Chemical properties of fatty acid derivatives as inhibitors of DNA polymerases

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In this study, the chemical properties of organic acids as DNA polymerase inhibitors were examined. In total, we assayed the inhibitory activities of 23 compounds. We found that the DNA synthesis activity of DNA polymerase was usually reduced to less than 50% in the presence of 100 μM monoprotic acids, which have a Clog *P* value greater than 7.0 and a pK_a value less than 5.4. With a minor modification these chemical properties applied to several organic fatty acids previously reported as DNA polymerase inhibitors. Moreover, we also examined the inhibitory activities of perfluorooctadecanoic acid (PFOdA) and perfluorooctanesulfonic acid (PFOS) against DNA polymerase β in detail. These compounds inhibited the polymerase activity of pol β competitively with template–primer DNA, and non-competitively with dNTPs. In addition, the 8 kDa domain-defective pol β was also sensitive to these compounds. Our results suggest that the inhibitory mode of action of PFOdA and PFOS is different from that mediated by the classic fatty acid inhibitors against DNA polymerase β.

Introduction

DNA polymerases are essential enzymes for DNA replication, repair and recombination. Eukaryotic cells contain three replicative DNA polymerases (pol α, δ and ε), a mitochondrial DNA polymerase (pol γ) and at least 10 repair-types of other DNA polymerases (pol β, δ, ε, ζ, η, θ, κ, λ, μ and ν).[1,2] Recently, DNA polymerase inhibitors have been a major focus of research as candidate anti-cancer agents.[3–6] In particular, repair-type DNA polymerases, including pol β, could be considered both as markers of tumorigenesis and as potential drug targets for chemotherapy.[6]

We previously reported that long-chain fatty acids, such as stearic acid (**1**) and nervonic acid (**2**), inhibit the activities of a mammalian pol α and pol β *in vitro*, but that the fatty acids did not influence the activities of prokaryotic DNA polymerases or other DNA metabolic enzymes at a concentration below 100 μM.[7–13] Lineweaver–Burk plots of the inhibition of pol α by fatty acids indicate non-competitive inhibition with both the template DNA and the dNTPs.[8,9] By contrast, inhibition of pol β was competitive with both the template DNA and the dNTPs.[8,9] Moreover, our previous NMR study and docking simulation found that nervonic acid (**2**) would form a 1 : 1 complex with the 8 kDa domain of pol β through interaction with four amino acids of pol β (Leu11, Lys35, His51, and Thr79).[10,13] The hydrophobic region, which contains Leu11, His51 and Thr79, might interact with the side-chain of **2**, and the hydrophilic Lys35, might interact with the carboxylic group of **2**. The structure–activity relationships of fatty acids for inhibition of DNA polymerases revealed three major findings: (1) fatty acids with C18 or more carbon atoms inhibit the polymerases; (2) the carboxylic acid group is important for inhibition; (3) unsaturated fatty acids with the *cis*-configuration at the double bond display a stronger inhibition than those with the *trans*-configuration.

During the course of our investigation of DNA polymerase inhibitors, we became interested in the unique physical, chemical and biological properties of perfluorinated alkyl substances (PFAS). PFAS have a variety of applications, including acting as surfactants or additives for polymers and plastics.[14–17] The amphipathic, thermal, photochemical and chemical properties of PFAS, combined with their biological inertness, account for the versatile uses of these compounds for over 50 years.[14] However, PFAS are environmentally persistent and bioaccumulative.[14–16] In previous reports, PFOS was shown to display biological toxicity by targeting specific functions such as peroxisome proliferation and lipid metabolism.[14,17]

In this study, the inhibition assay was carried out with 23 organic acids to examine the structure–activity relationships in detail. On the basis of these results, we investigated the correlation between the chemical properties of the organic acids and the inhibitory activities against mammalian polymerases. We focused on the calculated log *P* value (partition coefficients for octanol–water) and pK_a of the organic acid as the chemical properties. Moreover, we examined the enzymatic inhibition of PFOdA and PFOS against DNA polymerases by kinetic analysis. A model for the inhibition by these compounds is also discussed.

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Materials and methods

General (chemistry)

^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrometer (Avance DRX-400), using $CDCl_3$ solutions, unless otherwise noted. Chemical shifts were expressed in δ (ppm) relative to Me_4Si or residual solvent resonance, and coupling constants (J) were expressed in Hz. Melting points were determined with Yanaco MP-3S and were uncorrected. Infrared (IR) spectra were recorded on a Jasco FT-IR-410 spectrometer using NaCl (neat) or KBr pellets (solid), and were reported as wavenumbers (cm^{-1}). Mass spectra (MS) were obtained on an Applied Biosystems mass spectrometer (API QSTAR pulsar i) under high resolution conditions. Column chromatography was carried out on Fuji Silisia PSQ100B. Analytical thin-layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates, and compounds were visualized by UV illumination (254 nm) or heating at 150 °C after spraying with phosphomolybdic acid in ethanol. HPLC was performed on an Agilent 1100 Series using a Mightysil Si 60 column (KANTO Inc.) with a 30 min linear gradient elution from 100 : 0 to 85 : 15 (hexane–*i*-PrOH with 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL min^{-1} with UV detection at 254 nm. THF was distilled from sodium–benzophenone. CH_2Cl_2 was distilled from P_2O_5. DMSO was distilled from CaH_2. All other solvent and reagents were obtained from commercial sources and used without further purification. Organic extracts were dried over Na_2SO_4, filtered, and concentrated using a rotary evaporator. Involatile oils and solids were vacuum dried.

Organic acids

Compounds **1**, **2**, **3**, **5**, **6**, **13**, **14**, **15**, **16**, **17**, **22**, and **23** were obtained from commercial sources and used without further purification. Compounds **4**,[18] **7**,[19] **8**,[20] **9**,[21] **10**,[22] **12**,[23] **18**,[24] **19**,[24] **20**[25] and **21**[25] were prepared by literature procedures with slight modifications and characterized by NMR spectra. Compound **11** was prepared from the known compound **24**[26] (Scheme 1).

Synthesis of 5-*tert*-butyldimethylsilyloxy-4-oxo-4*H*-pyran-2-carbaldehyde (25)

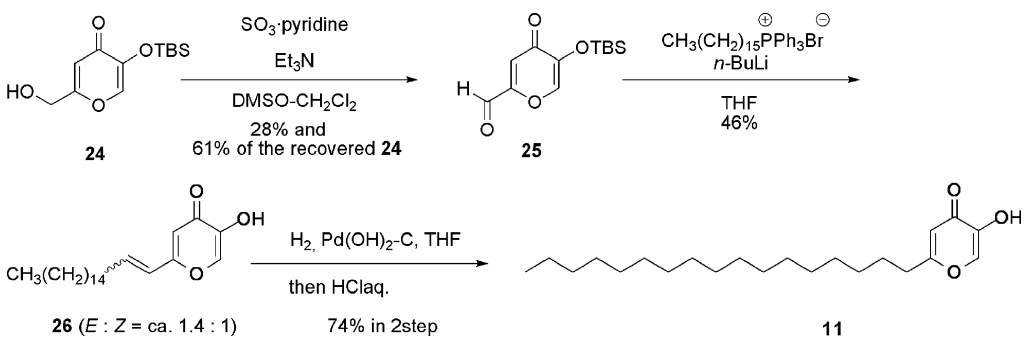
To a solution of **24** (114.7 mg, 0.50 mmol) and Et_3N (0.5 mL, 3.6 mmol) in CH_2Cl_2–DMSO (4 : 1, 5 mL) was added SO_3·pyridine (363.6 mg, 2.3 mmol) at 0 °C. The mixture was stirred at rt for 10.5 h. Then the mixture was quenched by the addition of sat. $NaHCO_3$ aq. and diluted with EtOAc. The layers were separated. Then the organic layer was washed with H_2O and brine, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by silica gel chromatography (hexane–EtOAc = 4 : 1) to yield **25** (31.4 mg, 28%) and the recovered **24** (68.8 mg, 61%). **25**: ^{1}H NMR (400 MHz) δ 0.25 (6H, s), 0.97 (9H, s), 7.00 (1H, s), 7.80 (1H, s), 9.67 (1H, s).

