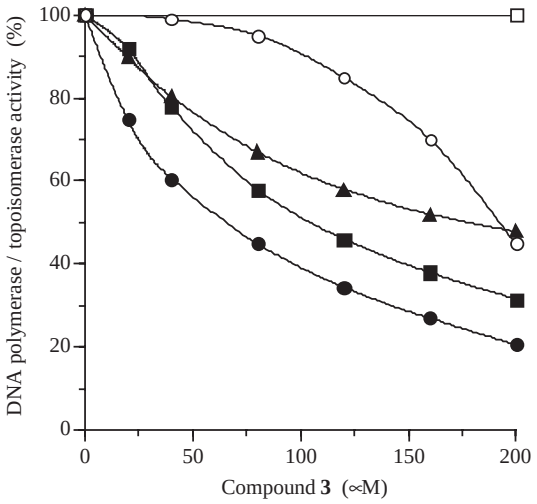

Fig. 2. Dose–response curves for the inhibition of mammalian DNA polymerases and human DNA topoisomerases by compound **3**. The enzymes used were calf pol α (closed circle), rat pol β (closed triangle), human pol λ (closed square), human topo I (open square) and human topo II (open circle). The pol and topo activities were measured as described in the Materials and methods section. The enzyme activity in the absence of the compound was taken as 100%.

The compound was effective at inhibiting calf pol α, rat pol β, human pol λ and human topo II activities and the inhibition was dose-dependent, with 50% inhibition for pol α, β, λ and topo II observed at doses of 64.0, 177, 103 and 190 μM, respectively. The inhibitory activity by compound **3** was strongest toward pol α among the DNA metabolic enzymes tested (Fig. 2). Since the IC_{50} value of aphidicolin, which was an inhibitor of replicative pols such as pols α, δ and ε, for pol α was 20–40 μM under the same conditions (Ikegami et al., 1978), compound **3** seemed not to be such a strong inhibitor. On the other hand, the compound did not influence the activity of human topo I. These observations indicated that compound **3** was a selective inhibitor of mammalian pols, especially pol α.

*Mode of DNA polymerase α inhibition by compound **3***

To elucidate the inhibitory mechanism, the extent of inhibition was measured as a function of the concentration of either the DNA template-primer or deoxyribonucleoside triphosphates (dNTP) in the absence or presence of compound **3** (Fig. 3). 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 compound **3**-mediated inhibition of the pol α activity was non-competitive with both the DNA template-primer and nucleotide substrate. In the case of the DNA template-primer, 35.7% and 62.5% decreases in maximum velocity (V_{max}) were observed in the presence of 25 and 50 μM compound *3*, respectively, whereas the apparent Michaelis constant (K_m) was unchanged at 13.0 μM (Fig. 3A). The K_m for the nucleotide substrate was unchanged at 1.65 μM and the V_{max} for the nucleotide substrate decreased from 29.2 to 17.2 pmol/h in the presence of 50 μM of compound **3** (Fig. 3B). When activated DNA and dNTP were used as the DNA template-primer and nucleotide substrate, respectively, the mode of inhibition of pol α by compound **3** was the

