Synthesis and Antitumor Activity of 2',3'-Didehydro-3'-deoxy-thymidine and Its Derivative

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In an effort to enhance the lipophilicities, thereby, the penetration into the cell membrane and to increase the antitumor activities of modified derivatives of 2',3'-didehydro-3'-deoxythymidine (d4T, **1**), derivatives of **1** were designed and synthesized. Starting from thymidine, **1**, 2',3'-didehydro-3'-deoxythymidine-5'-phosphate, disodium salt (d4T-p, **7**), and two nicotinate esters of **1**; 2',3'-didehydro-3'-deoxy-5'-O-(3-pyridinylcarbonyl)thymidine (d4T-NA, **5**) and 2',3'-didehydro-3'-deoxy-5'-phosphoryl-O-(3-pyridinylcarbonyl)thymidine (d4T-p-NA, **8**) were synthesized. The lipophilicities of the synthesized compounds were measured by P-values and antitumor activities of those were estimated against *mouse leukemia P388, murine mammary carcinoma FM3A,* and *human histiocytic lymphoma U937 tumor cells in vitro.* Although the lipophilicities of the nicotinate esters, **5** and **8** were increased 2.75- and 9.71-fold relative to that of **1** and **7**, respectively, the synthesized compounds, **1, 5, 7**, and **8** were found to be inactive against *P388* and *FM3A* cells except weak antitumor activity against *U937* cell.

Introduction

Traditional researches in the antimicrobial, antitumor, and antiviral agents have been focused on the nucleoside and nucleotide analogues for new therapies. Study for active drugs against HIV has shown nucleoside analogues to be efficacious. One common feature among the nucleoside derivatives which showed good *in vitro* activity was the lack of a 3'-OH group on the sugar part of the molecule, thereby enabling these substances to act as possible chain terminator of DNA synthesis.[1] Recently, 2',3'-dideoxynucleosides were found to be physiologically important[2] and to play an important role in protecting cells against the cytopathic effect of HIV.[3] In fact the synthesis of dideoxynucleosides from ribonucleoside has been studied extensively.[4] However, many difficulties were envisioned in adapting deoxynucleosides because it required many steps, needed expensive reagents, and had the problems of stereospecificity. Moreover the biochemical action mode of 2',3'-didehydro-3'-deoxythymidine (d4T, **1**) was not clear. Thus we turned our attention to the improved route suggested by Horwitz *et al.*[5] so that we synthesized **1** which was one of 2',3'-dideoxy nucleoside derivatives. And in order to study the biochemical action mode, the monophosphate of **1**, 2',3'-didehydro-3'-deoxythymidine-5'-phosphate, disodium salt (d4T-p, **7**) was synthesized by a modified method of Yoshigawa *et al.*[6] and Hong *et al.*[7] In an attempt to investigate the use of nicotinic acid as a covalent carrier of cytotoxic groups, to enhance the lipophilicity (enhance the penetration ability into cell membrane[8]), and to increase the antitumor activity, two nicotinate esters of **1** and **7**, 2',3'-didehydro-3'-deoxy-5'-O-(3-pyridinylcarbonyl)thymidine (d4T-NA, **5**) and 2',3'-didehydro-3'-deoxy-5'-phosphoryl-O-(3-pyridinylcarbonyl)thymidine (d4T-p-NA, **8**) were also synthesized. Then the partition coefficient which was essentially a measure of lipophilicity[9] was measured and the evaluation for antitumor activity was carried out[10] against three tumor cell lines; *mouse leukemia P388, murine mammary carcinoma FM3A*, and *human histiocytic lymphoma U937* cells by MTT assay.

Materials and Methods

Synthesis. All solvents were purified by normal procedure.[14] Thin layer chromatography was performed using Silica Gel 60 F_{254} (Merck) and TLC spots were detected by irradiating with a short wave UV (254 nm) and/or by charring after spraying with anisaldehyde.[15] All evaporations were carried out *in vacuo* by rotary evaporator or by short-path distillation into a dryice-acetone cooled receiver under high vacuum. Column chromatography was performed using Silica Gel 60 (70-320 mesh, Merck). Melting points were measured with electrothermal capillary melting point apparatus. ^{1}H NMR spectra were obtained on Bruker AM 300 spectrometer with tetramethylsilane as an internal reference. ^{13}C NMR spectra were recorded on Bruker AM 300 spectrometer. Infrared spectra were recorded on a Bomen spectrometer as potassium bromide pellets.