Synthesis of *E*- and *Z*-5-*tert*-butyldimethylsilyloxy-2-(1-heptadecenyl)-4-oxo-4*H*-pyran (26)

To a solution of the known phosphonium salt, hexadecyltriphosphenylphosphonium bromide,[27] (40.2 mg, 0.071 mmol) in THF (2 mL) was added *n*-BuLi (125 μL of 1.56 M solution in hexanes, 0.20 mmol) at 0 °C. The mixture was stirred at rt for 10 min. Then a solution of **25** (31.4 mg, 0.12 mmol) in THF (1.5 mL) was added to the mixture at 0 °C and the mixture was stirred at rt for 3 h. The mixture was quenched by the addition of sat. NH_4Cl aq. and diluted with EtOAc. The layers were separated. Then the organic layer was washed with H_2O and brine, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by silica gel chromatography (hexane–EtOAc = 20 : 1) to yield **26** (15.1 mg, 46%) as a 1.4 : 1 mixture of *E*- and *Z*-isomers. *E*-isomer: ^{1}H NMR (400 MHz) δ 0.22 (6H, s), 0.88 (3H, t, J = 7.0 Hz), 0.95 (9H, s), 1.26 (24H, brs), 1.46 (2H, m), 2.21 (2H, q, J = 7.0 Hz), 6.02 (1H, d, J = 15.8 Hz), 6.16 (1H, s), 6.55 (1H, dt, J = 15.8 Hz, 7.0 Hz), 7.59 (1H, s). *Z*-Isomer: ^{1}H NMR (400 MHz) δ 0.24 (6H, s), 0.88 (3H, t, J = 7.0 Hz), 0.96 (9H, s), 1.26 (24H, brs), 1.46 (2H, m), 2.44 (2H, m), 5.92–5.93 (2H, m), 6.24 (1H, s), 7.64 (1H, s).

Synthesis of 2-heptadecyl-5-hydroxy-4*H*-pyran-4-one (11)

A solution of **26** (15.1 mg, 0.033 mmol) and $Pd(OH)_2$–C (4.4 mg) in THF (2 mL) was stirred under a H_2 atmosphere for 6 h. Then the solution was filtered through Celite and washed with THF. The filtrate was treated with 0.5 mL of 1 N HCl aq. and the mixture was stirred for 10 min. The mixture was quenched by the addition of sat. $NaHCO_3$ aq. and diluted with EtOAc. The layers were separated. Then the organic layer was washed with H_2O and brine, dried (Na_2SO_4), and evaporated *in vacuo*. The residue was purified by silica gel chromatography (hexane–EtOAc = 20 : 1) to yield **11** (8.5 mg, 74%) as a white solid. Mp = 85 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (3H, t, J = 6.8 Hz), 1.25 (26H, brs), 1.30 (2H, m), 1.64 (2H, m), 2.53 (2H, t, J = 7.6 Hz), 6.27 (1H, s), 7.79 (1H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 22.7, 27.0, 28.9, 29.2, 29.35, 29.42, 29.56, 29.61, 29.65 (×3), 29.68 (×3), 31.9, 33.9, 110.3,



Scheme 1 Synthesis of **11**.

137.4, 145.1, 170.4, 174.2. IR (KBr) 3224, 2919, 2849, 1734, 1702, 1653, 1620, 1585, 1461, 1378, 1226, 1186, 1149, 917, 892, 835, 757, 722, 701 cm^{-1}. ESIMS: calcd for $C_{22}H_{38}O_3Na$ ($[M + Na]^+$) 373.2713, found 373.2711. HPLC analysis: t_R = 9.9 min. Anal. calcd for $C_{22}H_{38}O_3 \cdot H_2O$: C, 71.70; H, 10.94. Found: C, 72.05; H, 10.93%.

Materials for DNA polymerase assay

Calf thymus DNA, deoxynucleotide triphosphates and synthetic polynucleotides such as poly(dA), oligo(dT)$_{12-18}$, and [^{3}H]-2′-deoxythymidine 5′-triphosphate (dTTP) (43 Ci $mmol^{-1}$) were purchased from GE Healthcare Bio-Sciences Corp (Piscataway, NJ 08855, USA). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Enzymes

DNA polymerase α (pol α) was purified from the calf thymus by immuno-affinity column chromatography as described previously.[28] Recombinant rat DNA polymerase β (pol β) and human terminal deoxynucleotidyl transferase (TdT) were purified from *Escherichia coli* BL21 (DE3) as described previously.[29] The 31 kDa domain of pol β (residues 88–335) was cloned into the pET28a expression vector (Novagen), and purified by the same method as for the whole pol β.

DNA polymerase assays

The DNA polymerase activities were measured based on the methods described previously.[30] Briefly, the reaction mixture (25 μl) contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% (v/v) glycerol, 10% (v/v) dimethyl sulfoxide, 50 μg ml^{-1} poly(dA)–oligo(dT)$_{12-18}$, 0.5 μM dTTP, 0.1 μM [^{3}H]-dTTP, 4.1 fmol of pol α, β or TdT, and at least five different concentrations of each compound. When activated calf thymus DNA (100 μg ml^{-1}) was used as the DNA template, the reaction mixture was supplemented with 100 mM each of dATP, dGTP and dCTP, and dTTP was removed from it. After incubation at 37 °C for an hour, the reaction mixture was spotted onto a Whatmann DE81 ion-exchange filter, air-dried, and washed 3 times with 5% (w/v) Na_2HPO_4, 3 times with distilled water, and twice with ethanol. The radioactivity of each dried filter was measured in a toluene-based scintillator. The GraphPad Prism program (GraphPad Software Inc., San Diego, CA) was used to calculate the IC_{50} value for each compound, and to determine the inhibitory mechanisms of compounds **3** and **17** by global fitting models.

Results and discussion

Enzymatic inhibitory activity of organic acids against DNA polymerases

The structures of all the compounds tested in this study are shown in Fig. 1. The calculated log *P* (Clog *P*), pK_a, and the IC_{50} values of each compound are summarized in Table 1. Most of the Clog *P* and pK_a values of the organic acids were obtained from the calculated properties in SciFinder Scholar, which were originally calculated using Advanced Chemistry Development (ACD/Lab) Software V8.14 for Solaris (ACD/Labs).

We previously reported that compound **1** inhibited the activity of pol α and pol β with IC_{50} values of 9 μM and 91 μM, respectively. In order to examine the effect of the alkyl moiety on the inhibitory activity of fatty acids against DNA polymerase, we first changed the alkyl group of **1**. Replacement of the octadecyl group in **1** with a perfluorooctadecyl moiety (**3**), thereby increasing the overall hydrophobicity and acidity of the molecule (Clog *P* = 16.64, pK_a = 0.37), gave more potent inhibitory activity. The IC_{50} values of perfluorooctadecanoic acid (PFOdA, **3**) against pol α, pol β and TdT were 0.71 μM, 0.63 μM and 5.4 μM, respectively. Since the CMC of **3** is reported to be 80 μM, **3** would not form a micelle at the concentration at which **3** inhibited DNA polymerases.[31] Replacement of the alkyl group with pentaethylene glycol (**4**) increased the hydrophilicity (Clog *P* = −2.45), but 100 μM of compound **4** had no effect on DNA polymerase activity. Compound **5**, which has a Clog *P* value of 2.9, also lacked inhibitory activity. By contrast, compound **6**, which is considerably more hydrophobic (Clog *P* = 7.75), inhibited pol α and TdT with IC_{50} values of 56 μM and 62.5 μM, respectively. These results suggested that the hydrophobicity of the alkyl moiety of the fatty acids was closely related to the inhibitory activity against DNA polymerases. This proposal is supported by our previous NMR study, in which the hydrophobic interaction between the hydrophobic region (Leu11, His51 and Thr79) in pol β and the side-chain in **2** had an important role in the binding between pol β and **2**.[10,13]

The branched chain fatty acid (**7**) was a slightly weaker inhibitor of pol α activity (IC_{50} = 63.4 μM) than the straight chain fatty acid (**1**). However, the IC_{50} value for **7** against pol β (82.2 μM) was similar to that of **1**. Because compound **7** has the same level of hydrophobicity (Clog *P* = 8.03) and acidity (pK_a = 4.82) as that of **1**, any differences in inhibitory activity against pol α can be attributed to molecular shape. Compound **8** exhibited more potent inhibition than **7** against pol α, β and TdT with IC_{50} values of 19.6 μM, 36.1 μM and 33.6 μM, respectively. Indeed compound **8** displayed similar activities to compound **9**. Thus the α-carboxyl substituents increased the inhibitory activity against the DNA polymerases.

