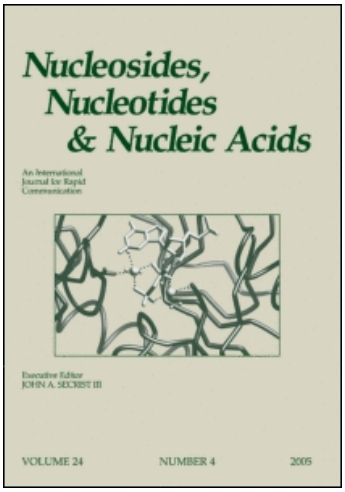

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New Neplanocin Analogues II. Enzymatic One-Step Synthesis and Antitumor Activity of 6'-(3-sn-Phosphatidyl)Neplanocin a Derivatives

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NEW NEPLANOCIN ANALOGUES II. ENZYMATIC ONE-STEP SYNTHESIS AND ANTITUMOR ACTIVITY OF 6'-(3-*sn*-PHOSPHATIDYL)NEPLANOCIN A DERIVATIVES†,[1]

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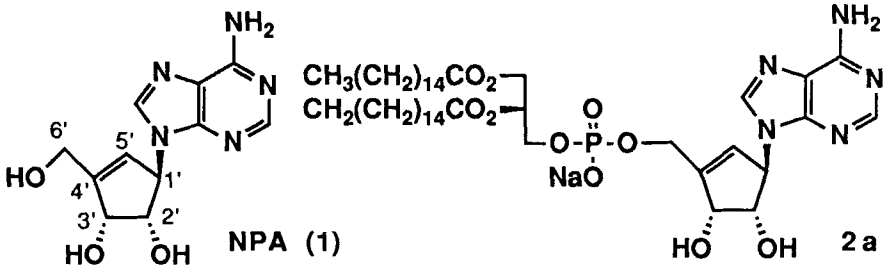
ABSTRACT: A novel series of neplanocin analogues, 6'-(3-*sn*-phosphatidyl)neplanocin As bearing a variety of fatty acyl or alkyl residues in the glyceride moiety (**2b-2h**), were synthesized by means of phospholipase D-catalyzed transphosphatidylation. Among them, **2b, 2c**, and **2e** each exhibited significant antitumor effect against P388 leukemia in mice, which evidently surpassed that of parent compound neplanocin A.

INTRODUCTION

Neplanocin A (NPA, **1**), a carbocyclic adenine nucleoside, was originally isolated from the culture filtrate of the soil fungus *Ampullariella regularis* in our laboratory.[2] This compound has been noticed because of the important biological property and the unique chemical structure in which the ribose of adenosine is replaced by the hydroxylated cyclopentene ring.



† This paper is dedicated to the memory of the Professor Tohru Ueda.

Although NPA exhibited significant antitumor effects against leukemia (L1210 or P388)[2d,4d] and ascites tumor (Sarcoma-180 or Ehrlich carcinoma)[5] in mice, this compound was almost inactive against mouse solid tumor (Meth A fibrosarcoma or Lewis lung carcinoma).[5] This result may suggest that NPA may not be a sufficient drug for the clinical treatment of cancer. However, NPA would be the compound of vital importance as a prototype for designing novel antitumor agents because of its characteristic biological property and structure.

In the biological mode of action of NPA as an antitumor agent, significant suggestions have been disclosed: NPA is rapidly converted into an inactive inosine congener by adenosine deaminase *in vivo*,[2d] its antitumor effects would be mediated through its phosphorylation by adenosine kinase.[6,7]