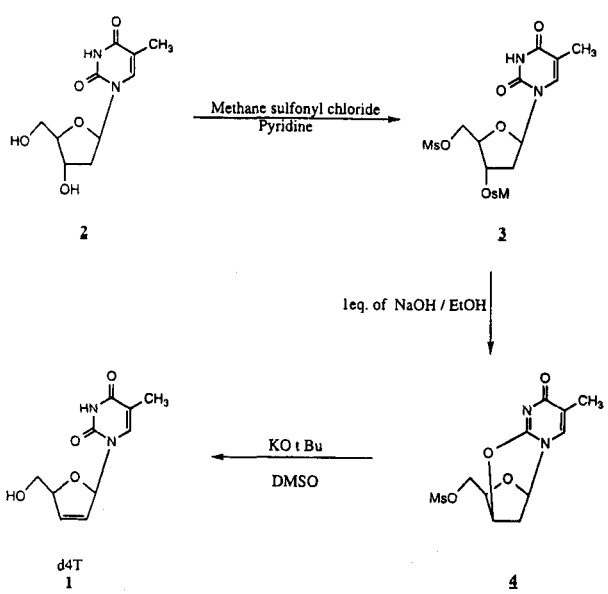
Partitioning. Synthesized compounds were partitioned between the phases of mixture containing equal volumes of 1-octanol and 0.1 M sodium phosphate, pH 7.0.[12] The phases were separated by centrifugation and the concentrations of nucleoside and nucleotide compounds in each phase were determined by absorbance measurements at the wavelength determined from spectral studies[13] using Shimadzu UV-250 UV-Visible spectrophotometer.

Antitumor activity test. Antitumor activities of the synthesized compounds were assayed by measuring the concentration that inhibited 50% of the cell growth (IC_{50} in μg/m*l*) using the modified procedure of Mosmann *et al.*[10] *P388, FM3A*, and *U937* cells were suspended in growth medium (Dulbecco's modified Egele's medium) and loaded to microplate well at a density of 2×10^4 cells/well in the presence of varying concentration of the test compounds. After incubation for 4 days at 37 °C under 5% CO_2, the supernatant of the culture media was discarded and 20 μ*l* of MTT solution[11] were added. At the end of a further 4 hr in-

cubation period, the solution was removed and 100 μl of DMSO were added to solubilize the formazan formed by the cellular reduction of MTT. Absorbance was recorded at 570 and 650 nm using ELISA Processor II Microplate reader and IC (μg/ml) was determnd.

Experimental

Synthesis of 2',3'-didehydro-3'-deoxythymidine (d4T, 1). A round-bottomed flask equipped with a mechanical stirrer, thermometer, and nitrogen inlet was charged with dry DMSO (400 mL) and 1-[3',5'-anhydro-2-deoxy-β-D-threo-pentofuranosyl)thymidine (**4**, 90.0 g, 0.042 mol). To this solution was added 97% KOtBu (74.0 g, 0.643 mol) in 1.5 g portions over 25 min. The solution was stirred for further 1h and poured cold toluene, resulting in the precipitation of a beige solid. The temperature of the mixture rose to 7 °C upon addition of the DMSO solution. The mixture was occasionally swirled over 20 min and then filtered on a Buchner funnel. The collected yellowish oily solid was washed twice with cold toluene and allowed to dry under suction for 1h. The solid was dissolved in 300 mL of water, whereupon two layers were formed. The upper layer (containing residual toluene) was discarded and the aqueous solution was cooled to 10 °C. Conc. HCl was added dropwise to pH 7 and a precipitate was formed. Potassium chloride (70 g) was added to this thick mixture and stirred under vacuum for 2h, and then air-dried for 16h. The solid was crushed, slurried in hot acetone(300ml), and then filtered. The combined filtrate was concentrated to dryness to give 51.5 g (57%) of **1** as white solid (Scheme 1): mp 165-166 °C; ^{1}H NMR ($CDCl_3$) δ 10.40 (s, 1H, NH), 6.92 (s, 1H, H-6), 6.19 (d, 1H, H-1'), 5.64 (d, 1H, H-3'), 5,12 (dd, 1H, H-2'), 4.09 (m, 1H, OH), 3.68 (s, 1H, H-4'), 2.97 (dd, 2H, H-5'), 1.05 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 163.92 (C-4), 150.73 (C-2), 136.36 (C-6), 134.45 (C-3'), 125.72 (C-2'), 109.23 (C-5), 88.76 (C-1'), 87.05 (C-4'), 62.28 (C-5'), 12.10 (CH_3); IR (KBr) 3466, 3031, 2823, 1677, 1462, 1255, 1094 cm^{-1}.