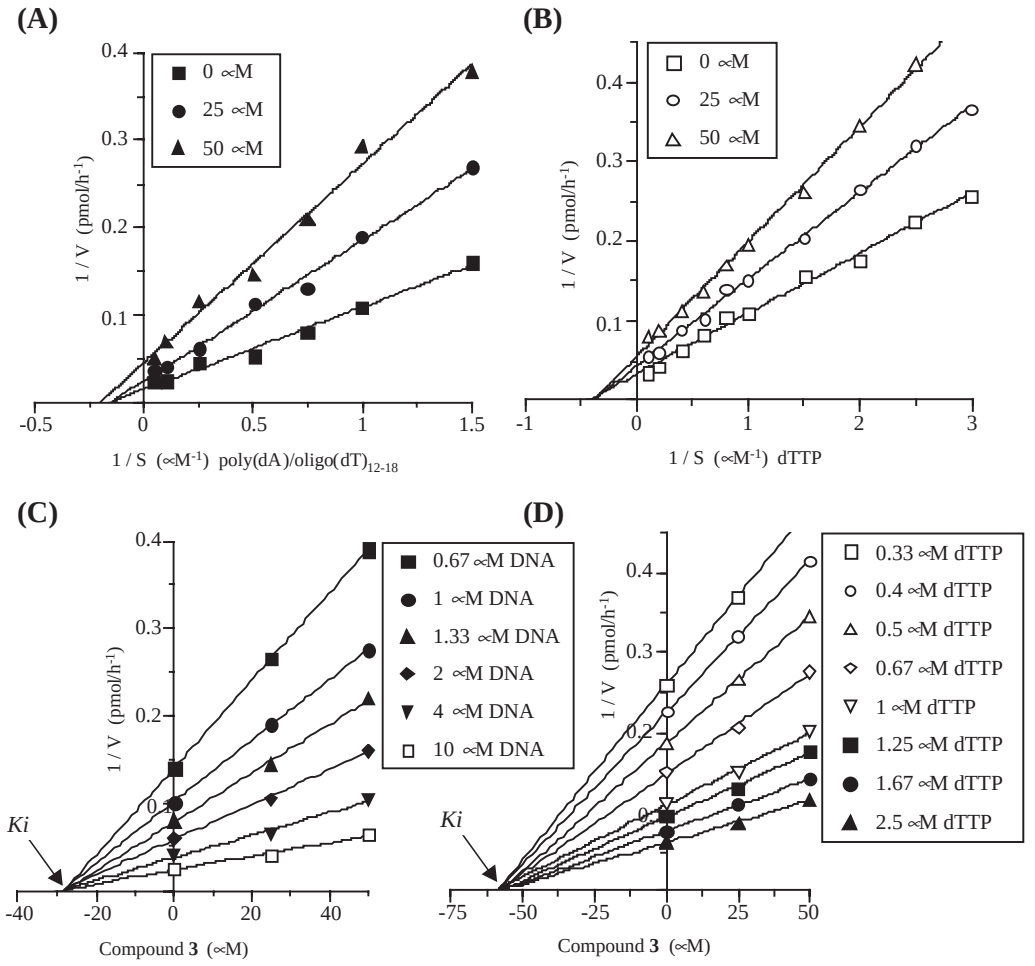

Fig. 3. Kinetic analyses of the inhibition of DNA polymerase α by compound **3**. (A and B) Lineweaver–Burk double-reciprocal plots obtained by varying DNA template-primer concentrations (A) and nucleotide substrate concentrations (B). Activity of human pol α was assayed in the absence (square) or presence of 25 (circle) and 50 (triangle) μM of compound **3**. (C and D) The inhibition constant (K_i) was determined as 27.5 and 57.0 μM from a Dixon plot made on the basis of the data for A and B, respectively. The amount of human pol α in the assay mixture was 0.05 units.

same as when poly(dA)/oligo(dT)$_{12–18}$ and dTTP were used (data not shown). These findings suggested that the site where compound **3** binds to pol α is different from the DNA template-primer and nucleotide substrate binding sites of pol α.

The inhibition constant (K_i) for pol α, obtained from the Dixon plots in Fig. 3C and D, was found to be 27.5 and 57.0 μM for the DNA template-primer and nucleotide substrate, respectively.

Effects of isosteviol and related compounds on cultured human cancer cells

The pols and topos have recently emerged as important cellular targets for chemical intervention in the development of anti-cancer agents. The stevioside, isosteviol and related compounds could, therefore, be

useful in chemotherapy. We tested the cytotoxic effect of the twelve compounds against a human T cell acute lymphoblastic leukemia cell line, MOLT-4, in vitro.

As shown in Fig. 4A, 200 μM of compound **3** had a potent growth inhibitory effect on this cancer cell line, whereas the other compounds did not prevent cell growth. The MOLT-4 cell growth inhibition by compound **3** was dose-dependent and the concentration required for the LD_{50} was 84 μM (Fig. 4B).