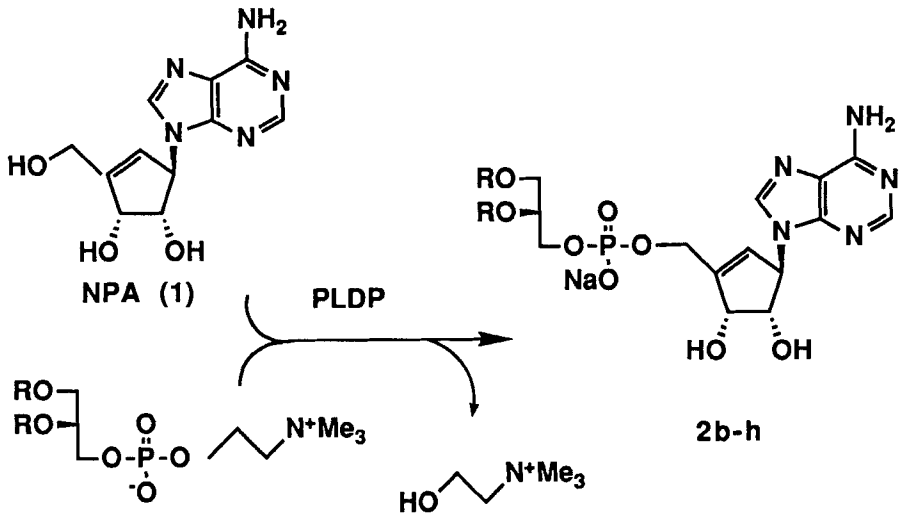
Recently we reported that the 5'-(3-*sn*-phosphatidyl) derivatives of antitumor nucleoside analogues (*e.g.*, 5-fluorouridine, arabinosyl-5-fluorocytosine), which are glycerophospholipids having a nucleoside as a polar head group, possessed improved antitumor effects compared with the corresponding parent nucleoside analogues.[4a] Compound **2a**, a 6'-phosphatidyl derivative of NPA[8] bearing palmitoyl groups in the glyceride moiety, also surpassed NPA in the antileukemic potency in mice.[4a]

6'-(3-*sn*-Phosphatidyl)NPA contain a phosphatidyl residue as a nontoxic carrier moiety, which have potential advantages as antitumor agents: being probably protected from inactivation by adenosine deaminase, the potential ability to penetrate into cells due to the high affinity for cell membranes, the prospect of improved pharmacokinetics, and the possibility to be activated intracellularly by enzymes (*e.g.*, phospholipases, phosphodiesterase, or lysophospholipase).

In an effort to develop more efficient antitumor agents, we have synthesized various 6'-(3-*sn*-phosphatidyl)NPAs and evaluated them in P388 leukemia system in mice. Then, compounds showed significant activity against P388 leukemia, including **2a**,[9] have been evaluated for the antitumor activity against s.c.-implanted Meth A fibrosarcoma in mice.

CHEMISTRY

In the last decade, several chemical syntheses of 5'-(3-*sn*-phosphatidyl)nucleosides have been reported because of their biological importance.[10] However, these methods have not been general, or been complicated. This would be due to the multi-functional unique structures of



SCHEME 1

them, in which a polar 5'-nucleotide group and a lipophilic diacyl glycerol group (or a nucleoside group and a phosphatidic acid group) coexist.

Recently we have developed a novel method for preparing 5'-(3-*sn*-phosphatidyl)nucleosides from phosphatidylcholines (PC) and nucleosides in one-step reaction, in which phospholipase D-catalyzed transphosphatidylation, namely, the regiospecific transfer reaction of the phosphatidyl residue from 3-*sn*-phosphatidylcholine to the 5'-hydroxyl group of nucleoside, was utilized.[4] This could be applied to the synthesis of phosphatidyl derivatives of unusual nucleoside analogues including NPA, while the chemical phosphorylation of the 6'-hydroxyl group of NPA was often troublesome because of its unusual cyclopentenyl structure.[11] Therefore we have planed to synthesize a variety of 6'-(3-*sn*-phosphatidyl)NPAs by this enzymatic method.

In the presence of NPA (10 eq to phosphatidylcholine) "diacyl-type" phosphatidylcholines (**3b-3e**)[12] were treated with phospholipase D from

TABLE 1. Yields and Physical Data for 6'-(3-*sn*-Phosphatidyl)NPAs

Compound	Yield[a]	UV λ_{max} (nm)[b]	FAB-MS (m/z)	Formula[c]
2b	64	260	882 (MNa_2-H)	$C_{42}H_{71}N_5O_{10}PNa \cdot 1/2H_2O$
2c	87	260	972 (MNa)	$C_{50}H_{87}N_5O_{10}PNa \cdot 1/2H_2O$
2d	59	260	1006 (MH), 1028 (MNa)	$C_{54}H_{95}N_5O_{10}PNa \cdot H_2O$
2e	53	260	968 (MH)	$C_{50}H_{83}N_5O_{10}PNa \cdot 2/3H_2O$
2f	41	260	866 (MH),888 (MNa)	$C_{46}H_{83}N_5O_8PNa \cdot 2H_2O$
2g	67	260	922 (MH), 944 (MNa)	$C_{50}H_{87}N_5O_8PNa \cdot 2H_2O$
2h	49	260	962 (MNa_2-H),	$C_{50}H_{91}N_5O_8PNa \cdot 3/2H_2O$