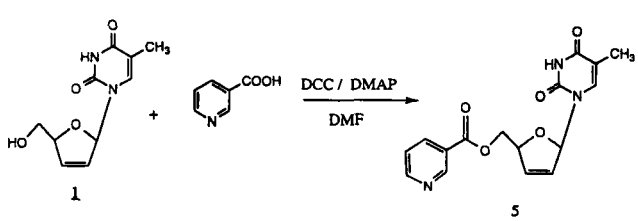


Scheme 1.

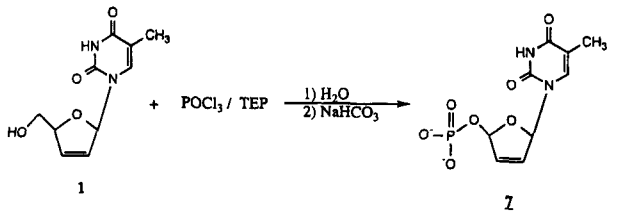
Synthesis of 1-[2'-deoxy-3',5'-bis(methylsulfonyl)-β-D-erythro-pentofuranosyl] thymidine (3). Thymidine (50 g, 0.205 mol, **2**) was dissolved in pyridine (180 mL). The mixture was stirred and then cooled in an ice bath to 0-3 °C and the dropping funnel was charged with methanesulfonyl chloride (51.8 g, 35 mL, 0.45 mol). The methanesulfonyl chloride was then added dropwise over 40 min during which the temperature rose to 10 °C. The mixture was cooled to 0 °C and stirred for 1h, and then stored at 5 °C for 18h. The resulting light brown mixture was poured onto rapidly stirred water containing ice. The desired product was crystallized immediately. After stirring for 0.5h, the product was collected by filtration and washed with water. The white solid was dried under vacuum overnight (crude weight, 80.5 g, 98%). The product was recrystallized from hot acetone to give **3** (66.7 g, 81%) as a white solid (Scheme 1): mp 169-173 °C; ^{1}H NMR (DMSO-d_6) δ 11.40 (s, 1H, NH), 7.50 (s, 1H, H-6), 6.21 (t, 1H, H-1'), 5.29 (m, 1H, H-3'), 4,45 (m, 2H, H-5'), 4.35 (m, 1H, H-4'), 3.31 (s, 6H, SO_2CH_3), 2.50 (m, 2H, H-2'), 1.78 (s, 3H, CH_3).

Synthesis of 1-[3',5'-anhydro-2-deoxy-β-D-threo-pentofuranosyl)thymidine (4). **3** (2.5 g, 62 mmol) was added in portions to a stirred solution of sodium hydroxide (0.75 g, 18 mmol) in water (150 mL) and heated to reflux for 45 min. After cooling of the reaction mixture to room temperature, conc. hydrochloric acid (0.5 mL) was added. Approximately 10 mL of water were removed to give a white slurry, which was left to cool in an ice water, vacuum dried, and recrystallized from ethanol to give **4** (1.04 g, 74%) (Scheme 1): mp 188-190 °C; ^{1}H NMR (DMSO-d_6) δ 11.35 (s, 1H, NH), 8.01 (s, 1H, H-6), 6.49 (t, 1H, H-1'), 5.47 (m, 1H, H-3'), 4.88 (m, 1H, H-5'), 4.67 (dd, 1H, H-4'), 3.97 (d, 1H, H-5'), 2.47 (m, 2H, H-2'), 1.77 (s, 3H, CH_3).