We next turned our attention to the carboxylic group of stearic acid (**1**). Compounds **10–12**, having similar pK_a values, are bioisosteres of **1**. The IC_{50} value of **10** against pol α (8.6 μM) is similar to that of **1**, whereas the IC_{50} value of **10** against pol β (7 μM) is much lower than that of **1**. Although compound **11** is a weaker inhibitor of pol α than **1**, inhibition of pol β by **11** was significantly more potent than that by **1** (IC_{50} values of **11**: IC_{50} = 31.1 μM against pol α; IC_{50} = 26.3 μM against pol β). Both **10** and **11** did not affect the activity of TdT. Interestingly, the diprotic sulfonamide **12** showed no inhibitory activity against any DNA polymerase at a concentration less than 100 μM. These results indicate that the acidic functional group affected not only inhibitory activity, but also selectivity against the enzymes.

The inhibitory activities of compounds **13–17**, all of which have pK_a values much lower than that of **1**, were also examined. The diprotic phosphoric acid **13** lacked inhibitory activity against pol α, pol β and TdT. Murofushi *et al.* reported that PHYLPA, a monoprotic lysophosphatidic acid, specifically inhibited pol α.[32,33] Thus, the type of protic acid might be a crucial factor for inhibition

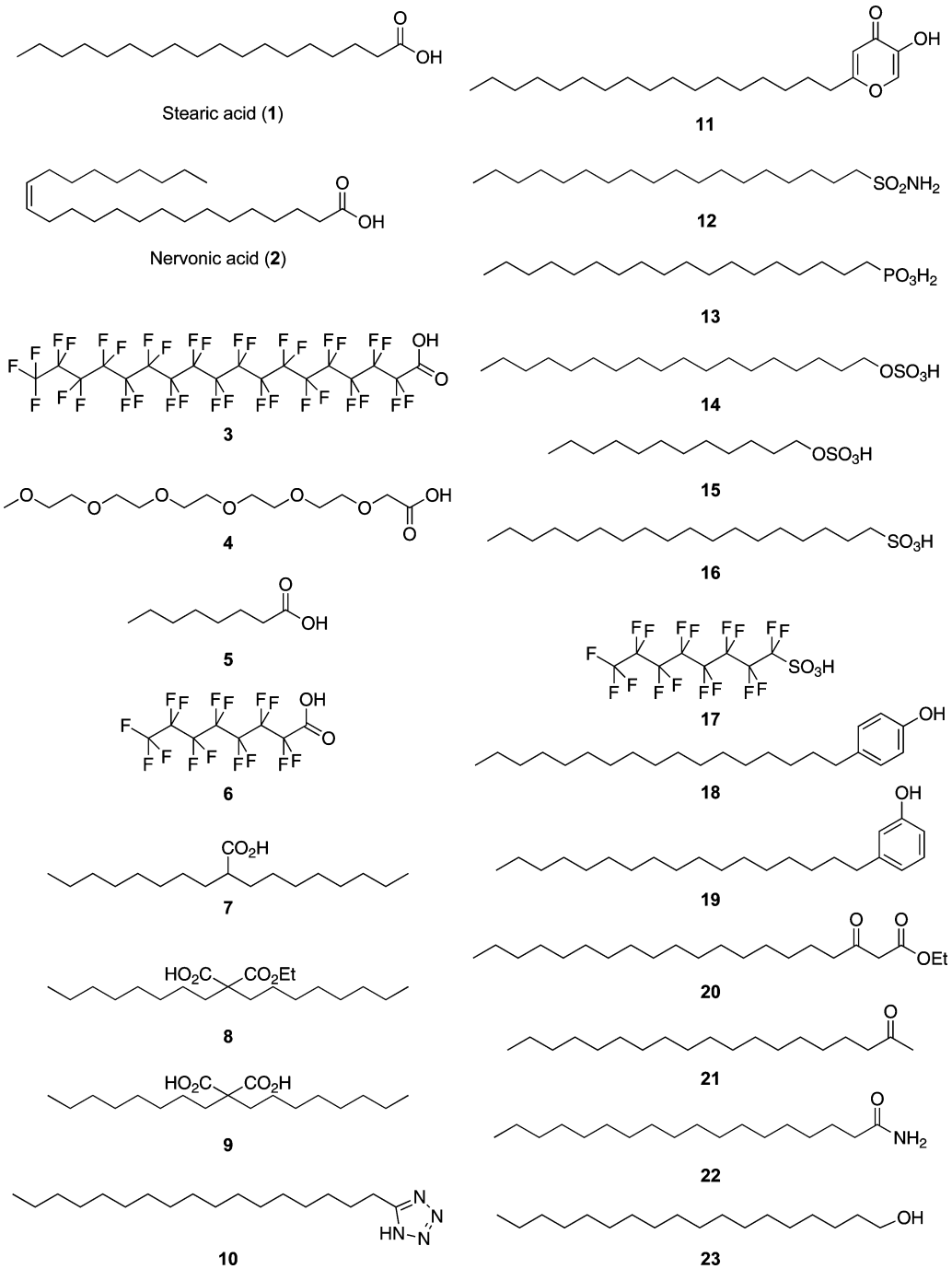


Fig. 1 Chemical structures of compounds tested as DNA polymerase inhibitors.

against DNA polymerases. Octadecyl sulfate (**14**) and dodecyl sulfate (**15**) are alkyl sulfonates, which have the same pK_a value of −3.29. Although **14** efficiently inhibited all three polymerases (IC_{50} = 5.8 μM against pol α; IC_{50} = 12.8 μM against pol β; IC_{50} = 18.3 μM against TdT), **15** did not display any inhibitory activity even at a concentration of 100 μM. Octadecyl sulfonic acid **16** inhibited the α and β polymerase activities with a similar dose to that of **14** (IC_{50} values of **16**: IC_{50} = 12.9 μM against pol α; IC_{50} = 21 μM against pol β). PFOS (**17**) inhibited the activity of these polymerases in the submicromolar range (IC_{50} = 24.5 μM against pol α; IC_{50} = 46.4 μM against pol β; IC_{50} = 31.3 μM against TdT). Since it is reported that the CMC of **17** ranges from 0.88 mM to 1.7 mM, **17** would not form micelles at the concentration used in this study.[34,35] These results suggest that (1) the type of protic acid as well as the acidic functional group is important for inhibition against polymerases; (2) the hydrophobicity of the side-chain moiety (Clog *P*) is important for the inhibitory activity against DNA polymerases.