[a]Yields were based on phosphatidylcholine used. [b]Measured in MeOH. [c]Compounds were analyzed for C, H, and N and were within ±0.4% of the theoretical value.

TABLE 2. Antileukemic Activities of 6'-(3-*sn*-Phosphatidyl)NPAs and Related Compounds against i.p.-Implanted P388 Leukemia in Mice.

Dose[a]	ILS (%)[b]									
(mg/kg/day)	**2a**[c]	**2b**	**2c**	**2d**	**2e**	**2f**	**2g**	**2h**	**4**	NPA
3	57	69	21	22	48	6	19	15	2	56
10	73	149	50	31	102	12	16	17	17	68
30	180	102	128	41	51	35	31	26	26	toxic

[a]Administered i.p., day 1-5. [b]Percent increase in life span. The control mice died in 7-10 d. [c]Data taken from S. Shuto *et al.*, (ref. 4a)

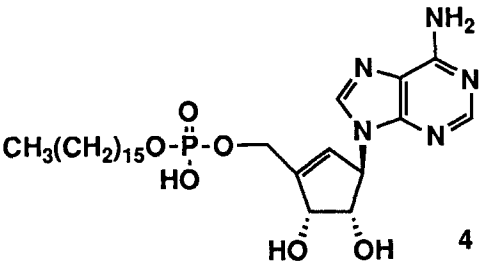
Streptomyces sp. AA 586 (PLDP)[13] in a two-phase system of chloroform and acetate buffer. After usual purification and the treatment with cation-exchange resin, the desired 6'-(3-*sn*-phosphatidyl)NPAs (**2b-2e**) were obtained as sodium salts. Unreacted NPA was readily recovered from the aqueous phase of the reaction mixture.

"Dialkyl-type" derivatives (**2f-2h**) were also prepared in the similar way with the corresponding "dialkyl-type" phosphatidylcholines (**3f-3h**)[14] as phosphatidyl donors, however in these cases, much more PLDP was required to allow the reaction to proceed than in the reaction with "diacyl-type" phosphatidyl cholines as donors. The catalytic efficiency of PLDP may be changed by the phosphatidyl donors used in the reaction.

ANTITUMOR ACTIVITY

Antileukemic activities of i.p.-administered 6'-(3-*sn*-phosphatidyl)NPAs (**2**) and NPA (**1**) against i.p.-implanted P388 leukemia in mice are summarized in Table 2. Newly synthesized "diacyl-type" 6'-phosphatidyl derivatives **2b, 2c**, and **2e** showed notable ILS values (> 100%) at their optimum doses, which are analogous to the result on **2a** reported previously.[4a] These effects were clearly superior to that obtained with NPA, the parent compound. On the other hand, "diacyl-type" derivative **2d**, having eicosanoyl groups in the glyceride moiety, and all of "dialkyl-type" derivatives **2f, 2g,** and **2h** evaluated, possessed only moderate effects upon life spans (ILS_{max} Ca. 30 %).

We also evaluated NPA 6'-cetylphosphate (**4**),[11] a simple lipophilic analogue of NPA 6'-phosphate, to compare its antitumor effect with those of 6'-phosphatidyl derivatives **2**. However, the cetylphosphate **4** exhibited only an insignificant effect in this system. This result suggests the importance of diacylglycerol moiety, the essential component of natural glycerophospholipids, as the carrier moiety.



