Synthesis and Biological Evaluation of Radioiodinated Phospholipid Ether Stereoisomers

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Radioiodinated phospholipid ethers have shown the remarkable ability to selectively accumulate in a variety of animal tumors as well as in human tumor xenografts. It has been suggested that this tumor avidity may arise as a consequence of metabolic differences between tumor and corresponding normal tissue. One such compound, 1-*O*-[12-(*m*-iodophenyl)dodecyl]-2-*O*-methyl-*rac*-glycero-3-phosphocholine (NM-294), contains a chiral center at the *sn*-2 position. The unnatural *S*- and natural *R*-enantiomers (**4** and **5**, respectively) of NM-294 were synthesized in order to provide further information on the mechanism(s) responsible for the tumor avidity of phospholipid ethers. *In vitro* cytotoxicity studies demonstrated a lack of stereospecificity. Biodistribution studies in rats bearing the Walker 256 tumor demonstrated the *S*- and *R*-isomers to have similar tissue uptake at 24 and 48 h after administration. Tumor-to-blood ratios at 24 h were 11.1 and 11.0 for the *S*- and *R*-isomers, respectively. In addition, γ-camera scintigrams of tumor-bearing rats at various time points after iv administration of the *S*- and *R*-isomers did not show any qualitative differences in the distribution of radioactivity. Prior studies have shown that *rac*-NM-294 was not a substrate for phosphatidylcholine specific phospholipase C, but was a substrate for two forms of phospholipase D (PLD). Therefore, metabolism studies with **4** and **5** with various forms of PLD were performed. PLD from cabbage demonstrated a degree of stereoselectivity. In the presence of 1% ethanol, the *R*-isomer was metabolized to the greatest extent, followed by *rac*-NM-294 and the *S*-isomer. PLD isolated from *Streptomyces chromofuscus* failed to demonstrate any stereoselectivity. The results suggest that the mechanism(s) of retention of these compounds in tumors may not involve a highly stereoselective component.

Introduction

In the late 1960's, Snyder and co-workers[1,2] performed a series of experiments designed to evaluate the lipid composition of normal and neoplastic tissues. It was discovered that both animal and human tumor tissue contained much larger quantities of naturally-occurring ether lipids relative to corresponding normal tissues. When following studies demonstrated that tumor tissue had less than normal amounts of the enzyme *O*-alkylglycerol monooxygenase (E.C. 1.14.16.5, AGMO),[3] it was proposed that differences in the tissue concentration of this ether cleavage enzyme were responsible for the accumulation of ether lipids in tumors. More recent evidence, however, has argued against this as the only reason since certain phospholipid ether analogs which are not substrates for this enzyme also have the capacity to accumulate in tumors.[4]

Phospholipid ethers occur naturally in cell membranes in very small quantities, yet they play a very important role in a host of biological activities. One of the most potent phospholipid ether mediators known is platelet-activating factor (PAF) which has detectable biological responses at concentrations as low as 10^{-14} M.[5] Biological responses caused by PAF range from aggregation and degranulation of platelets to cellular effects such as chemotaxis, superoxide formation, and tumor necrosis factor production. Several investigators have shown that the biological effects of PAF result via a receptor-mediated process.[5-10] It has also been demonstrated that the pharmacologic effects of PAF are strongly influenced by the stereochemistry of the *sn*-2 position.[6-10] For example, PAF is highly potent when present in the natural *R*-configuration, whereas the

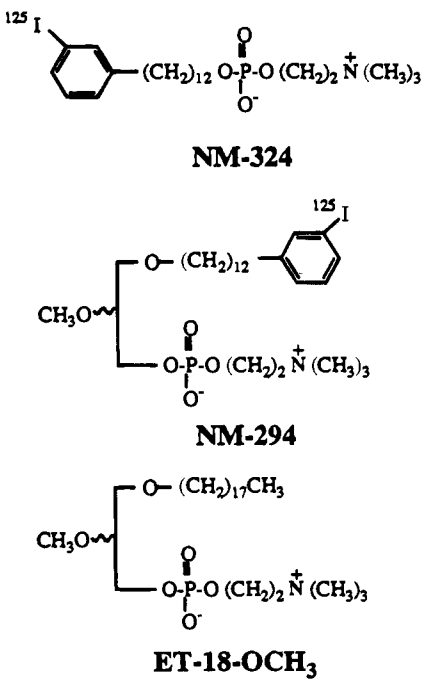

Figure 1. Phospholipid ether ET-18-OCH_3 and radioiodinated phospholipid ethers NM-324 and NM-294.

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unnatural *S*-enantiomer has greatly reduced to negligible biological activity.

ET-18-OCH_3 (Figure 1) is a phospholipid ether (PLE) analog which lacks PAF-like activity and has been widely studied for its antitumor and antimetastatic activities. Both *in vitro* and *in vivo* cytotoxicity studies have shown this agent to inhibit growth of a variety of human and murine tumor cells.[11,12] Clinical phase I trials have been completed with ET-18-OCH_3 and multiinstitutional phase II studies are underway.[13]

We have previously described the remarkable capacity of certain radioiodinated PLE analogs (Figure 1) to be selectively retained by a variety of rodent and human cell lines.[14-17] Moreover, this property made it possible to obtain images of these tumors in rabbits, rats, and mice using γ-camera scintigraphy. On the basis of these and other preliminary results, one of these radioiodinated analogs, 12-(*m*-iodophenyl)dodecylphosphocholine (NM-324; Figure 1), is currently undergoing clinical evaluation in human cancer patients as a radiodiagnostic imaging agent.