Compounds **18–23** have greater pK_a values than that of **1**. The phenolic compounds (**18** and **19**), β-ketoester (**20**), ketone (**21**), amide (**22**) and alcohol (**23**) used in this study showed no inhibitory activity against pol α, pol β and TdT. These results

Table 1 The Clog *P* value and calculated pK_a value of organic acids **1–23**, and the IC_{50} and logIC_{50} values for enzymatic inhibition of DNA polymerase α and β, and TdT by the compounds **1–23**. The IC_{50} and log IC_{50} values were determined by using the GraphPad Prism program. The IC_{50} values were obtained by taking the antilog of the log IC_{50} values. Unless otherwise noted, both the Clog *P* values and the pK_a values of organic acids were obtained from the calculated properties in SciFinder Scholar, which were originally calculated using Advanced Chemistry Development (ACD/Lab) Software V8.14 for Solaris (ACD/Labs)

			$IC_{50}/10^{-6}$ M			Log IC_{50}/M		
Compounds	Clog *P*	pK_a	Pol α	Pol β	TdT	Pol α	Pol β	TdT
1	8.21	4.78	9	91	ND			
2	10.9	4.78	2	8	ND			
3	16.64	0.37	0.71	0.63	5.4	−6.15 ± 0.05	−6.20 ± 0.07	−5.27 ± 0.07
4	−2.45	3.39	>100	>100	>100			
5	2.9	4.78	>100	>100	>100			
6	7.75	0.35	56	>100	62.5	−4.25 ± 0.02		−4.20 ± 0.02
7	8.03	4.82	63.4	82.2	>100	−4.20 ± 0.05	−4.08 ± 0.01	
8	8.45	3.66	19.6	36.1	33.6	−4.71 ± 0.03	−4.44 ± 0.01	−4.47 ± 0.01
9	7.58	2.07	22.4	57.7	54.3	−4.65 ± 0.02	−4.24 ± 0.01	−4.27 ± 0.02
10	7.42	5.35	8.6	7	>100	−5.07 ± 0.01	−5.16 ± 0.02	
11	5.15[a]	5.15[b]	31.1	26.3	>100	−4.51 ± 0.02	−4.58 ± 0.03	
12	7.75	10.81 −5.27	>100	>100	>100			
13	7.01	2.42	>100	>100	>100			
14	8.58	−3.29	5.8	12.8	18.3	−5.23 ± 0.02	−5.89 ± 0.07	−4.74 ± 0.02
15	5.4	−3.29	>100	>100	>100			
16	7.14	1.84	12.9	21	>100	−4.89 ± 0.09	−4.68 ± 0.05	
17	7.03	−3.27	24.5	46.4	31.3	−4.61 ± 0.05	−4.33 ± 0.03	−4.51 ± 0.02
18	10.44	10.14	>100	>100	>100			
19	10.44	10.06	>100	>100	>100			
20	9.22	10.67	>100	>100	>100			
21	8.34	>15	>100	>100	>100			
22	7.27	16.61 −0.49	>100	>100	>100			
23	8.31	15.19	>100	>100	>100			

Errors mean ± S.E.[a] The Clog *P* values were obtained using CS ChemDraw version 6.0 software (Cambridge Soft, USA). [b] The calculated pK_a value of compound **11** is substituted for that of 2-butyl-5-hydroxy-4*H*-pyran-4-one.

indicate that the acidic functional group is essential for inhibition.

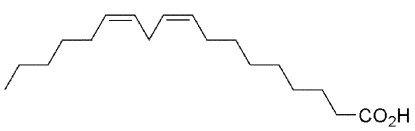
After conducting a series of enzymatic inhibitory assays we conclude that the most potent DNA polymerase inhibitors possess the following properties. Monoprotic acids, which have a Clog *P* value greater than 7.0 and a pK_a value less than 5.4, have a tendency to inhibit the activity of one or more DNA polymerases. Although the mechanism of DNA polymerase inhibition for these inhibitors is not fully examined, both hydrophobicity and acidity are essential for inhibitory activity against mammalian polymerases. Our previous NMR study showed that the carboxylic group of **2** interacted with the Lys35 hydrophilic region and the alkyl side chain of **2** interacted with the Leu11–His51 hydrophobic region.[10,13] Thus, the acidic functional group and hydrophobic moiety of the inhibitor are required for binding to the basic amino acid residues and hydrophobic regions of the DNA polymerase, respectively.

Most of the previously identified fatty acid inhibitors showed chemical properties similar to those of inhibitors found in the present studies. The known DNA polymerase inhibitors,[8,36–44] which have a Clog *P* value greater than 7.0 and a pK_a value less than 5.4, are summarized in Fig. 2. Actually some phenolic[45–49] and glycolipid[50–52] inhibitors of DNA polymerases do not possess the anticipated chemical properties. However, our predictions have wide applicability to lipid compounds, assuming that the Clog *P* value is defined as more than 5.3 for steroidal inhibitors. For instance, fomitellic acid A[53,54] (Clog *P* 5.3, pK_a 4.1), lithocholic acid (Clog *P* 6.7, pK_a 4.8),[55–57] vitamin A acid[58] (Clog *P* 6.8, pK_a 4.7), tormentic acid[59] (Clog *P* 6.2, pK_a 4.5) and euscaphic acid[59] (Clog *P* 6.2, pK_a 4.5) inhibit DNA polymerases. We also found that the shape of some lipid inhibitors influences the selective inhibition of pol α.[44] By contrast, these chemical property–activity predictions do not apply to nucleotide analogue based inhibitors, presumably reflecting a different mode of inhibition.

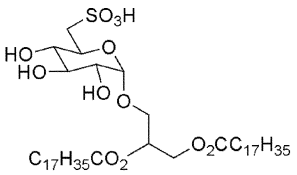
Inhibition of DNA polymerase activity by PFOdA and PFOS

Since PFOdA (**3**) and PFOS (**17**) have unique physical, chemical and biological properties, we investigated the mechanism of these inhibitors in detail.

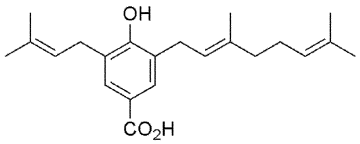
The mode of inhibition of pol β was examined by kinetic analysis (Fig. 3).[8,9] In these experiments, poly(dA)–oligo(dT)$_{12–18}$ and dTTPs were utilized as the DNA template–primer and nucleotide substrate, respectively. The V_{max} and K_m values of the polymerase reactions with and without **3** and **17** were obtained by fitting the data to the Michaelis–Menten equation with Graphpad Prism4 software (Table 2). Considering the template–primer DNA as substrate, the Michaelis constant (K_m) increased with increasing concentrations of **3** and **17**, but the apparent maximum velocity (V_{max}) was almost unaltered. Thus the inhibition of pol β by **3** and **17** is considered to be competitive with DNA template–primer. The Lineweaver–Burk plot analysis also supports this suggestion (Fig. 3, A and E). In contrast, measuring the activity over a range of dTTP concentrations as substrate, K_m was independent of the



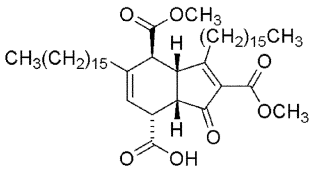
Linoleic acid (Clog P 7.2, pKa 4.78)



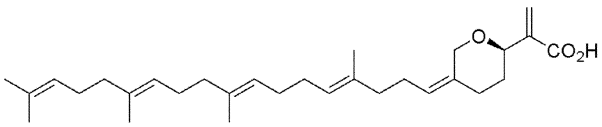
α-SQDG (Clog P 15.3, pKa 1.4)



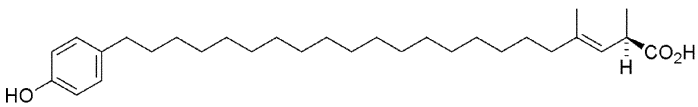
3-Geranyl-4-hydroxy-5-(3'-methyl-2'-butenyl) -benzoic acid (Clog P 7.6, pKa 4.7)



