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An Expedient Synthesis of Fluorescent Labeled Ceramide-1-phosphate Analogues¹

I. A. Boldyrev^a, R. E. Brown^b, and J. G. Molotkovsky^{a,2}

^aShemyakin–Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences, Moscow, 117997 Russian

^bHormel Institute, University of Minnesota, Austin, MN 55912 USA

Abstract

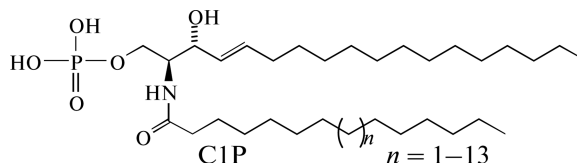
A synthesis for fluorescent analogs of ceramide-1-phosphate bearing 9-anthrylvinyl or 4,4-difluoro-3a,4a-diaza-*s*-indacene-8-yl (Me₄-BODIPY) fluorophore at co-position of fatty acid residue was carried out. The key stage of the synthesis is hydrolysis of corresponding sphingomyelins catalyzed by phospholipase D from *Streptomyces chromofuscus*, the enzymatic yield has been raised to 50–70% by appliance of organic solvent in the incubation medium.

Keywords

fluorescent lipid probes; ceramide-1-phosphate; BODIPY; anthrylvinyl; synthesis; phospholipase D

INTRODUCTION

Ceramide-1-phosphate (C1P) is a phospho-sphingolipid that is attracting increased interest as an important bioactive lipid molecule despite initial discovery over two decades ago in animal cells [1]. It is known now that C1P activates some enzymes, e.g. phospholipase A₂, and blocks the activity of others (e.g. caspases), regulates eicosanoid biosynthesis, and participates in membrane fusion and in other essential vital functions—see reviews [2–4].



Continued elucidation of new C1P functional and regulatory roles in cells is expected to benefit from enhanced ways of obtaining different molecular C1P species (in animal cells it may contain fatty acyls ranging from C14 to C26 [4]) and different type analogs—radioactive, fluorescent etc. Currently, several enzymatic syntheses of C1P are known,

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²Corresponding author: phone/fax: +7 (495) 330-66-01; jgmol@ibch.ru.

including a number of chemical syntheses, which more precisely should be named semi-chemical syntheses because all start from natural compounds, sphingosine or ceramides.

Byun et al. [5] realized a 'classical' lipid rout that involved several stages and use of different protective groups and specific reagents. Szulc et al. developed a simplified synthesis that relied on phosphorylation of the primary HO group of ceramide or *N*-Boc-sphingosine in the presence of secondary one [6], but rather harsh conditions for dimethylphosphate group removal at the end of synthesis raise concerns about its effectiveness for obtaining C1P analogs with labile labels. Nussbaumer and co-authors described a simple way for obtaining C1Ps with different *N*-acyl species by direct acylation of sphingosine-1-phosphate [7]; the last is available but rather expensive. References to other C1P chemical syntheses are cited in the above papers [5–7].

In contrast to organic syntheses, C1P enzymatic syntheses currently involve two strategies: i—phosphorylation of ceramide with ATP and ceramide kinase, and; ii—hydrolytic splitting of sphingomyelin (SM) by phospholipase D (PLD). Schneider and Kennedy first obtained C1P in 1973 by phosphorylation of ceramide with ATP and ceramide kinase from *E. coli* [8]. Preiss et al. observed C1P formation when platelet lipids were treated with ATP and ceramide kinase [9]. On the basis of this reaction, Don and Rosen developed a method for C1P activity measurement [10]. Although this approach is convenient for preparation of ³²P-labeled C1P [1], the complexity of the method limits its use for preparative purposes (see e.g. [12]).

Depending on the source of PLD, C1P production by hydrolysis of SM also can be inefficient. It is well known that PLD substrate specificity is limited to glycerophospholipids when this enzyme is obtained from various plant, animal or microbial sources [13]. However, Davidson and Long reported [14] that PLD from cabbage leaves splits SM, albeit at low rate. Analogous low activity was found in several microbial PLDs [15]. In contrast, moderate activity toward SM by PLD from the fungus *Streptomyces chromofuscus* has been reported [16–18]. Since we were looking for an efficient and direct way to obtain fluorescent-labeled C1Ps needed as probes for studies of lipid transfer mechanisms, our goal was to elaborate and improve the use of *S. chromofuscus* PLD to hydrolyze fluorescent anthrylvinyl (AV) and Me₄BODIPY-labeled SM analogs [19, 20]. In this communication, the synthesis of fluorescent C1P analogs bearing residues of 12-(9-anthryl)-11*E*-dodecenic, 7-(Me₄-BODIPY)heptanoic, and 15-(Me₄-BODIPY)pentadecanoic acids, through PLD-mediated hydrolysis of the corresponding SMs is described.