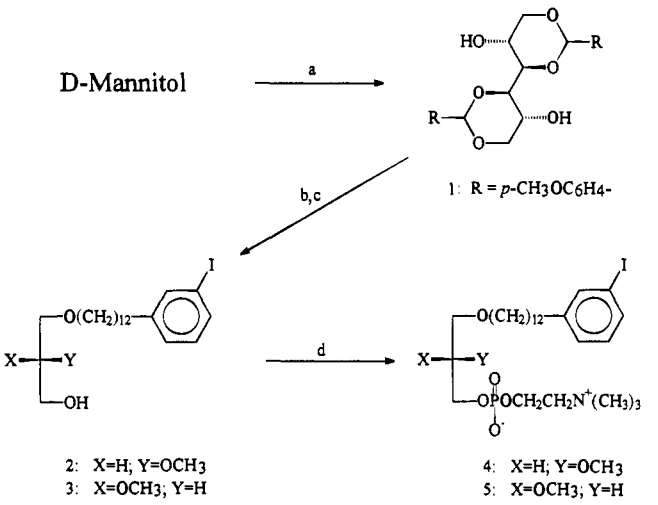
We have synthesized a large number of PLE analogs in an effort to elucidate the mechanism(s) whereby these substances are selectively retained in tumors. One compound we have evaluated is 1-*O*-[12-(*m*-iodophenyl)-dodecyl]-2-*O*-methyl-*rac*-glycero-3-phosphocholine (NM-294; Figure 1), a close structural analog of ET-18-OCH_3. Prior studies have demonstrated that *rac*-NM-294, similar to NM-324, has the capacity to be selectively retained in tumor tissue.[15-17]

The prevailing hypothesis is that phospholipid ethers such as NM-294 become trapped in tumor membranes because of their inability to become metabolized and cleared. Support for this hypothesis was found when extraction of tumors following administration of radioiodinated PLE to tumor-bearing animals showed only the presence of the intact agent.[18] However, similar analysis of urine and feces revealed only the presence of metabolites. If metabolic factors are involved in the cellular clearance of NM-294, the enzymes phospholipase C (E.C. 3.1.4.3, PLC) and phospholipase D (E.C. 3.1.4.4, PLD) are logical candidates, since previous studies have demonstrated that similar structures are not substrates for AGMO.[4] Moreover, if metabolism by either PLC or PLD is involved, previous reports indicate a degree of substrate stereospecificity.[19-21]

Prior studies in our laboratory have shown that *rac*-NM-294 is not a substrate for PLC.[18] This finding was consistent with the studies of Bishop and co-workers[22] who analyzed the various routes of metabolism for the ether lipid analogs, SRI 62-834, and hexadecylphosphocholine by ^{31}P-NMR spectroscopy. Their work revealed that various ether lipids were unmetabolized by PLC but readily broken down by PLD. As a result of these findings, PLD became the focus of our attention.

Accordingly, we synthesized the *S*- and *R*-isomers of NM-294 in order to determine the effect of chirality on (1) tumor retention and/or cytotoxicity and (2) metabolism by PLD. This report considers these issues by comparing the enantiomers and *rac* NM-294 for their biodistribution and retention in tumor-bearing rats, their *in vitro* cytotoxicity, and their susceptibility to metabolism by various sources of PLD.

Scheme 1. Synthesis of *S*- and *R*-Enantiomers of NM-294 (**4** and **5**, respectively)[a]



[a] Reagents: (a) *p*-MeO-C_6H_4-CHO, $HC(OMe)_3$, $\cdot H_2SO_4$ (cat.), DMF; (b) ref 23; (c) $AlCl_3$–PhOMe, CH_2Cl_2; (d) 2-chloro-2-oxo-1,3,2-dioxaphospholane, Et_3N, benzene, then Me_3N, CH_3CN, 60 °C.

Chemistry

Our approach to the synthesis of *S*- and *R*-enantiomers of NM-294 (**4** and **5**, respectively) was based on the construction of both compounds from a single starting material, D-mannitol, as shown in Scheme 1. This synthetic strategy represented a modification of the procedure described by Pinchuk et al.[23] for the synthesis of straight chain chiral ether lipids. Based on the regioselective cleavage of *p*-methoxybenzylidene acetals, the reaction sequence maintained the stereochemistry at the *sn*-2 carbon atom of the glycerol backbone. This allowed for the isolation of final compounds with high optical purity. Moreover, this approach allowed for the preparation of the key intermediate 1,3:4,6-bis-*O*-(*p*-methoxybenzylidene)-D-mannitol (**1**) in multigram quantities unlike the process previously described.[24] The purification of **1** took advantage of the selective extraction of byproducts using boiling chloroform followed by crystallization rather than purification by column chromatography. The only major difference between the present procedure and that described previously by Pinchuk et al.[23] was the use of $AlCl_3$–anisole for cleavage of the *p*-methoxybenzyl protective group. This reagent proved milder than $AlCl_3$–*N*,*N*-dimethylaniline and has been reported to not adversely affect methyl ethers.[25]

For the final conversion of alcohols **2** and **3** into the corresponding phosphocholines **4** and **5**, use was made of 2-chloro-2-oxo-1,3,2-dioxaphospholane in benzene followed by Me_3N cleavage of the intermediate cyclic phosphotriester. This procedure has been reported to provide high yields in instances where the compound does not contain labile groups susceptible to breakdown with continuous heating in Me_3N.[26]