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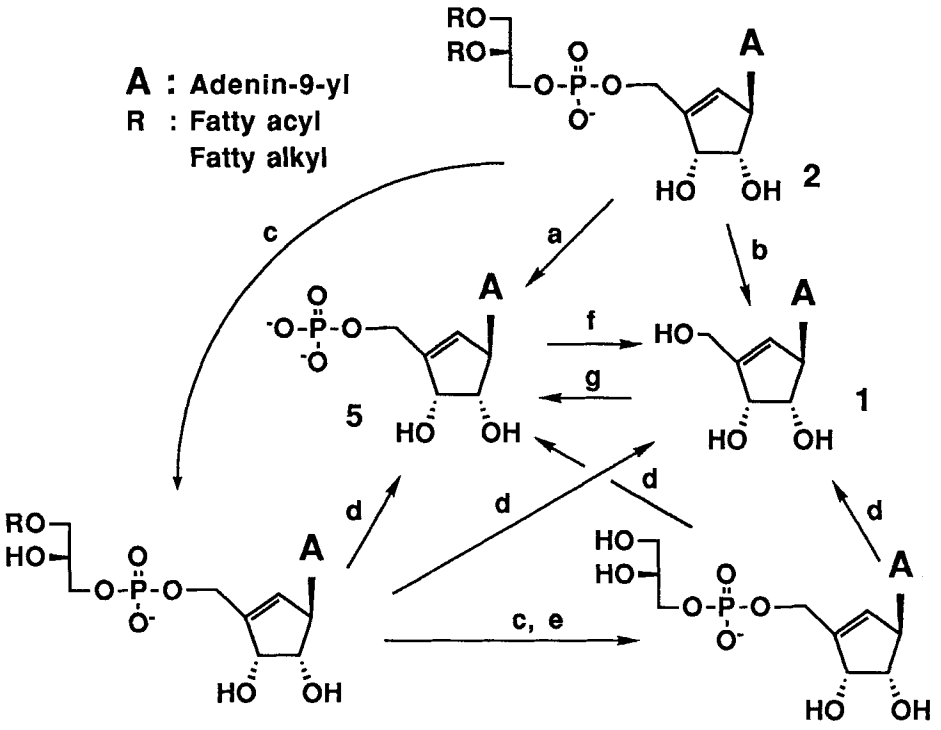
Antitumor activity of **2a**,[9] **2c, 2e,** and NPA (**1**) against solid tumor was investigated with s.c-implanted Meth A fibrosarcoma in mice, and the result is shown in Table 3. Compounds **2c** and **2e**, administered i.p., exhibited

TABLE 3. Antitumor Activities of 6'-(3-*sn*-Phosphatidyl)NPAs and NPA against s.c.-Implanted Meth A Fibrosarcoma in Mice.

Dose[a] (mg/kg/day)	T/C (%)[b] 2a	2c	2e	NPA
3	66	71	93	98
10	59	60	75	toxic
30	39[c]	55	54	not tested

[a]Administered i.p., day 1-5. [b]Antitumor activity was evaluated from the average tumor volume of treated mice (T) over that of control mice (C). [c]Significantly different from the control at $p<0.01$.



a) phospholipase C b) phospholipase D c) phospholipase A
d) phosphodiesterase e) lysophospholipase f) phosphatase, 5'-nucleotidase
g) adenosine kinase

SCHEME 2. Conceibable Activation Pathways for 6'-(3-*sn*-Phosphatidyl)NPA by cellular enzymes.

TABLE 4. ^{1}H-NMR Data for 6'-(3-*sn*-Phosphatidyl)NPAs.[a]