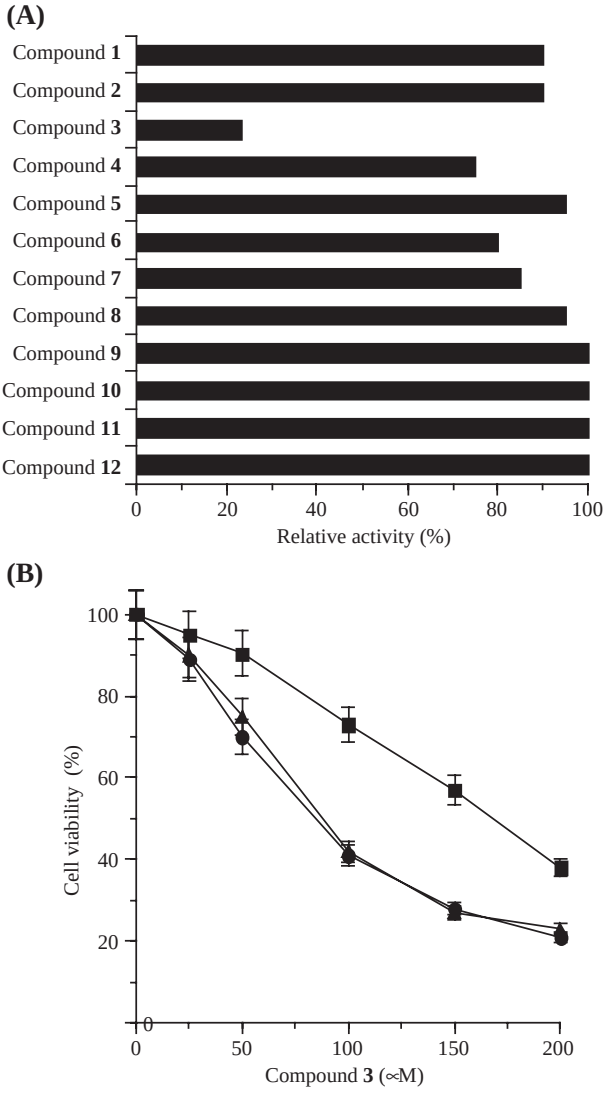

Fig. 4. Effects of isosteviol and related compounds on proliferation of human cancer cells. (A) Inhibition of cell growth of the human T cell acute lymphoblastic leukemia cell line, MOLT-4, by twelve isosteviol and related compounds (200 μM each). (B) Dose–response curves for the inhibition of human cancer cell growth by compound **3**. MOLT-4 (circle), BALL-1 (human B cell acute lymphoblastoid leukemia cell line, triangle) and NUGC-3 (human gastric cancer cell line, square). The assays were carried out under the conditions described in Materials and methods with the indicated concentrations of compound **3**. The rate of survival was determined by MTT assay (Mosmann, 1983). Values are shown as the mean ± SEM for three independent experiments.

Compound **3** also inhibited the cell growth of BALL-1 which is a human B cell acute lymphoblastoid leukemia cell line and NUGC-3 which is a human gastric cancer cell line, with an LD_{50} of 87 and 167 μM, respectively. The dose–response curve for MOLT-4 was almost the same shape as that for BALL-1, indicating that they do not selectively inhibit either B cells or T cells (Fig. 4B). The inhibitory effect for the non-adherent cell lines (i.e., MOLT-4 and BALL-1 cells) was stronger than that for an adherent cell line (i.e., NUGC-3 cells). The LD_{50} value of compound **3** for lymphocyte cells was similar to the IC_{50} value in vitro for pol α. The inhibition curves of the pol enzymes and cell growth showed parallel dose-dependent reductions. These observations suggested that the cell growth inhibition occurred in a manner dependent on the enzyme inhibition. Compound **3** was, therefore, suggested to inhibit the activity of pols, especially replicative pol α, in the intact cell. Since compound **3** also arrested the cells at S-phase (data not shown), the compound might distribute in nucleus and influences the progression of the cell cycle.

*Effect of anti-inflammatory activity by compound **3***

Using the mouse inflammatory test, we examined the anti-inflammatory activity of compound **3**. An application of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (0.5 μg) to the mouse ear induced edema, the weight increase of an ear disk 7 h after the application being 241%. As shown in Table 2, pretreatment with 250 and 500 μg of compound **5** resulted in the strongest reduction in TPA-induced inflammation among the compounds tested, with an inhibitory effect (IE) of 25.8% and 53.0%, respectively. Because the IE of compound **3** (500 μg) was more than 50%, this compound is suggested to have an effect on the inflammation. TPA is a compound that promotes tumorigenesis (Hecker, 1978) and is generally used as an artificial inducer of inflammation (Fujiki and Sugimura, 1987; Nakamura et al., 1995). TPA-induced inflammation can be distinguished from acute inflammation and is accompanied by fibroblastic proliferation and granulation. These results suggested that the molecular basis of the so-called promotion process in oncogenesis also contains a biochemical process which requires pols.

Three-dimensional structures of isosteviol and related compounds

To obtain information about the molecular basis for the differential inhibition exhibited by stevioside, isosteviol and the ten related compounds, computational analyses were performed using molecular simulation and surface analysis software (Fig. 5). The three-dimensional molecular structures of the compounds are shown in Fig. 5(1–12). A comparison of the electrostatic potential of these compounds revealed a remarkable difference in their overall disposition and rapport. The electrostatic potential at

Table 2
Anti-inflammatory activities of compound **3** in the mouse ear inflammation test

Compound	Inhibitory effect (%) (± S.E.)	
	250 μg/ear	500 μg/ear
3	25.8 (± 5.4)*	53.0 (± 4.8)*

S.E. is shown in parentheses. A sample (250 or 500 μg) was applied to one ear and, after 30 min, TPA (0.5 μg) was applied to both ears of the mouse. The edema was evaluated after 7 h, the inhibitory effect being expressed as a percentage ratio of the edema. Five mice were used for each experiment. *Significantly different, $P<0.05$, using Student's *t*-test.