Biology

The influence of phospholipid ether stereochemistry on cytotoxicity was assessed in the Walker 256 carcinosarcoma cell lines using the MTT assay.[27] Cytotoxicity is expressed as a percentage of control samples which received vehicle only. Figure 2A shows the effects of various concentrations of *rac*-ET-18-OCH_3 and its

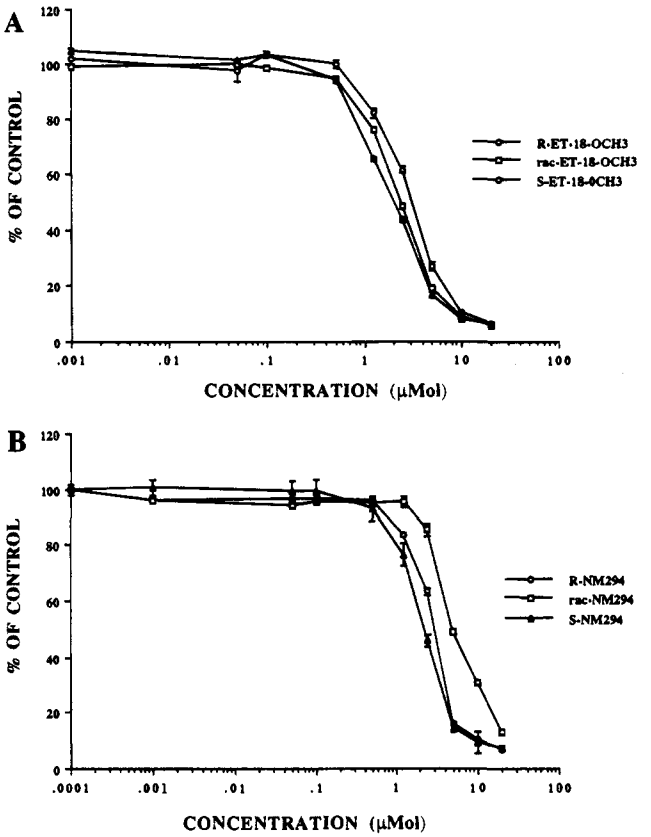


Figure 2. Cytotoxicity of phospholipid ethers as measured by the MTT assay in the Walker 256 carcinosarcoma cell line. Data are expressed as percent of control ± standard error. Percent of control is calculated by dividing the optical density of the mean of eight replicates per drug concentration divided by the optical density of the mean of eight replicates receiving vehicle only. (A) Cytotoxicity of the stereoisomers of ET-18-OCH_3. (B) Cytotoxicity of the stereoisomers of NM-294.

stereoisomers after a 48 h incubation. Figure 2B shows the effects of various concentrations of NM-294 and its stereoisomers. The IC_{50} values for all six compounds ranged from 2 to 5 μmol.

Tissue distribution analysis of radioactivity was performed in Walker tumor-bearing rats following administration of the radioiodinated PLE analogs via tail-vein injection. As shown in Table 1, the tissue levels of radioactivity are expressed as percent of injected dose/g at 24 and 48 h after administration for *S*- (**4**), *R*- (**5**), and *rac*-NM-294. As opposed to other tissues (e.g., liver and blood), the uptake of radioactivity in the tumor continued to increase with time (earlier times not shown) and reached a maximum at 24 h for all three compounds. At 48 h, the levels of **4** and **5** in tumor were 0.45 and 0.43% dose/g, respectively, whereas *rac* NM-294 levels in the tumor were 0.56% dose/g.

Table 2 shows the tumor-to-blood ratios and tumor-to-liver ratios for all three compounds at 24 and 48 h after administration. For compounds **4** and **5**, the tumor-to-blood ratios were approximately 11 and 15 at 24 and 48 h, respectively. Tumor-to-liver ratios for **4**, **5**, and *rac* -NM-294 were much lower than their corresponding tumor-to-blood ratios and reached a maximum of 2.0 at 48 h after administration.

Scintigraphic images of rats were obtained at various time points following tail-vein injection. Administration of **4** and **5** provided excellent images of the Walker 256 tumor in the hind limb. At 24 h after injection, a high background activity was seen in the abdominal area which partially cleared by 72 h. The γ-camera scintiscans obtained following administration of the two enantiomers were essentially identical at all time points. Figure 3 shows scintigrams of **4** and **5** at 72 h after iv injection.

Table 1. Biodistribution of the Stereoisomers of NM-294 in Walker Tumor-Bearing Rats

