Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 18, pp. 2177-2182, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00389-7

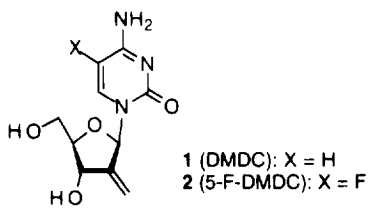
NUCLEOSIDES AND NUCLEOTIDES. 155. SYNTHESIS, ANTITUMOR EFFECTS, AND POSSIBLE ENZYMATIC ACTIVATION MECHANISM OF 5'-PHOSPHATIDYL-2'-DEOXY-2'-METHYLENECYTIDINE (DMDC)[1]

Satoshi Shuto,[a] Hirokazu Awano,[a]
Akihiro Fujii,[b] Keiji Yamagami,[b] and Akira Matsuda[a,*]

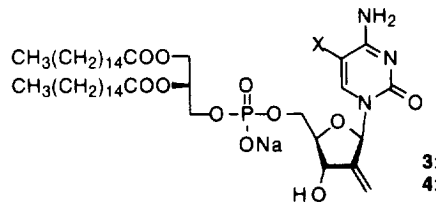
[a]*Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-12, Nishi-6, Kita-ku, Sapporo 060, Japan*
[b]*Research Laboratories, Yoshitomi Pharmaceutical Industries, Ltd., 3-7-25, Koyama, Iruma 358, Japan*

Abstract: 2'-Deoxy-2'-methylenecytidine (DMDC, **1**) and its 5-fluoro congener (5-F-DMDC, **2**), potent antitumor nucleosides developed by us, were efficiently converted to their 5'-phosphatidyl derivatives bearing palmitoyl residues (**3** and **4**, respectively) as novel antitumor phospholipids by phospholipase D-catalyzed trans-phosphatidylation. These phospholipids **3** and **4**, administered i.p., remarkably prolonged the life-span of mice which were i.p.-inoculated with M5076 sarcoma, and the effects were clearly superior to that of DMDC. Compound **3** was a good substrate for phospholipase A_2 from bovine pancreas as well as phospholipase D from *Streptomyces*, while it was slightly hydrolyzed by phospholipase C from *Bacillus cereus*. Copyright © 1996 Elsevier Science Ltd

Conversion of antitumor nucleosides to their phospholipid derivatives has been extensively studied because of their possible advantages over their parent compounds.[2] We recently synthesized 5'-phosphatidyl derivatives of antitumor nucleosides with the same backbone as natural phospholipids, and these had more significant antitumor activities against various mouse tumors than the respective parent nucleosides.[3] We have also demonstrated that 5'-phosphatidylnucleosides given orally can be absorbed via the deacylation-reacylation cycle, a specific pathway for natural phospholipids, to be transported to the lymph.[3d] This finding suggests that 5'-phosphatidylnucleosides can be used clinically in targeting chemotherapy for lymphoma or lymphatic metastases of certain tumors.



1 (DMDC): X = H
2 (5-F-DMDC): X = F



3: X = H
4: X = F

On the other hand, we have also performed synthetic studies of new antitumor nucleosides and found that 2'-deoxy-2'-methylenecytidine (DMDC, **1**) and its 5-fluoro congener (5-F-DMDC, **2**) are potent

antitumor nucleosides with a new mechanism of action, which potently inhibit the growth of various human tumor cells both *in vitro* and *in vivo*.[4] These findings suggested that it would be interesting to determine the biological effects of 5'-phosphatidyl derivatives of DMDC and 5-F-DMDC. In this communication, we describe the synthesis and antitumor effects of 5'-phosphatidyl-DMDC (**3**) and -5-F-DMDC (**4**), as well as their susceptibility to phospholipases which would modify their biological effects.