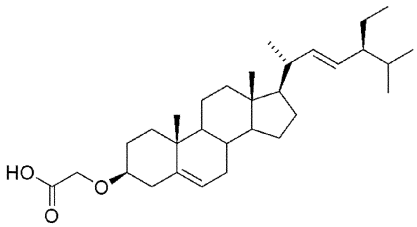
Manzamenone A (Clog P 17.2, pKa 3.93)



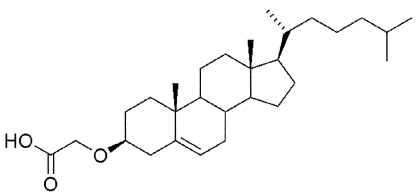
Hippospongic acid A (Clog P 9.8, pKa 3.6)



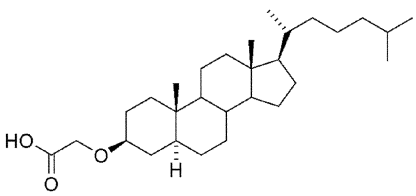
Elenic acid (Clog P 11.7, pKa 4.4)



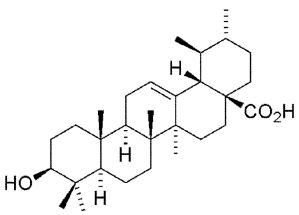
Stigmasterol derivative (Clog P 10.0, pKa 3.4)



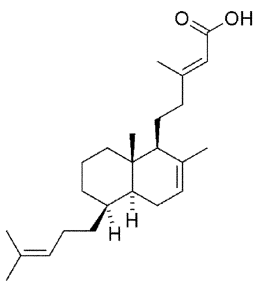
Cholesterol derivative (Clog P 9.7, pKa 3.4)



Cholestanol derivative (Clog P 10.0, pKa 3.5)



Ursolic acid (Clog P 9.0, pKa 4.7)



Kohamaic acid (Clog P 9.8, pKa 5.10)

Fig. 2 Known DNA polymerase inhibitors that have a Clog *P* value greater than 7.0 and a pK_a value less than 5.4.

concentration of **3** and **17**, although V_{max} was reduced in the presence of these compounds, suggesting that **3** and **17** inhibit the DNA synthesis by pol β non-competitively with a nucleotide substrate. This result was also consistent with that obtained from the Lineweaver–Burk plot (Fig. 3, B and F).

The melting temperature of double-stranded DNA was unchanged in the presence of 100 μM **3** or **17** (data not shown). Thus, compounds **3** and **17** must inhibit the enzymatic activity by interacting with the enzyme in the reaction mixture. In order to analyze the mode of inhibition further, the inhibitory activity of **3** and **17** against the 31 kDa domain of pol β was examined (Table 3). Tryptic digestion of pol β liberates an N-terminal domain (8 kDa), which is responsible for single-strand DNA binding activity, and a C-terminal domain (31 kDa), which catalyzes DNA synthesis.[60,61] Compounds **3** and **17** inhibit the activity of the 31 kDa domain of pol β with an IC_{50} value of 4.4 μM and 51.1 μM, respectively.

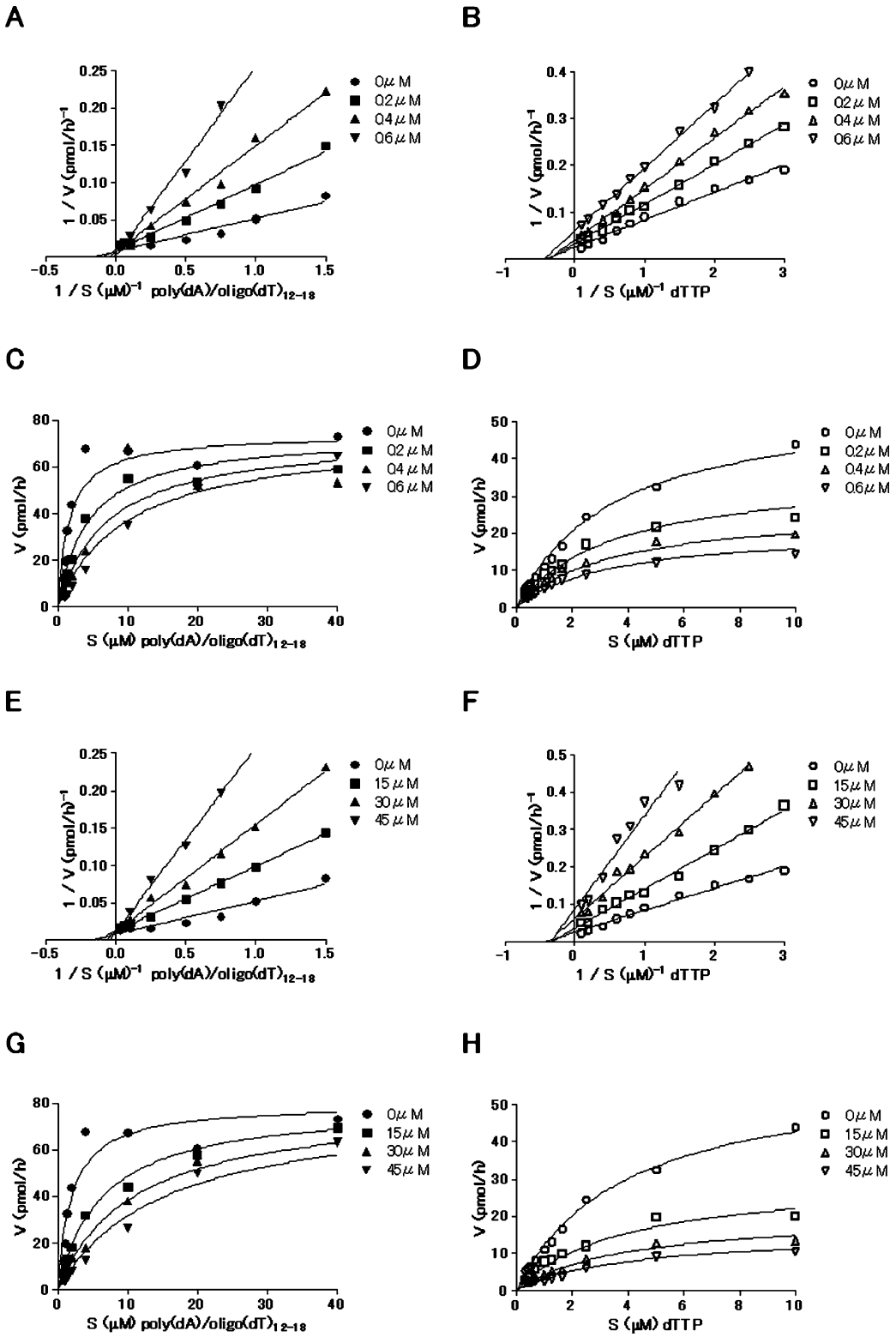


Fig. 3 Kinetic analysis of the inhibitory effects of compounds **3** (A–D) and **17** (E–H) on the activities of DNA polymerase β, as a function of the DNA template–primer dose and the nucleotide substrate concentration. (A) and (C): Pol β activity was measured in the absence or presence of the indicated concentrations of compound **3** using varying concentrations of the DNA template–primer (A: Lineweaver–Burk plots, C: Michaelis–Menten curves). (B) and (D): Pol β activity was measured in the absence or presence of the indicated concentrations of compound **3** using varying concentrations of the substrate dTTPs (B: Lineweaver–Burk plots, D: Michaelis–Menten curves). (E) and (G): Pol β activity was measured in the absence or presence of the indicated concentrations of compound **17** using varying concentrations of the DNA template–primer (E: Lineweaver–Burk plots, G: Michaelis–Menten curves). (F) and (H): Pol β activity was measured in the absence or presence of the indicated concentrations of compound **17** using varying concentrations of the substrate dTTPs (F: Lineweaver–Burk plots, H: Michaelis–Menten curves).