Synthesis of 2',3'-didehydro-3'-deoxy-5'-O-(3-pyridinylcarbonyl)thymidine (5).[16] A mixture of **1** (10 g, 44.6 mmol), nicotinic acid (7.8 g, 63.4 mmol), 1,3-dicyclohexyl carbodiimide (13.6 g, 66.0 mmol), and 4-(dimethylamino)pyridine (0.85 g) in 75 mL of DMF was stirred for 12h. The precipitated dicyclohexylurea was filtered off and the filter cake was washed with DMF. The filtrate was evaporated and the residue was extracted with $CHCl_3$, washed with water, dried (Na_2SO_4), and evaporated. Chromatography of the residue over a silicagel column using 1% $CHCl_3$ in EtOAc gave 15.5 g (74%) of the product (Scheme 2): mp 172-175 °C; ^{1}H NMR (DMSO-d_6) δ 9.04(s, 1H, pyridine H-2), 8.80 (d, 1H, pyridine H-6), 8.25 (m, 1H, pyridine H-4), 7.56 (m, 1H, pyridine H-5), 7.11 (s, 1H, NH), 6.80 (d, 1H, H-1'), 6.51 (m, 1H, H-2'), 6.03 (m, 1H, H-3'),



Scheme 2.

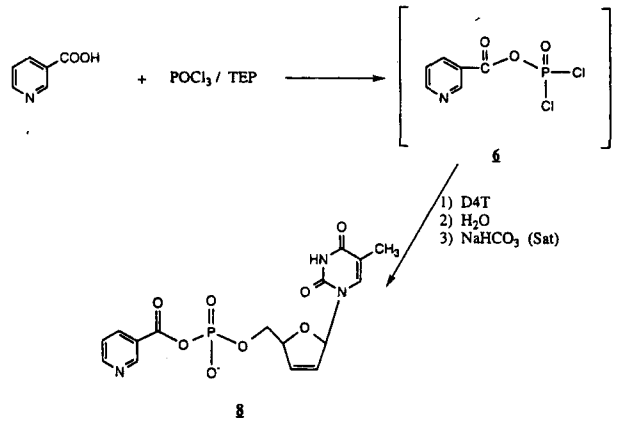


Scheme 3.

5.11 (m, 1H, H-4'), 4.55 (m, 1H, H-5'), 1.39 (s, 3H, CH_3); IR (KBr) 3600, 3494, 2948, 1726, 1694, 1663, 1440, 1301, and 1280 cm^{-1}.

Synthesis of 2',3'-didehydro-3'-deoxythymidine-5'-phosphate (disodium salt, 7). A solution of **1** (40 mg, 0.179 mmol) in triethylphosphate (0.5 mL) was treated with phosphorus oxychloride (150 mg, 1.61 mmol) at 0 °C and then stirred at room temperature for 2.5h under argon. The mixture was decomposed by addition of a cold sodium bicarbonate solution (1 M, pH 7.4, 20 mL). The reaction mixture was concentrated *in vacuo* (bath temperature should be kept below 30 °C) and the residue was triturated with diethyl ether (3×15 mL). The ether solutions were discarded and the semisolid material was dissolved in a minimum amount of water, and purified on a silica gel column packed with $CHCl_3$: MeOH : H_2O (65 : 25 : 4, v/v). Column was eluted with $CHCl_3$: MeOH : H_2O (65 : 25 : 4, v/v) and solvent was removed *in vacuo* to provide white solid residue. Residue containing the title compound as a disodium salt was 24 mg (33%) (Scheme 3): mp 283-285 °C; ^1H NMR (D_2O) δ 7.48 (s, 1H, H-6), 6.75 (s, 1H, H-1'), 6.34 (d, 1H, H-3'), 5.79 (dd, 1H, H-2'), 4.85 (s, 1H, H-4'), 4.34 (dd, 2H, H-5'), 1.70 (s, 3H, CH_3); ^{13}C NMR (D_2O) δ 157.03 (C-4), 151.49 (C-2), 148.55 (C-6), 124.01 (C-3'), 86.98 (C-2'), 86.37 (C-5), 71.41 (C-1'), 69.57 (C-4'), 62.64 (C-5'), 12.32 (CH_3); IR (KBr) 3456, 1672, 1253, 1171, 1045, 975 cm^{-1}.