We have developed an efficient enzymatic method for preparing 5'-phosphatidylnucleosides from a nucleoside and a 3-*sn*-phosphatidylcholine (PC) by a one-step reaction which uses phospholipase D-catalyzed transphosphatidylation, i.e., the regiospecific transfer of the phosphatidyl residue from PC to the 5'-hydroxyl of a nucleoside.[3a,b,f] Using this enzymatic method, 5'-phosphatidyl derivatives of DMDC and 5-F-DMDC were readily synthesized.[5] In the presence of 1.2 equiv of dipalmitoyl phosphatidylcholine (DPPC, **5**), DMDC was treated with phospholipase D from *Streptomyces* sp. AA 586 (PLDP) in a two-phase system of chloroform and acetate buffer (pH 4.5) at 40 °C. After the usual work-up and treatment with a cation-exchange resin, the desired 5'-phosphatidyl-DMDC (**3**) was isolated as a sodium salt in a pure form.[6] 5'-Phosphatidyl-5-F-DMDC (**4**) was enzymatically synthesized in a similar manner.[7]

Scheme I

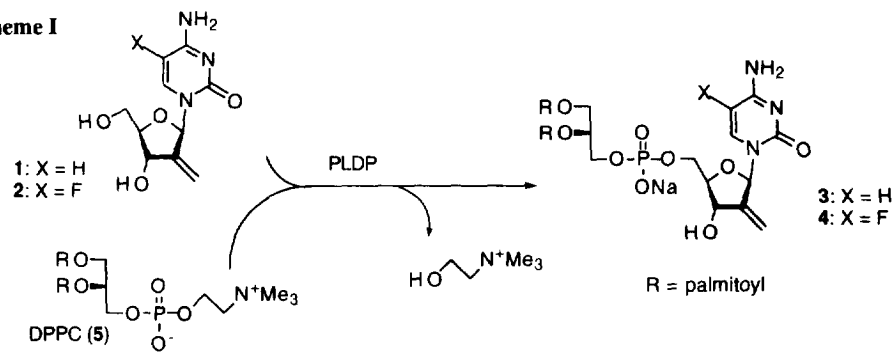


Table I. Antitumor Activities of **3**, **4**, and DMDC (**1**) against i.p.-Implanted L1210 Leukemia and M5076 Sarcoma in Mice

Compound	L1210 leukemia		M5076 sarcoma		
	Dose[a] (mmol/kg/day)	ILS[b] (%)	Dose[c] (mmol/kg/day)	ILS (%)	60-Day survivor
3	0.125	64	0.125	121	1/4
			0.40	>179	4/4
4	0.125	96	0.125	>179	3/4
			0.40	toxic	-
1 (DMDC)	0.125	68	0.125	44	0/4
			0.40	116	0/4

[a]Compounds were given p.o. on Days 1-5. [b]Percent increase in life-span. [c]Compounds were given i.p. on Days 1-5.

We first evaluated the antitumor effects of 5'-phosphatidyl derivatives **3** and **4** with i.p.-implanted L1210 leukemia in mice, and compared these effects with that of DMDC. The compounds were administered p.o. on Days 1-5 at a dose 0.125 mmol/kg/day, and the results are summarized in Table I. 5'-Phosphatidyl-DMDC **3** had an excellent antileukemic effect (ILS 64%) which was comparable to that of DMDC (ILS 68%). The corresponding 5-fluoro derivative **4** increased the life-span of mice even more (ILS 96%).

We next examined the antitumor effect with M5076 mouse reticulum cell sarcoma implanted i.p., by the i.p.-administration of these compounds on Days 1-5 in mice. M5076 cells are known to be sensitive to alkylating agents and nitrosoureas, but apparently are unresponsive to antimetabolites such as methotrexate and 5-fluorouracil.[8] Both of 5'-phosphatidyl derivatives **3** and **4** had remarkable antitumor activities against this tumor. Note that with **3** at a dose 0.40 mmol/kg/day, all of the mice survived for 60 days. Three of the four mice suvived for 60 days with compound **4** at a dose of 0.125 mmol/kg/day, while it was toxic (ILS < 0%) at 0.40 mmol/kg/day. On the other hand, none of the mice survived for 60 days with either dose of DMDC. This result clearly demonstrates that the antitumor effects of the nucleosides can be improved by converting them to their corresponding 5'-phosphatidyl derivatives.