Table 2 Kinetic analysis of the inhibitory effects of compounds **3** and **17** on the activity of DNA polymerase β as a function of DNA template–primer dose and nucleotide substrate concentration. These data were obtained from global fitting models using the GraphPad Prism program. Errors mean ± S.E

Substrate	Concentration /μM	K_m/μM	V_{max}/pmol min^{-1} ng^{-1}	K_i/μM	Inhibitory mode
Compound ***3***					
DNA Template–primer[a]	0	1.777 ± 0.563	0.795 ± 0.070	0.12 ± 0.03	Competitive
	0.2	3.970 ± 0.793	0.704 ± 0.045		
	0.4	5.415 ± 3.021	0.725 ± 0.136		
	0.6	18.24 ± 1.713	0.994 ± 0.043		
Nucleotide substrate[b]	0	4.432 ± 0.3391	0.655 ± 0.026	0.37 ± 0.02	Non-competitive
	0.2	2.509 ± 0.230	0.323 ± 0.013		
	0.4	2.737 ± 0.220	0.268 ± 0.010		
	0.6	2.462 ± 0.064	0.185 ± 0.002		
Compound ***17***					
DNA Template–primer[a]	0	1.777 ± 0.563	0.795 ± 0.070	6.8 ± 1.2	Competitive
	15	6.812 ± 0.658	0.825 ± 0.028		
	30	14.79 ± 1.26	0.991 ± 0.037		
	45	27.75 ± 5.27	1.141 ± 0.115		
Nucleotide substrate[b]	0	4.432 ± 0.3391	0.655 ± 0.026	15.9 ± 0.7	Non-competitive
	15	2.652 ± 0.406	0.277 ± 0.019		
	30	3.193 ± 0.5181	0.195 ± 0.015		
	45	4.333 ± 0.8586	0.162 ± 0.016		

[a] *I.e.*, poly(dA)–oligo(dT)$_{12-18}$. [b] *I.e.*, dTTPs.

Table 3 The IC_{50} and log IC_{50} values of compounds **3** and **17** for enzymatic inhibition of the 31 kDa domain of DNA polymerase β. The IC_{50} and log IC_{50} values were determined by using the GraphPad Prism program. Errors mean ± S.E

Compound	$IC_{50}/10^{-6}$ M	Log IC_{50}/M
3	3.9	−5.41 ± 0.05
17	51.1	−4.29 ± 0.04

By contrast, **1** and **2** had almost no effect on the activity of the 31 kDa domain.[9] We therefore conclude that the perfluorinated chain of **3** and **17** enhances the hydrophobic interaction between these compounds and the 31 kDa domain of pol β. However, the sensitivity of the 31 kDa domain to **3** was reduced by approximately 6-fold, whereas **17** inhibited the activity of both the 31 kDa domain and the full-length pol β equally. These results indicate that compound **17** interferes competitively with the interaction between the template–primer DNA and the active site of the 31 kDa domain of pol β, but non-competitively with incoming dNTPs. By contrast, compound **3** appears to display moderate affinity to the 8 kDa domain in addition to binding to the 31 kDa domain of pol β.

Conclusion

We have investigated the chemical properties of organic acids as DNA polymerase inhibitors. Monoprotic acids, which have a Clog *P* value greater than 7.0 and a pK_a value less than 5.4, were generally found to inhibit the activity of one or more DNA polymerases at concentrations lower than 100 μM. By contrast, compounds with a Clog *P* value less than 7.0 and with a pK_a value greater than 5.4 generally had little influence on the polymerase activity. With a minor modification these chemical properties of DNA polymerase inhibitors also apply to several lipid compounds. Moreover, the molecular shape was suggested to be important for both inhibition and selectivity. In the present study, we found that DNA polymerases are sensitive to PFOdA and PFOS. Furthermore, our kinetic analysis suggests that the perfluorinated chain might enhance the hydrophobic interaction between these compounds and the 31 kDa domain of pol β. These observations will provide insights not only for identifying new DNA polymerase inhibitors from natural or synthetic compounds, but for providing information on inhibitor–enzyme interactions in order to develop inhibitors of therapeutic utility.

Abbreviations

PFAS, perfluorinated alkyl substance; PFOdA, perfluorooctadecanoic acid; PFOS, perfluorooctanesulfonic acid; pol, DNA polymerase; TdT, terminal deoxynucleotide transferase; Clog *P*, calculated log *P*; IC_{50}, 50% inhibition concentration; dNTP, deoxynucleotide 5′-triphosphate; CMC, critical micelle concentration.

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References

1 U. Hübscher, G. Maga and S. Spadari, Eukaryotic DNA polymerases, *Annu. Rev. Biochem.*, 2002, **71**, 133–163.
2 K. Benek and T. A. Kunkel, Functions of DNA polymerases, *Adv. Protein Chem.*, 2004, **69**, 137–165.
3 L. A. Loeb, S. S. Agarwal, D. K. Dube, K. P. Gopinathan, E. C. Travaglini, G. Seal and M. A. Sirover, Inhibitors of mammalian DNA polymerases: possible chemotherapeutic approaches, *Pharmacol. Ther., Part A:*, 1977, **2**, 171–193.
4 S. Miura and S. Izuta, DNA polymerases as targets of anti-cancer nucleotides, *Curr. Drug Targets*, 2004, **5**, 191–195.
5 Y. Mizushina, N. Kasai, H. Iijima, F. Sugawara, H. Yoshida and K. Sakaguchi, Sulfo-quinovosyl-acyl-glycerol (SQAG), a eukaryotic DNA