tissue	(*R*)-NM-294 (**5**)	*rac*-NM-294	(*S*)-NM-294 (**4**)
	24 h		
blood	0.05 ± 0.00[a]	0.07 ± 0.01	0.07 ± 0.00
duodenum	0.73 ± 0.08	1.12 ± 0.23	1.04 ± 0.02
inj site	0.23 ± 0.02	0.24 ± 0.03	0.26 ± 0.02
kidney	0.33 ± 0.02	0.46 ± 0.03	0.37 ± 0.04
liver	0.52 ± 0.02	0.81 ± 0.03	0.53 ± 0.02
lung	0.29 ± 0.01	0.34 ± 0.04	0.35 ± 0.03
muscle	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.00
ovary	0.28 ± 0.01	0.37 ± 0.01	0.31 ± 0.02
plasma	0.06 ± 0.00	0.10 ± 0.01	0.06 ± 0.00
spleen	0.37 ± 0.01	0.42 ± 0.03	0.43 ± 0.05
thyroid	23.81 ± 5.74	47.22 ± 19.99	10.33 ± 1.35
tumor	0.55 ± 0.06	0.85 ± 0.07	0.78 ± 0.12
	48 h		
blood	0.03 ± 0.00	0.05 ± 0.00	0.03 ± 0.00
duodenum	0.34 ± 0.04	0.44 ± 0.03	0.23 ± 0.02
inj site	0.25 ± 0.03	0.17 ± 0.01	0.24 ± 0.03
kidney	0.20 ± 0.03	0.24 ± 0.03	0.16 ± 0.01
liver	0.25 ± 0.01	0.35 ± 0.01	0.23 ± 0.01
lung	0.14 ± 0.01	0.20 ± 0.00	0.14 ± 0.01
muscle	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
ovary	0.14 ± 0.01	0.27 ± 0.01	0.14 ± 0.01
plasma	0.03 ± 0.00	0.05 ± 0.01	0.03 ± 0.00
spleen	0.10 ± 0.04	0.17 ± 0.01	0.10 ± 0.04
thyroid	24.11 ± 2.73	24.44 ± 1.63	13.69 ± 0.57
tumor	0.43 ± 0.08	0.56 ± 0.04	0.45 ± 0.06

[a] Biodistribution of PLE analogs (5–10 μCi) in Sprague–Dawley rats with the Walker 256 carcinosarcoma tumor implanted intramuscularly in the thigh at 24 and 48 h after iv injection. Tissues were dissected and weighed. The radioactivity (cpm/g) was counted and corrected for decay. The percentage of the injected dose/g (% ID/g) of tissue was calculated from these data. The results reflect the mean % ID/g ± SEM of three rats.

Table 2. Tumor-to-Blood and Tumor-to-Liver Ratios for Phospholipid Ether Analogs at 24 and 48 h following iv Administration

time (h)	**4**	**5**	*rac*-NM-294
	tumor-to-blood		
24	11.1[a]	11.0	12.1
48	15.0	14.3	11.2
	tumor-to-liver		
24	1.5	1.1	1.1
48	2.0	1.7	1.6

[a] Data are expressed as the % ID/g of tissue in the tumor divided by the % ID/g of either liver or blood.

Enzymatic studies were performed to determine the susceptibility of these radioiodinated PLE analogs to breakdown by PLD. Table 3 depicts the percent of radioactivity recovered and the identity of metabolites after incubation of **4** and **5** with PLD from *Streptomyces chromofuscus*. Both compounds were broken down to form a phosphatidic acid derivative (PA) as well as a dialkylglycerol (DAG) metabolite. In the presence of PLD, the *S*-isomer was hydrolyzed to form (59.4 ± 2.5 (*S*)-PA and (37.5 ± 1.6)% (*S*)-DAG, respectively. Similarly, in the presence of PLD, the *R*-isomer was hydrolyzed to form (59.5 ± 0.2)% (*R*)-PA and (38.6 ± 0.2)% (*R*)-DAG, respectively. In the presence of ethanol, a substance known to stimulate cabbage PLD activity, [28] the *S*-isomer was hydrolyzed to form (57.1 ± 13.7)% (*S*)-

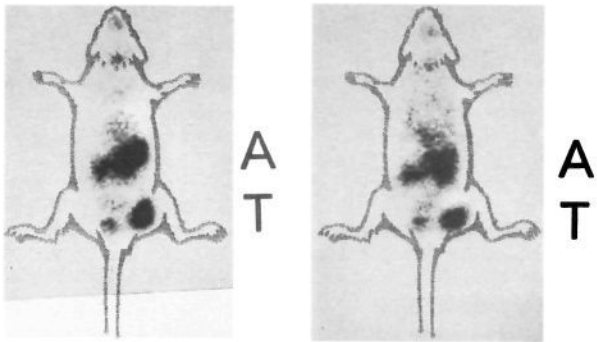


Figure 3. γ-Camera scintigraphy of (*S*)- and (*R*)-NM-294 at 72 h after tail-vein administration. A = abdominal activity; T = tumor.

Table 3. Metabolism of the Stereoisomers of NM-294 by Phospholipase D from *S. chromofuscus* after a 30 min Incubation

	control	control no. 2	PLD	PLD + ETOH
(*R*)-NM-294	99.25 ± 0.01[a]	99.26 ± 0.02	2.28 ± 0.81	9.28 ± 8.71
PA	ND	ND	59.43 ± 2.53	57.13 ± 13.65
DAG	0.47 ± 0.04	0.74 ± 0.02	37.49 ± 1.57	33.09 ± 4.77
(*S*)-NM-294	99.76 ± 0.24	99.61 ± 0.22	1.63 ± 0.35	0.81 + 0.07
PA	ND	ND	59.48 ± 0.16	43.81 ± 2.07
DAG	ND	ND	38.57 ± 0.24	54.76 ± 2.25

[a] Values represent percent of the total region ± the standard error for each radioactive substance as determined by radio-TLC. Three samples were incubated under each of the conditions above. Abbreviations: PA, phosphatidic acid derivative (PLD product); DAG, dialkylglycerol derivative; ND, not detected.

PA and (33.1 ± 4.77)% (*S*)-DAG, whereas the *R*-isomer was converted to (43.8 ± 2.1)% (*R*)-PA and (54.8 ± 2.3)% (*R*)-DAG.