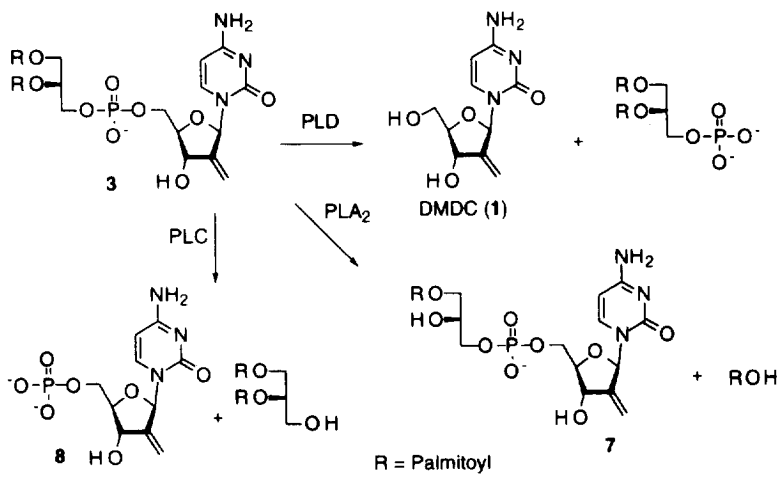
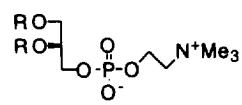


Figure I. Possible Pathways for Metabolism of 5'-Phosphatidyl-DMDC (**3**) by Phospholipases.



Natural phospholipids are metabolized by phospholipase A_2, C, and D (PLA_2, PLC, and PLD, respectively). Thus, the antitumor effects of phospholipid derivatives could depend on their metabolism by phospholipases which recognize them as substrates. The possible metabolic pathways of **3** for regiospecific hydrolysis by phospholipases are shown in Figure I. We investigated the susceptibility of **3** to phospholipases, together with a natural phospholipid, dioleoyl-phosphatidylcholine (DOPC, **6**), as a control. PLA_2 from bovine pancreas, PLC from *Bacillus cereus*, and

PLD from *Streptomyces chromofuscus*[9] were used in this study,[10] and the time courses of the reactions monitored by HPLC are shown in Figure II.

First, the susceptibility to PLA_2 was tested. It is very important to know whether or not 5'-phosphatidyl nucleosides can be recognized as a substrate by PLA_2, especially when these compounds are administered p.o. because phospholipids are absorbed from the intestinal tract only after being digested by PLA_2 in pancreatic juice. When the compounds (2.0 μmol) were incubated with PLA_2 (0.1 unit) at 37 °C,[11] 5'-phosphatidyl-DMDC **3** was rapidly hydrolyzed by the enzyme to give the corresponding lyso-derivative **7**,[12] as shown in Figure IIA. Note that its $t_{1/2}$ (15 min) was shorter than that of the natural substrate **6** (20 min), which suggests the efficient p.o.-absorption of **3**. This supports our previous result that 5'-phosphatidyl nucleosides can be absorbed from the intestinal tract via deacylation by PLA_2 and subsequent reacylation by acyltransferase, which is the specific absorption mechanism for natural phospholipids.[3d]