polymerase inhibitor and anticancer agent, *Curr. Med. Chem.: Anti-Cancer Agents*, 2005, **5**, 613–625.
6 M. R. Albertella, A. Lau and M. J. O'Connor, The overexpression of specialized DNA polymerases in cancer, *DNA Repair*, 2005, **4**, 583–592.
7 Y. Mizushina, H. Yagi, N. Tanaka, T. Kurosawa, H. Seto, K. Katsumi, M. Onoue, H. Ishida, A. Iseki, T. Nara, K. Morohashi, T. Horie, Y. Onomura, M. Narusawa, N. Aoyagi, K. Takami, M. Yamaoka, Y. Inoue, A. Matsukage, S. Yoshida and K. Sakaguchi, *J. Antibiot.*, 1996, **49**, 491–492.
8 Y. Mizushina, N. Tanaka, H. Yagi, T. Kurosawa, M. Onoue, H. Seto, T. Horie, N. Aoyagi, M. Yamaoka, A. Matsukage, S. Yoshida and K. Sakaguchi, Fatty acids selectively inhibit eukaryotic DNA polymerase activities *in vitro*, *Biochim. Biophys. Acta*, 1996, **1308**, 256–262.
9 Y. Mizushina, S. Yoshida, A. Matsukage and K. Sakaguchi, The inhibitory action of fatty acids on DNA polymerase β, *Biochim. Biophys. Acta*, 1997, **1336**, 509–521.
10 Y. Mizushina, T. Ohkubo, T. Date, T. Yamaguchi, M. Saneyoshi, F. Sugawara and K. Sakaguchi, Mode analysis of a fatty acid molecule binding to the N-terminal 8 kDa domain of DNA polymerase β. A 1 : 1 complex and binding surface, *J. Biol. Chem.*, 1999, **274**, 25599–25607.
11 Y. Mizushina, F. Sugawara, A. Iida and K. Sakaguchi, Structural homology between DNA binding sites of DNA polymerase β and DNA topoisomerase II, *J. Mol. Biol.*, 2000, **304**, 385–395.
12 M. Oda, T. Ueno, N. Kasai, H. Takahashi, H. Yoshida, H. Sugawara, K. Sakaguchi, H. Hayashi and Y. Mizushina, Inhibition of telomerase by linear-chain fatty acids: a structural analysis, *Biochem. J.*, 2002, **367**, 329–334.
13 N. Kasai, Y. Mizushina, F. Sugawara and K. Sakaguchi, Three-dimensional structural mode analysis of the binding site of an inhibitor, nervonic acid, of both DNA polymerase β and HIV-1 reverse transcriptase, *J. Biochem.*, 2002, **132**, 819–828.
14 J. Giesy and K. Kannan, Perfluorochemical surfactants in the environment, *Environ. Sci. Technol.*, 2002, **36**, 146A–152A.
15 K. Prevedouros, I. T. Cousins, R. C. Buck and S. H. Korzeniowski, Sources, fate and transport of perfluorocarboxylates, *Environ. Sci. Technol.*, 2006, **40**, 32–44.
16 J. Verreault, M. Houde, G. W. Gabrielsen, U. Berger, M. Haukas, R. J. Letcher and D. C. G. Muir, Perfluorinated alkyl substances in plasma, liver, brain and eggs of glaucous gulls (*Larus hyperboreus*) from the Norwegian Arctic, *Environ. Sci. Technol.*, 2005, **39**, 7439–7445.
17 C. Lau, J. L. Butenhoff and J. M. Rogers, The developmental toxicity of perfluoroalkyl acids and their derivatives, *Toxicol. Appl. Pharmacol.*, 2004, **198**, 231–241.
18 (*a*) CIBA Ltd., *Polyglycol ethers*, GB 779 829 19 570 724, 1957; (*b*) K. Yutaka, T. Ashizawa, K. Kawashima, S. Ikeda and T. Tamaoki, *Bioorg. Med. Chem. Lett.*, 2003, **13**, 455–458.
19 (*a*) D. H. Hunter and R. A. Perry, Synthetic application of crown ethers; the malonic ester synthesis, *Synthesis*, 1977, 37–39; (*b*) P. Bühlmann, M. Badertscher and W. Simon, Molecular recognition of creatinine, *Tetrahedron*, 1993, **49**, 595–598.
20 T. Kawai, M. Shimamura, K. Obata, Preparation of hydroxamic acids and elastase inhibitors containing them, *Jpn. Kokai Tokkyo Koho*, Appl. JP 93-345168, 1995. See also ref. 47.
21 (*a*) P. Geneste, A. Guida, C. Reminiac, G. Amblard and C. Gavach, Transport des cations a travers des membranes lipidiques biomoleculaires par des nouveaux macrocyles di et tetraesters polyetheres hydrophobes, *Tetrahedron Lett.*, 1981, **22**, 1397–1398; (*b*) K. Neimert-Anderson, E. Blomberg and P. Somfai, Stereoselective synthesis of polyhydroxyl surfactants. Stereochemical influence on Langmuir monolayers, *J. Org. Chem.*, 2004, **69**, 3746–3752.
22 S. Fürmeier and J. O. Metzger, Synthesis of new heterocyclic fatty compounds, *Eur. J. Org. Chem.*, 2003, 885–893.
23 (*a*) M. Akiyama, H. Akashi, T. Shiga, T. Matsui, Y. Hayashi, T. Kimura, H. Kobayashi, *Jpn. Kokai Tokkyo Koho*, Appl. JP 78-76988, 1980; (*b*) C. Fruit and P. Müller, Intramolecular asymmetric amidations of sulfonamides and sulfamates catalyzed by chiral dirhodium (II) complex, *Helv. Chim. Acta*, 2004, **87**, 1607–1615.
24 S. Junno and T. Okita, Synthesis and structure–activity relationships of phaffiaol and related antioxidant, *Chem. Pharm. Bull.*, 1998, **46**, 1688–1694.
25 F. Bergel, A. Jacob, R. Todd and T. S. Work, Vitamin E. IV. Synthetic experiments in the coumaran and chroman series. The structure of tocopherol, *J. Chem. Soc.*, 1938, 1375–1382.
26 T. Kamino, K. Kuramochi and K. Kobayashi, A concise approach to 5-substituted-4-pyrones from kojic acid, *Tetrahedron Lett.*, 2003, **44**, 7349–7351.
27 R. F. Keyes, W. M. Golebiewski and M. Cushman, Correlation of anti-HIV potency with lipophilicity in a series of cosalane analogs having normal alkenyl and phosphodiester chains as cholestane replacements, *J. Med. Chem.*, 1996, **39**, 508–514.
28 K. Tamai, T. Kojima, T. Hanaichi, S. Masaki, M. Suzuki, H. Uekawa and S. Yoshida, Structural study of immunoaffinity-purified DNA polymerase α-DNA primase complex from calf thymus, *Biochim. Biophys. Acta*, 1988, **950**, 263–273.
29 Y. Uchiyama, J. Tagami, S. Kamisuki, N. Kasai, M. Oshige, H. Chiku, S. Ibe, O. Koiwai, F. Sugawara and K. Sakaguchi, Selective inhibitors of terminal deoxyribonucleotidyltransferase (TdT): baicalin and genistin, *Biochim. Biophys. Acta*, 2005, **1725**, 298–304.
30 K. Kuramochi, F. Saito, R. Takeuchi, T. Era, M. Takemura, J. Kobayashi, K. Sakaguchi, S. Kobayashi and F. Sugawara, Synthesis of plakevulin A and structure–activity relationships of its related compounds against DNA polymerases, *Tetrahedron*, 2006, **62**, 8006–8015.
31 A. F. Thüneman and H. Schnablegger, Monodisperse disk-shaped micelles of perfluorooctadecanoic acid, *Langmuir*, 1999, **15**, 5426–5428.
32 K. Murakami-Murofushi, M. Shioda, K. Kaji, S. Yoshida and H. Murofushi, Inhibition of eukaryotic DNA polymerase α with a novel lysophosphatidic acid (PHYLPA) isolated from myxoamoebae of *Physarum polycephalum*, *J. Biol. Chem.*, 1992, **267**, 21512–21517.
33 Y. Takahashi, Y. Shimada, M. Shioda, S. Yoshida, H. Murofushi and K. Murofushi-Murakami, Isolation of a new species of Physarum lysophosphatidic acid, PHLPA, and its effect on DNA polymerase activity, *Cell Struct. Funct.*, 1993, **18**, 135–138.
34 W. Schorr and H. Hoffmann, Electric birefringence measurements on micellar solutions of ionic surfactants, *J. Phys. Chem.*, 1981, **85**, 3160–3167.
35 D. P. Bossev, M. Matsumoto and M. Nakahara, ^{1}H and ^{19}F NMR study of the counterion effect on the micellar structures formed by tetraethylammonium and lithium perfluorooctylsulfonate. 1. Neat systems, *J. Phys. Chem. B*, 1999, **103**, 8251–8258.
36 S. Hanashima, Y. Mizushina, K. Ohta, T. Yamazaki, F. Sugawara and K. Sakaguchi, Structure–activity relationship of a novel group of mammalian DNA polymerase inhibitors, synthetic sulfoquinovosylacylglycerols, *Jpn. J. Cancer Res.*, 2000, **91**, 1073–1083.
37 Y. Mizushina, S. Miyazaki, K. Ohta, M. Hirota and K. Sakaguchi, Novel anti-inflammatory compounds from *Myrsine seguinii*, terpenobenzoic acids, are inhibitors of mammalian DNA polymerases, *Biochim. Biophys. Acta*, 2000, **1475**, 1–4.
38 F. Saito, R. Takeuchi, T. Kamino, K. Kuramochi, F. Sugawara, K. Sakaguchi and S. Kobayashi, Structure–activity relationships of untenone A and its derivatives for inhibition of DNA polymerases, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1975–1977.
39 Y. Mizushina, C. Murakami, H. Takikawa, N. Kasai, X. Xu, K. Mori, M. Oshige, T. Yamaguchi, M. Saneyoshi, N. Shimazaki, O. Koiwai, H. Yoshida, F. Sugawara and K. Sakaguchi, Molecular action mode of hippospongic acid A, an inhibitor of gastrulation of starfish embryos, *J. Biochem.*, 2003, **133**, 541–552.
40 Y. Mizushina, C. Murakami, K. Ohta, H. Takikawa, M. Mori, H. Yoshida, F. Sugawara and K. Sakaguchi, Selective inhibition of the activities of both eukaryotic DNA polymerases and DNA topoisomerases by elenic acid, *Biochem. Pharmcol.*, 2002, **63**, 399–407.
41 Y. Mizushina, A. Iida, K. Ohta, F. Sugawara and K. Sakaguchi, Novel triterpenoids inhibit both DNA polymerase and DNA topoisomerase, *Biochem. J.*, 2000, **350**, 757–763.
42 J. Z. Deng, S. R. Starck and S. M. Hecht, DNA polymerase β inhibitors from *Baeckea gunniana*, *J. Nat. Prod.*, 1999, **62**, 1624–1626.
43 Y. Mizushina, C. Murakami, K. Yogi, K. Ueda, T. Ishidoh, M. Takemura, M. Perpelescu, M. Suzuki, M. Oshige, T. Yamaguchi, M. Saneyoshi, H. Yoshida and K. Sakaguchi, Kohamaic acid A, a novel sesterterpenic acid, inhibits activities of DNA polymerases from deuterostomes, *Biochim. Biophys. Acta*, 2003, **1648**, 56–61.
44 M. Oshige, K. Kuramochi, K. Ohta, A. Ogawa, H. Kuriyama, F. Sugawara, S. Kobayashi and K. Sakaguchi, Molecular design of cholesterols as inhibitors of DNA polymerase α, *J. Med. Chem.*, 2004, **47**, 4971–4974.
45 Y. Mizushina, T. Ueno, M. Oda, T. Yamaguchi, M. Saneyoshi and K. Sakaguchi, The biochemical mode of inhibition of DNA polymerase β by α-rubromycin, *Biochim. Biophys. Acta*, 2000, **1523**, 172–181.