Compound	^{1}H-NMR data (δ)
2b[b]	8.24, 7.97 (each s, each 1H, H-2 and H-8), 6.01 (d, 1H, H-5', J=Ca. 0 Hz), 5.51 (m, 1H, H-1'), 5.25 (m, 1H, glycerol CH), 4.78-4.56 (m, 3H, H-3' and H-6'), 4.50-4.27 (m, 3H, H-2' and glycerol CH_2OCO), 4.00 (m, 2H, glycerol CH_2OPO), 2.30 (m, 4H, $COCH_2$), 1.7-1.2 (m, Myristoyl CH_2), 0.88 (t, 3H, CH_3).
2c[c]	8.26, 7.98 (each s, each 1H, H-2 and H-8), 6.01 (d, 1H, H-5', J=Ca. 0 Hz), 5.49 (m, 1H, H-1'), 5.26 (m, 1H, glycerol CH), 4.74 (d, 1H, H-3', J=5.4 Hz), 4.62 (m, 2H, H-6'), 4.50-4.18 (m, 3H, H-2' and glycerol CH_2OCO), 4.02 (t, 2H, glycerol CH_2OPO), 2.32 (m, 4H, $COCH_2$), 1.7-1.2 (m, stearoyl CH_2), 0.88 (t, 3H, CH_3).
2d[b]	8.23, 7.92 (each s, each 1H, H-2 and H-8), 6.00 (d, 1H, H-5', J=Ca. 0 Hz), 5.50 (m, 1H, H-1'), 5.24 (m, 1H, glycerol CH), 4.78-4.55 (m, 3H, H-3' and H-6'), 4.50-4.22 (m, 3H, H-2' and glycerol CH_2OCO), 4.00 (m, 2H, glycerol CH_2OPO), 2.32 (m, 4H, $COCH_2$), 1.7-1.2 (m, eicosanoyl CH_2), 0.88 (t, 3H, CH_3).
2e[c]	8.25, 7.96 (each s, each 1H, H-2 and H-8), 6.01 (bs, 1H, H-5'), 5.48 (m, 1H, H-1'), 5.35 (m, 4H, oleoyl CH=), 5.25 (m, 1H, glycerol CH), 4.74 (d, 1H, H-3', J=5.9 Hz), 4.62 (m, 2H, H-6'), 4.50-4.18 (m, 3H, H-2' and glycerol CH_2OCO), 4.03 (t, 2H, glycerol CH_2OPO), 2.31 (m, 4H, $COCH_2$), 2.01 (m, 8H, oleoyl **CH_2**CH=), 1.7-1.2 (m, oleoyl CH_2), 0.88 (t, 3H, CH_3).
2f[c]	8.26, 7.95 (each s, each 1H, H-2 and H-8), 5.99 (bs, 1H, H-5'), 5.48 (m, 1H, H-1'), 4.76 (d, 1H, H-3', J=5.9Hz), 4.63 (m, 2H, H-6'), 4.26 (dd, 1H, H-2', J=5.9, 4.4 Hz), 3.92 (m, 2H, glycerol CH_2OP), 3.64-3.43 (m, 7H, glycerol **CH(OCH_2-)CH_2OCH_2-**) 1.5-1.2 (m, cetyl CH_2), 0.88 (t, 3H, CH_3).
2g[c]	8.26, 7.97 (each s, each 1H, H-2 and H-8), 5.98 (d, 1H, H-5', J=1.5 Hz), 5.48 (m, 1H, H-1'), 4.76 (d, 1H, H-3', J=5.9Hz), 4.64 (m, 2H, H-6'), 4.26 (dd, 1H, H-2', J=5.9, 4.4 Hz), 3.94 (m, 2H, glycerol CH_2OP), 3.65-3.43 (m, 7H, glycerol **CH(OCH_2-)CH_2OCH_2-**) 1.5-1.2 (m, stearyl CH_2), 0.88 (t, 3H, CH_3).
2h[c]	8.23, 7.92 (each s, each 1H, H-2 and H-8), 5.97 (d, 1H, H-5', J=1.5 Hz), 5.49 (m, 1H, H-1'), 5.34 (m, 4H, oleyl CH=), 4.77 (d, 1H, H-3', J=5.4Hz), 4.62 (m, 2H, H-6'), 4.27 (dd, 1H, H-2', J=5.4, 1.5 Hz), 3.93 (m, 2H, glycerol CH_2OP), 3.64-3.42 (m, 7H, glycerol **CH(OCH_2-)CH_2OCH_2-**) 2.00 (m, 8H, oleyl **CH_2**CH=), 1.5-1.2 (m, cetyl CH_2), 0.88 (t, 3H, CH_3).

[a]Measured in $CDCl_3$:CD_3OD (3:1). [b]100 MHz. [c]400 MHz.

moderate effects in this tumor system. It is notable that i.p.-treatment with **2a** at the optimum dose of 30 mg/kg/day for 5 successive days, inhibited the growth of the solid tumor evidently (T/C 39%, significantly different from the control at $p<0.01$), with a slight weight loss in mice, in spite of the wholly ineffectiveness of NPA against the solid tumor.