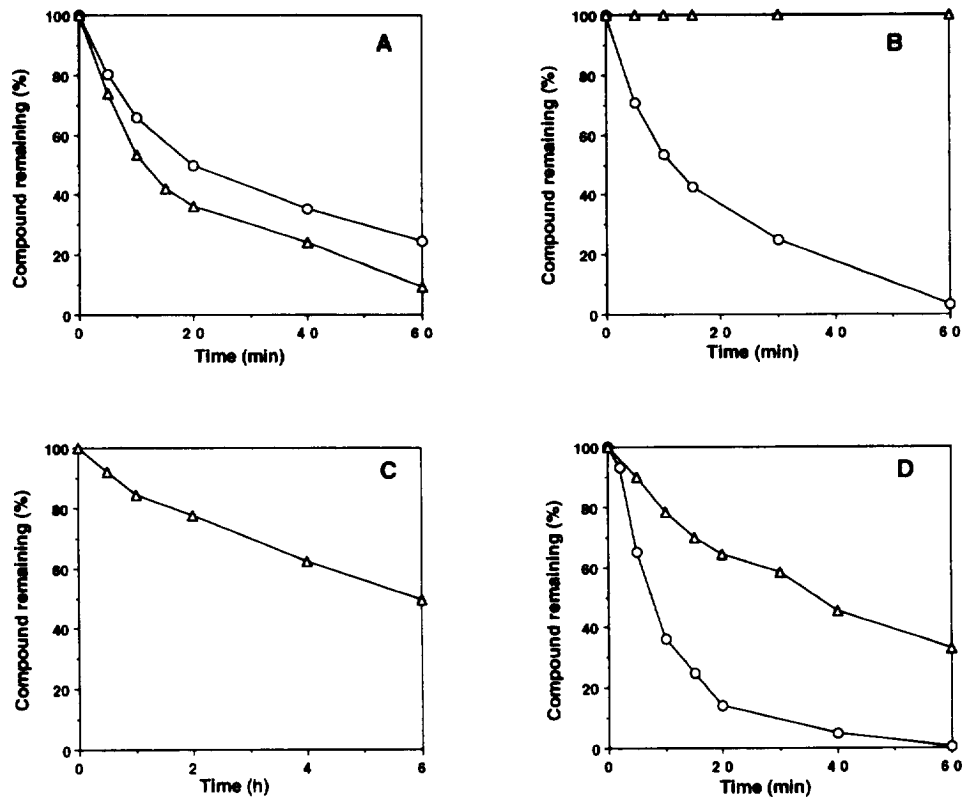


Figure II. Hydrolysis of **3** and DOPC by PLA_2 (A), PLC (0.1 units, B), PLC (5.0 units, C), and PLC (D): (Δ) **3**, (○) DOPC.

Next, the compounds were treated with PLC.[13] DMDC is believed to show antitumor activity after metabolic conversion by deoxycytidine kinase to its 5'-phosphate derivatives in tumor cells. If 5'-phosphatidyl-DMDC is recognized as a substrate for hydrolysis by PLC, DMDC 5'-monophosphate can be produced, and it should be able to overcome resistance to DMDC, since tumors cells resistant to DMDC are deficient in deoxycytidine kinase activity.[14] The compounds (2.0 μmol) were incubated with *Bacillus* PLC at 37 °C. In the presence of 0.1 units of PLC, hydrolysis of **3** was not observed (Figure IIB). DOPC was hydrolyzed effectively under these conditions ($t_{1/2}$ = 12 min). However, when 5.0 units of the enzyme was used, hydrolysis of **3** was detected (Figure IIC).

On the other hand, when 5'-phosphatidyl-DMDC **3** was treated with *Streptomyces* PLD,[15] it was effectively hydrolyzed to produce DMDC, as shown in Figure IID,[16] although the reaction rate was somewhat slower than that of DOPC.

These results for the enzymatic hydrolysis of **3** may suggest its possible metabolic pathway: **3** may be hydrolyzed by PLA_2 and/or PLD to give the lyso-derivative **7** and/or DMDC, while hydrolysis by PLC may be very slow.[14]

In conclusion, we enzymatically synthesized 5'-phosphatidyl derivatives of DMDC and 5-F-DMDC which had more significant antitumor effects than the parent compounds in mice. These results, together with our previous results,[4b,e,f] suggest that the derivatization of antitumor nucleosides to the corresponding 5'-phosphatidyl analogues would be an efficient method for improving the antitumor effects of the compounds. Although a preliminary investigation of the activation pathway of 5'-phosphatidyl nucleosides by phospholipases was performed in this study, further studies on the mode of action are needed.

Acknowledgment

We thank to Dr. H. Misaki of Asahi Chemical Industries and Dr. H. Nanba of Nihon Fine Chemical Co., Ltd. for their gifts of phospholipases and phosphatidylcholines, respectively.

References and Notes

1. Part 154: Obara, T.; Shuto, S.; Saito, Y.; Snoeck, R.; Andrei, G.; Balzarini, J.; De Clercq, E.; Matsuda, A. *J. Med. Chem.* in press.
2. a) Shuto, S.; Ueda, S.; Imamura, S.; Fukukawa, K.; Ueda, T. *Chem. Pharm. Bull.* **1988,** *36,* 5020-5023. b) Shuto, S.; Itoh, H.; Endo, E.; Fukukawa, K.; Tsujino, M.; Matsuda, A.; Ueda, T. *Chem. Pharm. Bull.* **1987**, *35*, 3523-3526. c) Turcotte, J. G.; Srivastava, S. P.; Meresak, W. A.; Rizkalla, B. A.; Louzon, F.; Wunz, T. P. *Biochim. Biophys. Acta* **1980**, *619*, 604-618. d) Hong, C. I.; An, S.-H.; Buchheit, D. J.; Nechaev, A.; Kirisits, A. J.; West, C. R.; Berdel, W. E. *J. Med. Chem.* **1986**, *29*, 2038-2044. e) Herrmann, R.; Berdel, W. E. *Cancer Res.* **1992**, *52*, 1865-1867. f) Neumann, J. -M.; Herve, M.; Debouzy, J. -C; Iglesias, G. F.; Gouyette, C.; Dupraz, B.; Huynh-Dinh, T. *J. Am. Chem. Soc.* **1989**, *111*, 4270-4377. g) Hostetler, K. Y.; Parker, S.; Sridhar, C. N.; Martin, M. J.; Li. J. -L.; Stuhmiller, L. M. van Wijk, G. M. T.; van den Bosch, H.; Gardner, M. F.; Aldern, K. A.; Richman, D. D. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11835-11839.
3. a) Shuto, S.; Ueda, S.; Imamura, S.; Fukukawa, K.; Tsujino, M.; Matsuda, A.; Ueda, T. *Tetrahedron Lett.* **1987**, *28*, 199-202. b) Shuto, S.; Itoh, H.; Ueda, S.; Imamura, S.; Fukukawa, K.; Tsujino, M.; Matsuda, A.; Ueda, T. *Chem. Pharm. Bull.* **1988**, *36*, 209-217. c) Shuto, S.; Itoh, H.; Obara, T.; Nakagami, K.; Yaso, M.; Yaginuma, S.; Tsujino, M.; Saito, T.; Matsuda, A.; Ueda, T. *Nucleosides*