46 Y. Mizushina, S. Kamisuki, T. Mizuno, M. Takemura, H. Asahara, S. Linn, T. Yamaguchi, A. Matsukage, F. Hanaoka, S. Yoshida, M. Saneyoshi, F. Sugawara and K. Sakaguchi, Dehydroaltenusin, a mammalian DNA polymerase α inhibitor, *J. Biol. Chem.*, 2000, **275**, 33957–33961.
47 Y. Mizushina, S. Kamisuki, N. Kasai, N. Shimazaki, M. Takemura, H. Asahara, S. Linn, S. Yoshida, A. Matsukage, O. Koiwai, F. Sugawara, H. Yoshida and K. Sakaguchi, A plant phytotoxin, solanapyrone A, is an inhibitor of DNA polymerase β and λ, *J. Biol. Chem.*, 2002, **277**, 630–638.
48 Y. Mizushina, S. Kamisuki, N. Kasai, T. Ishidoh, N. Shimazaki, M. Takemura, H. Asahara, S. Linn, S. Yoshida, O. Koiwai, F. Sugawara, H. Yoshida and K. Sakaguchi, Petasiphenol: a DNA polymerase λ inhibitor, *Biochemistry*, 2002, **41**, 14463–14471.
49 Y. Mizushina, M. Hirota, C. Murakami, T. Ishidoh, S. Kamisuki, N. Shimazaki, M. Takemura, M. Perpelescu, M. Suzuki, H. Yoshida, F. Sugawara, O. Koiwai and K. Sakaguchi, Some anti-chronic inflammatory compounds are DNA polymerase λ-specific inhibitors, *Biochem. Pharmacol.*, 2003, **66**, 1935–1944.
50 Y. Mizushina, L. Hanashima, T. Yamaguchi, M. Takemura, F. Sugawara, M. Saneyoshi, A. Matsukage, S. Yoshida and K. Sakaguchi, A mushroom fruiting body-inducing substance inhibits activities of replicative DNA polymerases, *Biochem. Biophys. Res. Commun.*, 1998, **249**, 17–22.
51 Y. Mizushina, N. Takahashi, A. Ogawa, K. Tsurugaya, H. Koshino, M. Takemura, S. Yoshida, A. Matsukage, F. Sugawara and K. Sakaguchi, The cyanogenic glucoside, prunasin (D-mandelonitrile-beta-D-glucoside), is a novel inhibitor of DNA polymerase β, *J. Biochem.*, 1999, **126**, 430–436.
52 Y. Mizushina, Y. Sugiyama, H. Yoshida, S. Hanashima, T. Yamazaki, S. Kamisuki, K. Ohta, M. Takemura, T. Yamaguchi, A. Matsukage, S. Yoshida, M. Saneyoshi, F. Sugawara and K. Sakaguchi, Galactosyldiacylglycerol, a mammalian DNA polymerase α-specific inhibitor from a sea alga, *Petalonia bingbamiae*, *Biol. Pharm. Bull.*, 2001, **24**, 982–987.
53 Y. Mizushina, N. Tanaka, A. Kitamura, K. Tamai, M. Ikeda, M. Takemura, F. Sugawara, T. Arai, A. Matsukage, S. Yoshida and K. Sakaguchi, The inhibitory effect of novel triterpenoid compounds, fomitellic acids, on DNA polymerase β, *Biochem. J.*, 1998, **330**, 1325–1332.
54 N. Tanaka, A. Kitamura, Y. Mizushina, F. Sugawara and K. Sakaguchi, Fomitellic acids, triterpenoid inhibitors of eukaryotic DNA polymerases from a basidiomycete, *Fomitella fraxinea*, *J. Nat. Prod.*, 1998, **61**, 193–197.
55 Y. Mizushina, T. Ohkubo, F. Sugawara and K. Sakaguchi, Structure of lithocholic acid binding to the N-terminal 8 kDa domain of DNA polymerase β, *Biochemistry*, 2000, **39**, 12606–12613.
56 Y. Mizushina, N. Kasai, F. Sugawara, A. Iida, H. Yoshida and K. Sakaguchi, Three-dimensional structural model analysis of the binding site of lithocholic acid, an inhibitor of DNA polymerase β and DNA topoisomerase II, *J. Biochem.*, 2001, **130**, 657–664.
57 Y. Mizushina, N. Kasai, K. Miura, S. Hanashima, M. Takemura, H. Yoshida, F. Sugawara and K. Sakaguchi, Structural relationship of lithocholic acid derivatives binding to the N-terminal 8 kDa domain of DNA polymerase β, *Biochemistry*, 2004, **43**, 10669–10677.
58 C. Murakami, M. Takemura, Y. Sugiyama, S. Kamisuki, H. Asahara, M. Kawasaki, T. Ishidoh, S. Linn, S. Yoshida, F. Sugawra, H. Yoshida, K. Sakaguchi and Y. Mizushina, Vitamin A-related compounds, all-*trans* retinal and retinoic acids, selectively inhibit activities of mammalian replicative DNA polymerases, *Biochim. Biophys. Acta*, 2002, **1574**, 85–92.
59 C. Murakami, K. Ishijima, M. Hirota, K. Sakaguchi, S. Yoshida and Y. Mizushina, Novel anti-inflammatory compounds from *Rubus sieboldii*, triterpenoids, are inhibitors of mammalian DNA polymerases, *Biochim. Biophys. Acta*, 2002, **1596**, 193–200.
60 A. Kumar, S. G. Widen, K. R. Williams, P. Kedar, R. L. Karpel and S. H. Wilson, Studies of the domain structure of mammalian DNA polymerase β. Identification of a discrete template binding domain, *J. Biol. Chem.*, 1990, **265**, 2124–2134.
61 A. Kumar, J. Abbotts, E. M. Karawya and S. H. Wilson, Identification and properties of the catalytic domain of mammalian DNA polymerase β, *Biochemistry*, 1990, **29**, 7156–7159.