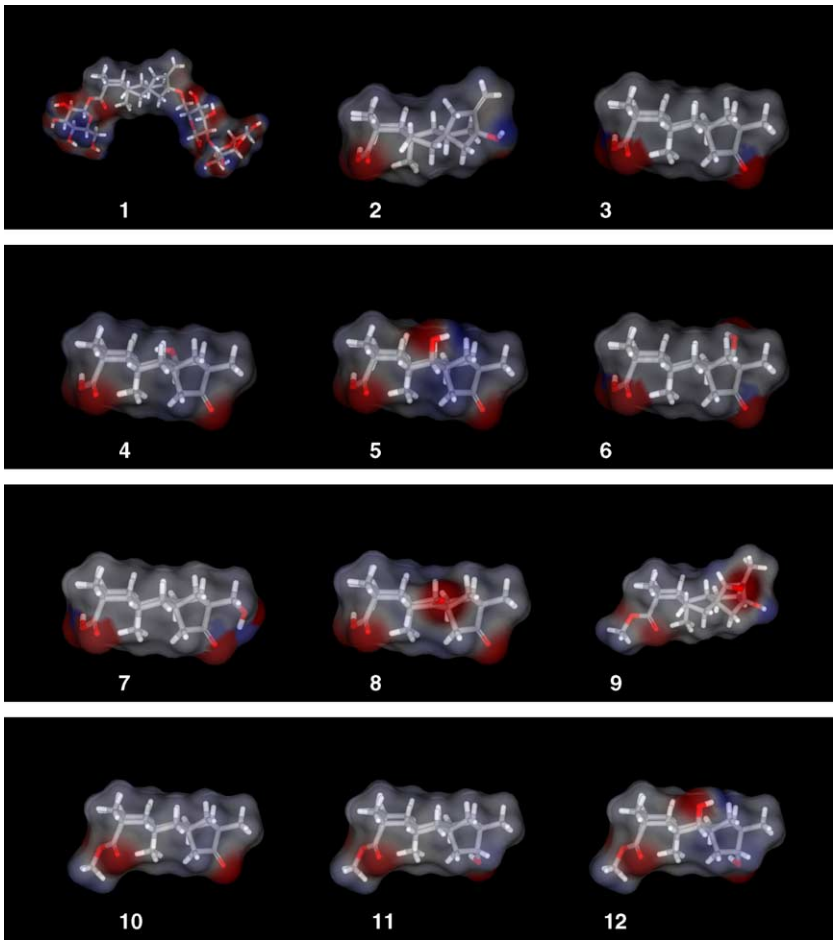

Fig. 5. Computer graphics of isosteviol and related compounds. Compound **1**: stevioside, compound **2**: steviol, compound **3**: isosteviol, compound **4**: 11β-hydroxyisosteviol, compound **5**: 7β-hydroxyisosteviol, compound **6**: 12β-hydroxyisosteviol, compound **7**: 17-hydroxyisosteviol, compound **8**: 7-oxoisosteviol, compound **9**: methyl *ent*-15β,16β-epoxy-13-hydroxykauran-19-oate, compound **10**: isosteviol methyl ester, compound **11**: methyl *ent*-16β-hydroxybeyeran-19-oate, and compound **12**: methyl *ent*-7α, 16β-dihydroxybeyeran-19-oate. Stick models of compounds **1**–**12** were built using the graphics program INSIGHT II (Accelrys Inc., San Diego, CA, USA). Carbons, oxygens and hydrogens are indicated in gray, red and white, respectively. Electostatic potentials over molecular surfaces were analyzed using WebLab ViewerLite software (version 3.2, Accelrys Inc., San Diego, CA, USA). Blue areas are positively charged, red areas are negatively charged, and white areas are neutral (see Materials and methods).

each point on a constant electronic density surface (approximating the van der Waals surface for each arrangement) was represented graphically with red corresponding to the regions where the electrostatic potential was most negative and blue corresponding to the most positive regions.

As shown in Fig. 5(3), isosteviol (compound **3**) consisted of a hydrophobic region (upper surface) and two negative electrostatic charges on the O atom in the carboxyl group (under-left) and ketone group (under-right). Other compounds have another negative or positive charge. The following molecular features might be important for the inhibition of pol activity, cytotoxicity and anti-inflammation; [1]

tetracyclic diterpenoid with a beyerane skeleton (i.e., hydrophobic region), [2] one carboxyl group, [3] one ketone group, and [4] no hydroxyl groups.

As described previously, sulfo-glycolipids, which inhibited the activity of mammalian pols equally (Hanashima et al., 2000; Ohta et al., 2000), were as effective as anti-cancer agents in vivo (Sahara et al., 1997, 2002). Compound **3** could, therefore, be considered a possible candidate for an anti-cancer agent.

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