PLD isolated from cabbage has been demonstrated to undergo the transphosphatidylation reaction in which a unique phosphatidylethanol metabolite is generated as the result of a substitution of choline with a short, straight chain alcohol.[28] As shown in Table 4, metabolism of **4**, **5**, and *rac*-NM-294 was similar in the presence of PLD alone. However, in the presence of 1% ethanol, the percent of parent compound remaining was (37.8 ± 0.8)%, (51.1 ± 2.8)%, and (72.9 ± 8.9)% for **5** (*R*), *rac*, and **4** (*S*) isomers, respectively.

Discussion

This report details the chemical synthesis of phospholipid ether stereoisomers with a chiral center at the *sn*-2 position of glycerol. The primary goal was to gain insight as to the potential mechanism(s) by which certain radioiodinated phospholipid ether analogs are selectively retained in tumor tissue.

Our initial cytotoxicity studies revealed that (*S*)-ET-18-OCH_3, (*R*)-ET-18-OCH_3, and *rac*-ET-18-OCH_3 had similar IC_{50}'s when incubated with the Walker 256 carcinosarcoma cell line as measured by the MTT test. Under the same conditions, **4**, **5**, and *rac*-NM-294 displayed a similar cytotoxicological profile. These results demonstrated that chirality at the *sn*-2 position had little effect on the ability of these compounds to inhibit growth. Such findings are consistent with a report by Lohmeyer and Workman[29] who demonstrated a lack of enantioselectivity in cytotoxicity and membrane-damaging capacity of the ether lipid SRI 62-834 in HT29 colon carcinoma cells. Berens et al.[30] also reported no observable stereoselectivity between (*S*)-ET-18-OCH_3 and its racemic form when incubated at various concentrations with various human glioma cell lines.[30] Moreover, *in vivo* experiments have shown that the optical isomers of a hexadecyl analog, ET-16-OCH_3, acted similarly in inhibition of growth of WEHI-3B and P388 tumors transplanted into BALB/C mice.[31]

Table 4. Metabolism of the Stereoisomers of NM-294 by Phospholipase D Isolated from Cabbage after a 1 h Incubation

compound	control	PLD	PLD + 1% EtOH
(*R*)-NM-294 (**5**)	98.7 ± 0.94[a]	63.02 ± 8.14	37.78 ± 0.80
PA	ND	8.03 ± 1.00	3.67 ± 1.29
PEt	ND	14.24 ± 3.93	51.11 ± 1.83
DAG	0.66 ± 0.42	11.28 ± 3.82	4.08 ± 1.09
rac-NM-294	93.31 ± 0.97	62.43 ± 8.11	51.10 ± 2.83
PA	ND	6.40 ± 2.27	3.71 ± 0.78
PEt	ND	9.74 ± 4.19	34.35 ± 2.29
DAG	4.63 ± 1.00	17.26 ± 2.11	7.91 ± 1.19
(*S*)-NM-294 (**4**)	97.52 ± 0.59	74.15 ± 3.69	72.88 ± 8.90
PA	ND	6.55 ± 1.96	3.42 ± 0.75
PEt	0.31 ± 0.16	4.03 ± 1.97	14.85 ± 5.72
DAG	ND	11.44 ± 1.34	5.35 ± 1.12

[a] Values represent percent of the total region ± the standard error for each radioactive substance as determined by radio-TLC. Three samples were incubated under each of the conditions above. Abbreviations: PA, phosphatidic acid derivative (PLD product); PET, phosphatidylethanol derivative; DAG, dialkylglycerol derivative; ND, not detected.

Biodistribution studies with the *S*-, *R*-, and racemic forms of NM-294 at 48 h after administration failed to demonstrate a significant difference in their percent uptake in tumor (Table 1). In addition, liver and kidney levels of the three radioiodinated PLE's were essentially the same. Likewise, the tumor-to-blood and tumor-to-liver ratios were essentially equivalent (Table 2).

Prior work with *rac*-NM-294 in Sprague–Dawley rats has demonstrated that the primary route of excretion is via the urinary tract.[18] In addition, when the urine and feces were extracted for radioactivity, only metabolites were detected suggesting that NM-294 is completely metabolized before being excreted. In the present study, liver and kidney levels of the *S*- and *R*-isomers were essentially identical suggesting that these two stereoisomers are cleared from tissues to a similar extent. γ-Camera scintigraphy of each of the three probes at 72 h after administration also confirmed these results. The images shown in Figure 3 were essentially identical to one another.

In studies with phospholipase D from cabbage in the presence of ethanol, the natural *R*-isomer of NM-294 (**5**) was broken down to the greatest degree followed by *rac*-NM-294 and (*S*)-NM-294 (**4**). Formation of the transphosphatidylation product followed the same trend. In contrast, incubation of the optical isomers **4** and **5** with phospholipase D from *S. chromofuscus* failed to show any stereoselectivity. In addition, when ethanol was added to the incubate, no phosphatidylethanol product was formed. Bruzik and Tsai [32,33] have investigated chiral phospholipids which were stereogenically labeled at phosphorus. They demonstrated that PLD isolated from cabbage preferred one of the phosphorus isomers (*R*) of dipalmitoylphosphatidylcholine. We were unable to find any prior reports investigating the importance of chirality at the *sn*-2 position of phospholipids or phospholipid ethers on the ability of PLC or PLD to metabolize such compounds.