The significant antitumor effect of **2** may be explained, at least in part, by unique intracellular release of NPA (**1**) or NPA 6'-phosphate (**5**) by cellular enzymes as shown in Scheme 2. The release of the latter **5** would be preferred since it would bypass the phosphorylation by adenosine kinase; the deficient activity of the enzyme in some NPA resistant cell lines have been known.[6] "Dialkyl-type" compounds (**2f, 2g,** and **2h**) were designed as phosphatidyl NPAs to be resistant to the hydrolysis by phospholipase A. The inferior activity of "dialkyl-type" derivatives to "diacyl-type"derivatives suggests that intracellular activation of **2** may be mediated by phospholipase A and phosphodiesterase mainly, not by phospholipase C or D. It also may be presumed that "diacyl-type" 6'-phosphatidyl-NPAs are better substrates for phospholipase C or D than corresponding "dialkyl-type" derivatives *in vivo*.

In conclusion, the derivatization of NPA to the corresponding 6'-phosphatidyl analogues resulted in a marked increase in antitumor effect of the compound, and it may also develop the antitumor activity spectrum.

EXPERIMENTAL

Melting points were determined on a Yanagimoto MP-3 micro-melting point apparatus and are uncorrected. The NMR spectra were recorded with a JEOL FX-100 or GSX-400 spectrometer with tetramethylsilane as an internal standard. Mass spectra were measured on a JEOL JMS-D300 spectrometer. Thin-layer chromatography was carried out on Merck precoated plate $60F_{254}$. Flash chromatography was conducted with Merck silica gel 9385. Neplanocin A and PLDP were prepared by Toyo Jozo Co.

General Procedure for the Synthesis of 5'-(3-*sn*-Phosphatidyl)NPA (2b-2h). PLDP (10 mg, 1860 units, for **2b, 2c, 2d,** and **2e**; 30 mg, 5580 units, for **2f, 2g,** and **2h**) and NPA (**1**, 1.32 g, 5.0 mmol) were dissolved in sodium acetate buffer (200 mM, pH5.7, 8 mL) containing $CaCl_2$ (250 mM), to which a $CHCl_3$ solution (20 mL) of 3-*sn*-phosphatidylcholine (**3**, 1.0 mmol) was added. The mixture was stirred at 45 °C for 6 h, then 2 N HCl (5 mL), MeOH (20 mL) and $CHCl_3$ (20 mL) were

added and the whole was shaken. The aqueous layer was concentrated to give a white precipitate of unreacted NPA (0.72-0.97 g). The organic layer was washed twice with water (10 mL) and evaporated to dryness. The residue was purified by flash chromatography (silica gel, $CHCl_3$/MeOH, 10:1, followed by 3:1), and fractions containing the desired product were evaporated. The residue was dissolved in a mixture of 2 N HCl (5 mL), MeOH (10 mL), and $CHCl_3$ (20 mL), and the resulting mixture was partitioned. The organic layer was washed twice with water (5 mL) and evaporated. The residue was dissolved in a mixture of $CHCl_3$:MeOH:water (10:5:1, 15 mL), and the solution applied to a column of Diaion WK-20 resin (2 x 8 cm, Na^+ form). The column was developed with the same solvent, and the eluate was concentrated to afford a white precipitate of **2** as the sodium salt.

Antitumor Assays in Mice. P 388 leukemia: The antileukemic assay was carried out as previously described.[4a] Meth A fibrosarcoma: Meth A fibrosarcoma cells were maintained by serial transplantation in BALB/c male mice (purchased from Charles River Japan, Inc.). Meth A fibrosarcoma cells (1 x 10^6) were inoculated subcutaneously into BALB/c male mice (average wight, 22 g). After 24 h, mice were randomized into groups of five and housed in shoebox cages. For administration to animals, compounds were sonicated in Tris buffered saline, and the preparations were administrated i.p. on day 1 through 5 (i.e., treatment was initiated 24 h after tumor inoculation). On day 14, the major axis and minor axis of the tumor were measured, and the volume was calculated according to the following equation; tumor volume (mm^3) = [minor axis (mm)]^2 x major axis (mm)/2.[15]

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