Synthesis of 2',3'-didehydro-3'-deoxy-5'-phosphoryl-O-(3-pyridinylcarbonyl) thymidine (8). $POCl_3$ (0.22 mL, 2.4 mmol) was added to a cooled mixture (−5 °C) of dried nicotinic acid (0.246 g, 2 mmol) and redistilled $(EtO)_3PO$ (10 mL). The mixture was stirred at 0-5 °C for 3h and then **1** (0.448 g, 2 mmol) was added to the reaction mixture. The mixture was stirred at 0-5 °C for 9h and the solution was poured slowly into ice-water (50 mL) containing $NaHCO_3$ (0.5 g). The suspension was then washed with 50 mL of Et_2O and the aqueous layer was neutralized to pH 7.0 with conc. NaOH. The resulting mixture was concentrated in vacuo and applied to a silicagel column prepacked with $CHCl_3$: MeOH : H_2O (65 : 25 : 4, v/v). Column was eluted with $CHCl_3$: MeOH : H_2O (65 : 25 : 4, v/v) and solvent was removed *in vacuo* to dryness and the white solid residue was treated with Me_2CO. The resulting white solid was filtered and washed with Me_2CO. Residue containing the title compound as a sodium salt was 0.596 g (66.6%) (Scheme 4): mp 221-223 °C (slowly dec.); ^1H NMR (DMSO-d_6) δ 11.35 (s, 1H, NH) 9.08 (s, 1H, pyridine H-2), 8.83 (d, 1H, pyridine H-6), 8.31 (d, 1H, pyridine H-4), 8.27 (s, 1H, H-6), 7.65 (m, 1H, pyridine H-5), 5.76 (d, 1H, H-1'), 5.54 (d, 1H, H-2'), 5.35 (d, 1H, H-3'), 4.54 (m, 1H, H-4'), 4.13 (s, 1H, H-5'), 2.49 (s, 3H, CH_3); ^{13}C NMR (DMSO-d_6) δ 164.60 (C-4), 163.03 (pyridine C-2),



Scheme 4.

153.85 (pyridine C-6), 150.56 (C-2), 150.05 (pyridine C-1), 140.99 (carbonyl C), 136.98 (C-6), 125.42 (C-3'), 123.99 (C-2'), 101.93 (C-5), 89.28 (C-1'), 80.86 (C-4'), 72.71 (pyridine C-4), 69.67 (pyridine C-5), 64.61 (C-5'), 12.01 (CH_3); IR (KBr) 3434, 3106, 1696, 1444, 1270, 1091 cm^{-1}.

Results and Discussion

Recently 2',3'-unsaturated nucleoside derivatives which 2- and 3-OH groups of sugar moiety were removed and had double bond showed good biological activity, so the chemical and medical study on them has been progressed actively. **1** was synthesized from thymidine using methanesulfonyl chloride, NaOH, and KOtBu as Scheme 1 in this study. 3',5'-OH group of **2** was protected as methylsulfonyl group using methanesulfonyl chloride. The synthesis of **3** was identified as a single peak of SO_2CH_3 group on the δ 3.31 position in the ^1H NMR spectrum. **3** was heated to reflux in NaOH solution for 40 min to obtain **4**. The single peak of SO_2CH_3 group in **3** was disappeared. **4** was dissolved in DMSO and reacted with KOtBu to synthesize **1**. According to Scheme 2, nicotinic acid was coupled to 5'-OH group of **1** when DCC was used as coupling reagent, added nucleoside, nicotinic acid, and 4-(dimethylamino)pyridine, and reacted in DMF solution.[16] **7** and **8** were synthesized according to Scheme 3 and 4, respectively, using the Hong *et al.* method[7] that steroid and alcohol derivatives were coupled to nucleoside. Coupling reagent and enzyme were not used in this reaction and **8** was synthesized as one step reaction which **1** was added to **6** formed in the reaction of nicotinic acid and $POCl_3$ under the $(EtO)_3PO$ solvent. This reaction was terminated in short time and special attention was needed because it was acidic condition, exothermal reaction, and acid-catalyzed hydrolysis reaction was processed. Nucleotide derivatives were identified as the original peaks of ^1H NMR and ^{13}C NMR and the strong P=O peaks of IR spectra in 1094 and 1091 cm^{-1} position.