In summary, we have synthesized the optical isomers of a phospholipid ether in order to further our under-

standing relating to the mechanism of tumor cytotoxicity and retention. (*R*)- And (*S*)-NM-294 were taken up and retained to a similar extent by the Walker 256 carcinosarcoma. In addition, these compounds were equipotent in an *in vitro* cytotoxicity assay. This is the first report examining the effects of chirality at the *sn*-2 position on the metabolism of PLE by PLD. Although PLD isolated from cabbage was found to display some degree of sterospecificity, this was not the case for the bacterial form of PLD. On the basis of these findings and previous results, it would appear that the PLE's are taken up, retained, and slowly cleared by tumor tissue in a nonstereospecific manner. Whether or not the excretory metabolites arise by stereospecific processes will have to await studies with mammalian tissues.

Experimental Section

^{1}H-NMR spectra were recorded on an AM-360 Bruker spectrometer using Me_4Si as an internal standard. Optical rotations were measured on a Perkin-Elmer polarimeter (Model 241) using a 1 dm cell. Melting points were measured using a Thomas-Hoover apparatus and are uncorrected. Thin-layer chromatography was performed using DC-Alufolien Kieselgel 60 F plates (E. Merck, Darmstadt, Germany). Visualization was achieved by UV light and/or charring following H_2SO_4 (5% in ethanol) spray. For flash chromatography, silica gel 32–63 μm (Fisher Scientific) was used. All chemicals were obtained from Aldrich Chemical Co. (Milwaukee, WI) except anhydrous trimethylamine which was from Fluka. Phospholipase D (type I from cabbage, 179 units/mg), phospholipase D (type VI from *S. chromofuscus*, 3000 units/mg), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thiazolyl blue) were purchased from Sigma Chemical Co. (St. Louis, MO). *rac* -ET-18-OCH_3 was purchased from Biomol (Plymouth Meeting, PA). (*R*)- and (*S*)-ET-18-OCH_3 were synthesized by one of the authors.[23]

Chemical Studies: 1,3(*R*):4,6(*R*)-Bis-*O*-(4-methoxybenzylidene)-D-mannitol (1). To a solution of D-mannitol (25 g, 137.2 mmol), 4-methoxybenzaldehyde (33 mL, 274.4 mmol), and trimethyl orthoformate (45 mL, 411.6 mmol) in DMF (90 mL) was added concentrated sulfuric acid (4 mL) with stirring. The reaction mixture was stirred for 2 h at 60 °C and then at 20 mmHg for 2 h at 20 °C to remove the water and facilitate product formation. The reaction mixture was poured with stirring into an ice-cold aqueous solution of potassium carbonate (15 g in 800 mL). The precipitate was collected by filtration and washed with hexane. The precipitate was rendered anhydrous by azeotropic distillation with benzene. $CHCl_3$ (80 mL) was added to the precipitate, and the mixture was boiled under reflux for 30 min and cooled to room temperature. The white precipitate was collected, washed with $CHCl_3$, and dried. It was recrystallized from MeOH to give the desired compound (19 g, 33%) as white crystals (mp 223–224 °C) (lit.[24] mp 224–225 °C). ^{1}H-NMR: δ 7.37 and 6.88 (2 dm, Ar-H, 8H, J = 8.8 Hz), 5.46 (s, Ar-CH, 2H), 4.37 (q, H-1_{eq} and H-6_{eq}, 2H, $J_{1ax,1eq}$ = 10 Hz, $J_{1eq,2}$ = 5 Hz), 4.25–4.16 (m, H-2 and H-5, 2H), 4.14 (dm, H-3 and H-4, 2H, $J_{2,3} = J_{4,5}$ = 9 Hz), 3.81 (s, OCH_3, 6H), 3.63 (t, H-1_{ax} and H-6_{ax}, 2H, $J_{1ax,1eq} = J_{1ax,2}$ = 10 Hz). Anal. ($C_{22}H_{26}O_8$) C, H.

3-*O*-[12-(*m*-Iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycerol (2). To a solution of 1-*O*-(*p*-methoxybenzyl)-2-*O*-methyl-3-*O*-[12-(*m*-iodophenyl) dodecyl]-*sn*-glycerol (1.014 g, 1.7 mmol) and anisole (0.92 mL, 8.5 mmol) in dry CH_2Cl_2 (20 mL) was added powdered aluminum chloride (905 mg, 6.8 mmol) in one portion at 0 °C. The reaction mixture was stirred for 15 min at 0 °C and then for 30 min at room temperature. The mixture was cooled in an ice bath, the reaction quenched with 0.1 **N** HCl (100 mL), and the mixture extracted with $CHCl_3$ (3 × 100 mL). The $CHCl_3$ extracts were washed with water and dried (Na_2SO_4), and the solvent was evaporated *in vacuo*. Silica gel chromatography with hexane–ethyl acetate (from 85:15 to 80:20) afforded the desired compound (793 mg, 98%) as an oil. $[\alpha]^{25}_D$ = +5.6° (c = 3% w/v, benzene). ^{1}H-NMR: δ 7.52 (m, Ar-*H*, 2H), 7.13 (m, Ar-*H*, 1H), 7.00 (t, 1H, Ar-*H*, J = 8 Hz), 3.76 and 3.65 (2 m, CH_2OH, 2H), 3.54 (m, $CHCH_2OCH_2$, 2H), 3.47 (s, OCH_3, 3H), 3.46–3.41 (m, 3H, $CHCH_2OCH_2$), 2.53 (t, 2H, Ar-CH_2, J = 8.0 Hz), 2.15 (br t, 1H, CH_2OH), 1.55 (m, Ar-CH_2CH_2 and OCH_2CH_2, 4H), 1.25 (m, $(CH_2)_8$, 16H). Anal. ($C_{22}H_{37}IO_3$) C, H.