Nucleotides **1992**, *11*, 437-446. d) Sakai, A.; Mori, N.; Shuto, S.; Suzuki, T. *J. Pharm. Sci.* **1993**, *82*, 575-578. e) Doi, K.; Oku, N.; Toyota, T.; Shuto, S.; Sakai, A.; Itoh, H.; Okada, S. *Biol. Pharm. Bull.* **1994**, *17*, 1414-1416. f) Shuto, S.; Itoh, H.; Sakai, A.; Nakagami, K.; Imamura, S.; Matsuda, A. *BioMed. Chem.* **1995**, *3*, 235-243. g) Shuto, S.; Awano, H.; Shimazaki, N.; Hanaoka, K.; Matsuda, A. *BioMed. Chem. Lett.* **1996**, *6*, 1021-1024.

4. a) Takenuki, K.; Matsuda, A.; Ueda, T.; Sasaki, T.; Fujii, A.; Yamagami, K. *J. Med. Chem.*, **1988**, *31*, 1063-1064. b) Matsuda, A.; Takenuki, K.; Tanaka, M.; Sasaki, T.; Ueda, T. *J. Med. Chem.*, **1991**, *34*, 812-819. c) Yamagami, K.; Fujii, A.; Arita, M.; Okumoto, T.; Sakata, S.; Matsuda, A.; Ueda, T. *Cancer Res.* **1991**, *51*, 2319-2323.
5. In a typical procedure, $CHCl_3$ solution (20 mL) of DPPC (**5**, 0.6 mmol) was added to a solution of PLDP (7 mg, 1220 units) and either DMDC or 5-F-DMDC (0.5 mmol) in sodium acetate buffer (200 mM, pH 4.5, 4 mL). The mixture was stirred at 40 °C for 6 h, and a mixture of H_2O (5 mL), MeOH (20 mL), and $CHCl_3$ (20 mL) was then added, and the mixture was shaken. The separated organic layer was washed with H_2O and evaporated to dryness. The residue was purified by flash chromatography (silica gel, $CHCl_3$/MeOH, 3:1) to give a phosphatidyl nucleoside as a white powder, which was treated with Diaion WK-20 resin (2 x 8 cm, Na^+ form) to give **3** or **4** as a sodium salt.
6. Compound **3**: yield 52%; mp >179 °C (decomp.); FAB-MS *m/z* 892 (MH^+); UV λmax 271 nm (MeOH); ^1H NMR ($CDCl_3/CD_3OD$, 3:1, 500 MHz) δ 7.82 (d, 1 H, J = 7.7 Hz), 6.67 (s, 1 H), 6.00 (d, 1 H, J = 7.3 Hz), 5.52 (br s, 1 H), 5.47 (br s, 1 H), 5.38-5.21 (m, 1 H), 4.79-4.76 (m, 1 H), 4.41 (dd, 1 H, J = 12.1, 3.3 Hz), 4.21-4.16 (m, 3 H), 4.02-3.95 (m, 2 H), 3.71-3.63 (m, 1 H), 2.32 (t, 2 H, J = 7.7 Hz), 2.31 (t, 2 H, J = 7.7 Hz), 1.43-1.60 (m, 4 H), 1.26 (m, 48 H), 0.88 (t, 6 H, J = 6.6 Hz). Anal. Calcd for $C_{45}H_{79}N_3O_{11}PNa \cdot 2/3H_2O$: C, 58.80; H, 8.99; N, 4.57. Found: C, 58.82; H, 8.94; N, 4.59.