The lipophilicities of nicotinate esters of **1** were enhanced significantly as indicated by the increase in corresponding P-values (Table 1). Thus the conjugations of nicotinic acid leading to **5** and **8** were associated with a 2.75- and 9.71-fold increase in P-value relative to that of **1** and **7**, respectively. This result showed that nicotinic acid was sufficiently good media to increase the lipophilicity for the

Table 1. Partition Coefficients(P)[a] of 2',3'-didehydro-3'-deoxythymidine (d4T, **1**) and its derivatives

Compound	P-value	P ratio[b]
d4T (**1**)	0.199[c]	
d4T-NA (**5**)	0.547	2.75
d4T-p (**7**)	0.463	
d4T-p-NA (**8**)	4.495	9.71

[a] Measured in 1-octanol-0.1 M Na_3PO_4, pH 7.0 by the method described in ref. 12. [b] $P_{ratio}=P_{NA}$ (**5** or **8**)/P (**1** or **7**).
[c] $P=\frac{[\mathbf{1}\text{ or }\mathbf{7}(1\text{-octanol})]}{[\mathbf{1}\text{ or }\mathbf{7}(0.1\text{ M }Na_3PO_4,\text{ pH }7.0)]}$

Table 2. IC_{50s}[a] (μg/mL) of 2',3'-didehydro-3'-deoxythymidine (d 4T, **1**) and its derivatives

Compound	IC_{50} (μg/mL) against tumor cell		
	P388[b]	*FM3A*[c]	*U937*[d]
d4T (**1**)	>100	>100	40
d4T-NA (**5**)	>100	>100	48
d4T-p (**7**)	>100	>100	74
d4T-p-NA (**8**)	>100	>100	55

[a] Concentration of drug resulting in 50% growth inhibition of cell. Determined by MTT assay described in ref. 10. [b] *Mouse leukemia P388* cell. [c] *Murine mammary carcinoma FM3A* cell. [d] *Human histiocytic lymphoma U937* cell.

easy penetration of pro-drug through the cell membrane. It was reported that 3'-OH deficient nucleoside analogues (for example, 3'-azido-3'-deoxythymidine, AZT) traversed the cell membrane chiefly by nonfacilitated diffusion,[6] therefore the increased lipophilic nature of the nicotinate esters of **1** might facilitate passive diffusion. These results suggest that 5'-O-nicotinate ester derivatives may be useful to enhance lipophilicity in the drug design but antitumor activities of those compounds are weak.[9] Synthesized compounds were *in vitro* evaluated for antitumor activity against three tumor cells. The synthesized compounds, **1**, **5**, **7**, and **8** were found to be inactive against *mouse leukemia P388* and *murine mammary carcinoma FM3A* cells as shown in Table 2, but sensitive to *human histiocytic lymphoma U937* cell. The highest IC_{50} value of **7** was thought to be due to the difficult transport into the cell membrane and this was resulted from the effect of negative charge of **7** because IC_{50} of **8** was decreased. This result suggest the selectivity of **1**, *i.e.*, **1** has good effect for the HIV reverse-transcriptase, but **1** shows no activity for tumor cell.[10]

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11. MTT was dissolved 5 mg/mL in phosphate bufferd saline (PBS; KCl 0.2 g, KH_2PO_4 0.2 g, Na_2HPO_4 1.15 g, $MgCl_2 \cdot 6H_2O$ 0.101 g/L, pH 7.4).
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