1-*O*-[12-(*m*-Iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycerol (3). This compound was prepared from **1** using $AlCl_3$–anisole[25] for removal of the *p*-methoxybenzyl protective group $[\alpha]^{25}_D$ = −5.3° (c = 3% w/v, benzene). The ^{1}H-NMR was identical to that obtained for **2**. Anal. ($C_{22}H_{37}IO_3$) C, H.

1-*O*-[12-(*m*-Iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycero-3-phosphocholine (5). To a solution of 1-O-[12-(*m*-iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycerol (**3**) (120 mg, 0.25 mmol) and triethylamine (0.05 mL, 0.33 mmol) in benzene (3 mL) was added 2-chloro-2-oxo-1,3,2-dioxaphospholane (24 μL, 0.26 mmol) in one portion. The reaction mixture was stirred for 16 h, the precipitated Et_3N·HCl was removed by filtration, and the filtrate was concentrated to dryness. The residue was dried *in vacuo* for several hours before adding 15%, w/v, anhydrous trimethylamine in CH_3CN (3 mL). The vessel was tightly closed and heated at 70 °C for 24 h. After cooling to room temperature, 8 N HCl (2 mL) was added to the reaction mixture. The reaction mixture was partitioned between $CHCl_3$ (40 mL), MeOH (40 mL), and ice-cold H_2O (35 mL). The $CHCl_3$ layer was separated, and the extraction was repeated (2 × 40 mL of $CHCl_3$). The organic layers were combined and dried (Na_2SO_4), and the solvent was removed *in vacuo*. Column chromatography using a $CHCl_3$–MeOH gradient (from 10:0 to 7:3) followed by $CHCl_3$–MeOH–H_2O (65:35:5) gave a semisolid (115 mg, 71%). $[\alpha]^{25}_D$ = −1.2° (c = 1% w/v, $CHCl_3$–MeOH, 1:1). ^{1}H-NMR ($CDCl_3$–CD_3OD–D_2O, 1:1:0.3): δ 7.52 (m, Ar-*H*, 2H), 7.16 (m, Ar-*H*, 1H), 7.02 (t, 1H, Ar-*H*, J = 8 Hz), 4.26 (m, $POCH_2CH_2$, 2H), 3.95 and 3.86 (2 m, $CHCH_2OPOCH_2$, 2H), 3.62 (m, CH_2N, 2H), 3.60–3.50 (m, $CHCH_2OCH_2$, 3H), 3.47 (s, OCH_3, 3H), 3.46 (t, $CHCH_2OCH_2$, 2H, J = 7 Hz), 2.55 (t, Ar-CH_2, 2H, J = 8 Hz), 1.6 (m, Ar-CH_2CH_2 and OCH_2CH_2, 4H), 1.30 (m, $(CH_2)_8$, 16H). Anal. ($C_{27}H_{49}INO_6P$·H_2O) C, H, N.

3-*O*-[12-(*m*-Iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycero-1-phosphocholine (4). This compound was synthesized as described for **5** from of 3-*O*-[12-(*m*-iodophenyl)dodecyl]-2-*O*-methyl-*sn*-glycerol (**2**) (127 mg, 0.27 mmol). Yield: 148 mg (86%). $[\alpha]^{25}_D$ = +1.2° (c = 1% w/v, $CHCl_3$–MeOH, 1:1). The phosphocholine **4** had the same ^{1}H-NMR spectrum as **5**. Anal. ($C_{27}H_{49}INO_6P$·H_2O) C, H, N.

Radioiodination of Phospholipid Ether Analogs. Radioiodination of the PLE analogs with iodine-125 was accomplished by an isotope exchange reaction as previously reported.[34] Radiochemical purity was established by radio-TLC with unlabeled material serving as a standard. Specific activity ranged from 0.05 to 41 Ci/mmol. Radiochemical purity exceeded 95% for all compounds evaluated.

Biological Studies: Cell Lines and Culture Conditions. Walker 256 carcinosarcoma cells, provided by Dr. James Varani of the Department of Pathology, University of Michigan, were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 200 units/mL penicillin, and 0.2 mg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Animals. Female Sprague–Dawley rats, 200–250 g (Charles River, Portage, MI), were housed in a temperature- and light-controlled room and had free access to food and water. The rats were inoculated with Walker 256 carcinosarcoma cells (5.0 × 10^6 cells) in 0.2 mL of saline in the right hind limb. The animals were used 6–10 days later when the tumor weight averaged 10 g. All procedures using animals conformed strictly to the guidelines set forth by the University of Michigan Unit of Laboratory Animal Medicine Group which reviewed and approved the experimental protocol.