7. Compound **4**: yield 49%; mp >178 °C (decomp.); FAB-MS *m/z* 910 (MH^+); UV λmax 281 nm (MeOH); ^1H NMR ($CDCl_3/CD_3OD$, 3:1, 500 MHz) δ 7.73 (d, 1 H, J = 6.2 Hz), 6.65 (s, 1 H), 5.53 (br s, 1 H), 5.47 (br s, 1 H), 5.38-5.21 (m, 1 H), 4.73 (m, 1 H), 4.46-4.42 (m, 1 H), 4.21-4.16 (m, 3 H), 4.02-3.98 (m, 2 H), 3.85 (m, 1 H), 2.36-2.28 (m, 4 H) 1.60 (m, 4 H), 1.43-1.27 (m, 48 H), 0.88 (t, 6 H, J = 6.6 Hz). Anal. Calcd for $C_{45}H_{78}FN_3O_{11}PNa \cdot 1/2H_2O$: C, 58.81; H, 8.66; N, 4.57. Found: C, 58.72; H, 8.64; N, 4.40.
8. Langdon, S. P.; Gescher, A.; Hickman, J. A.; Stevens, M. F. G. *Eur. J. Cancer Clin. Oncol.* **1984**, *20*, 699-705.
9. PLC and PLD from mammalian sources were unavailable.
10. Phospholipase D from *Streptomyces* sp. AA 586 (PLDP), PLD from *Streptomyces chromofusxus*, and PLC from *Bacillus cereus* are products of Asahi Chemical Industries. PLA_2 was purchased from Sigma.
11. The reaction mixture consisted of the substrate (20 mM/aq. 2% sodium deoxycholate, 100 μL), PLA_2 (2.0 units/mL H_2O, 200 μL), $CaCl_2$ (100 mM, 100 μL), 3,3-dimethylglutarate-NaOH buffer (pH 7.0, 200 mM, 200 μL), and H_2O (400 μL).
12. Compound **7** was isolated and its structure was confirmed as follows: FAB-MS *m/z* 654 (MH^+); UV λmax 271 nm (MeOH); ^1H NMR ($CDCl_3/CD_3OD$, 3:1, 500 MHz) δ 7.69 (d, 1 H, J = 7.5 Hz), 6.70 (s, 1 H), 5.93 (d, 1 H, J = 7.4 Hz), 5.51 (br s, 1 H), 5.45 (br s, 1 H), 4.77 (d, 1 H, J = 7.1 Hz), 4.17-4.11 (m, 4 H), 3.97-3.83 (m, 3 H), 3.36-2.33 (m, 2 H), 1.61 (m, 2 H), 1.37-1.17 (m, 24 H), 0.89 (t, 3 H, J = 6.4 Hz). Anal. Calcd for $C_{29}H_{49}N_3O_{10}PNa.1/4H_2O$: C, 52.92; H, 7.58; N, 6.38. Found: C, 52.84; H, 7.53; N, 6.16.
13. The reaction mixture consisted of the substrate (10 mM/aq. 2% sodium deoxycholate, 100 μL), PLC (2.0 or 50 units/mL H_2O, 200 μL), $CaCl_2$ (100 mM, 100 μL), 3,3-dimethylglutarate-NaOH buffer (pH 7.0, 200 mM, 200 μL), and H_2O (500 μL).
14. In a preliminary experiment, **3** was inactive against DMDC-resistant P388 leukemia, which is deficient in deoxycytidine kinase activity, in mice (Yamagami, K. *et al.* unpublished results). This result suggests the inefficient intracellular release of DMDC 5'-phosphate by PLC.
15. The reaction mixture consisted of the substrate (20 mM/aq. 5% Triton X-100, 100 μL), PLD (2.0 units/mL H_2O, 200 μL), $CaCl_2$ (100 mM, 100 μL), Tris-HCl buffer (pH 7.0, 200 mM, 200 μL), and H_2O (500 μL).
16. A time-dependent increase in DMDC was detected by HPLC.

(*Received in Japan* 25 *June* 1996; *accepted* 12 *August* 1996)