Tissue Distribution. The radiolabeled compounds were dissolved in absolute EtOH (50–500 μL), and Tween-20 (0.1 mL/mg of compound) was added to the solution. EtOH was removed by evaporation under a stream of nitrogen. Physiological saline was added, to give a 2–3% Tween-20 solution

which was subsequently mixed by vortex. The solubilized radiolabeled compound (5–10 μCi, 0.5 mL) was administered intravenously via tail vein to anesthetized tumor-bearing rats (n = 3/time point). The animals were sacrificed by exsanguination while under ether anesthesia at the various time points. The blood was collected via cardiac puncture, and selected tissues were removed and blotted to remove excess blood. Large organs were minced with scissors. Tissue samples from various organs (n = 2/tissue) were weighed, placed in 12 × 75 mm, 5 mL plastic γ-counter tubes (Sarstedt, Princeton, NJ), and counted with a Packard Minoxi Auto γ-5000 counter. The concentration of radioactivity in each tissue was expressed as a percentage of injected dose/g (% ID/g) of tissue.

γ-Camera Scintigraphy. Scanning of the animals was performed using an LEM mobile camera (Siemens Corp., Hoffman Estates, IL) with a high sensitivity–low energy collimator window optimized for iodine-125. Image acquisition and storage were accomplished with a Siemens MicroDELTA computer connected to a larger MicroVAX unit. Animals (n = 2/compound) were sedated with a premixed solution of 87 mg/kg ketamine and 13 mg/kg xylazine. Images were accumulated (100 000 counts/image) at various time points following tail-vein administration of radiolabeled compound (65–75 μCi, 0.5–1.0 mL).

MTT Assay. Cytotoxicity was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide test (MTT test) as previously described by Mossman.[27] Walker 256 cells were suspended in fresh RPMI media at a concentration of 2 × 10^5 cells/mL. Cells (200 μL) were added to individual wells in a 96-well plate. Test compounds were dissolved in absolute EtOH and added to give a final concentration of 0.001–60 μmol. Control wells contained cells and EtOH vehicle. Concentrations of EtOH were always <1%. After 48 h, 50 μL of a 2 mg/mL MTT solution was added to each well. Four hours later, plates were spun at 1200 rpm for 5 min to pellet the cells. Medium was removed by aspiration without disturbing cells or formazan crystals which had been created by the conversion of MTT by viable cells. DMSO (200 μL) was added to each well to dissolve the formazan crystals resulting from the metabolic conversion of MTT in viable cells. Optical density was read using a Microplate Autoreader EL311 (Biotek Instruments, Winooske, Vermont) 96-well plate reader at 600 nm with DMSO serving as a blank. All drug concentrations were done in replicates of eight and repeated at least twice. Results were obtained by dividing the mean of eight wells of a given drug concentration by the mean of eight control wells receiving EtOH only and are expressed as a percentage of control ± the standard deviation of the resulting ratio.

Phospholipase D from *S. chromofuscus*. Assay buffer (450 μL; 50 mmol Tris, 6.3 mmol $CaCl_2$ with 0.05% bovine serum albumin (BSA), pH 8.0) was added to borosilicate tubes containing radiolabeled PLE analogs. Controls were prepared similarly except for the presence of PLD. Reactions were started by the addition of 1.5 units of PLD dissolved in enzyme dilution buffer (10 mmol Tris with 0.05% BSA, pH 8.0) to adjust the final volume to 0.5 mL. Samples were incubated for 30 min in a shaking bath at 37 °C. Reactions were halted by the addition of 10 mL of $CHCl_3$:MeOH (2:1) followed by the addition of 2 mL of H_2O. Samples were mixed by vortex and spun for 5 min at 1000 rpm to separate the organic and aqueous layers. The organic and aqueous phases were separated. The lower organic layer was removed and evaporated to dryness under a nitrogen stream. The upper aqueous layer was brought to a total volume of 5 mL with $CHCl_3$:H_2O:MeOH (3:47:48) and analyzed for radioactivity. The residue from the upper organic phase was dissolved in 100 μL of $CHCl_3$:MeOH (1:1) and spotted on TLC plates (silica gel F; Merck). Plates were developed with a $CHCl_3$:MeOH:acetic acid:H_2O (75:25:8:3) or $CHCl_3$:MeOH:6 N NH_4OH (65:35:5) solvent system. TLC plates were allowed to air-dry and subsequently scanned using a Bioscan System 200 plate scanner (Bioscan Inc., Washington, DC).

Phospholipase D from Cabbage. The assay utilized was similar to that described elsewhere.[28] Assay buffer (80 μmol/L sodium acetate, 40 μmol/L $CaCl_2$, pH 5.6; (950 μL) was added to borosilicate glass tubes containing radiolabeled PLE. Phospholipase D from cabbage (179 units/mg) was diluted in 1.5 mL of deionized H_2O to make a solution of 119 units of enzyme/mL. Enzyme solution containing 10 units of PLD was added to each tube. EtOH or assay buffer was added to adjust the final volume to 1 mL. Control samples contained assay buffer and 1% absolute EtOH. Ether was washed three times with an equal volume of water to remove any ethanol contamination.[28] Washed ether (0.5 mL) was added to each tube to start the reaction. Samples were incubated in a shaker bath at room temperature for 60 min. Enzymatic activity was halted by addition of 100 μL of 1 **N** HCl. Ether:H_2O (4:1, 2 mL) was added to each sample. Samples were mixed by vortex and spun for 5 min at 1000 rpm to separate the organic and aqueous layers. The upper organic phase was removed and evaporated to dryness under a nitrogen stream at room temperature. The lower aqueous layer was brought to a total volume of 5 mL with H_2O and analyzed for radioactivity. The residue from the upper organic phase was dissolved in $CHCl_3$:MeOH (1:1) and spotted for TLC. Radio-TLC was performed on the organic residue as described above.

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