RESULTS AND DISCUSSION

The starting compound for synthesis of fluorescent C1P analogs was sphingosine-1-phosphocholine (**I**) that then was acylated with fluorescent-labeled acid to yield SM (**IIa–c**; see Scheme). Removal of the choline moiety from SM by PLD isolated from *S. chromofuscus* yielded the desired probes (**IIIa–c**).

The second stage of the synthesis, enzymatic hydrolysis of SMs, was complicated with certain difficulties. The hydrolysis activity toward SM by PLD from *S. chromofuscus*,

reportedly is only 20% of the rate for phosphatidylcholine [16, 17]. It also has been found that Triton X-100, BSA, fatty acids and diethyl ether activate hydrolysis, while SDS is inhibitory. The presence of Ca^{2+} ions is obligatory for *S. chromofuscus* PLD activity. Another feature distinguishing this enzyme from other PLDs is the hydrophobic site used by the protein to bind lipid substrate [16].

Our attempts to apply the procedure defined in the preceding works [16, 17] for hydrolysis of SMs (**IIa–c**), in a system containing Triton X-100 and CaCl_2 (it is also recommended by enzyme supplier, Sigma-Aldrich—see <http://www.sigmaaldrich.com>) could be considered as only partially successful, at best. The desired reaction proceeded slowly to produce C1P analogs in yields <15%, together with unidentified products (detailed data are not shown). Substantial improvement of the reaction rates and yields was obtained when we substituted organic solvent for detergent in the enzymatic hydrolysis system. The best effect was obtained with methylene chloride: yields of C1P analogs (**IIIa–c**) increased to 54–71%. The spectral characteristics, ^1H NMR, UV/visible and fluorescence, of the resulting probes were similar to those of other AV and Me_4BODIPY probes (see Experimental).

The reason(s) for the dramatic improvement in the enzymatic yield is a matter of conjecture. Imamura and Horiuti suggested that diethyl ether accelerates the PLD activity due to the favorable solvent effect on the enzyme conformation [16]. But El Kirat et al., after studying the enzymolysis of a series of phospholipids by PLD from *S. chromofuscus*, came to the conclusion that the interfacial tension at a water–lipid interface is a key regulator of the hydrolytic process [21]. Hirche and Ulbrich-Hofman analyzed different organic solvents for their ability to stimulate phosphatidylcholine hydrolysis catalyzed by PLD from cabbage and from *Streptomyces* [22]. The worst was isooctane and the best, diethyl ether with activities differing 300-fold. By comparing the effects of the solvents on the critical concentration of lipid micelle formation, the size of the aggregates, the water content of the organic phase, and the interfacial tension, they also found that the enzymatic activity correlated with the interfacial tension on the water system/organic solvent interface and concluded that the packing density of the phosphatidylcholine aggregates was a key parameter controlling PLD activity.

It is quite probable that the preceding considerations can be applied to our data as well, but we also consider the solvent effect on lipid solubility to be another important parameter that should not be overlooked. Based on our observations, methylene chloride saturated with water is a rather good solvent for SMs, natural and fluorescent-labeled. It has been ascertained that in systems containing detergent (e.g. Triton X-100), PLD interacts with substrate included in micelles. However, in systems where detergent is replaced by organic solvent, a favorable effect is observed [18]. In such a case, enhanced solubility of the lipid substrate is a substantial factor that positively influences the enzymatic rate.

In conclusion, we find that the modified C1P synthesis described here offers a facile and convenient route for production of C1P probes.

EXPERIMENTAL

Mass spectra were recorded on a ESI spectrometer Agilent 6224 TOF LC/MS (USA) and on an ESI-TOF mass spectrometer MX-5311 (IAP RAS, St. Petersburg); ^1H NMR spectra (δ , ppm, J , Hz) were recorded on a Bruker WM-700 spectrometer (United States). Electronic spectra of substances were measured on an SF-256 UVI spectrometer (LOMO Fotonika, St. Petersburg) in ethanol; fluorescence spectra (in ethanol) were recorded on a Hitachi F4000 spectrofluorimeter (Japan); the slit widths at excitation and emission were 3 nm.

DCC and DMSO were from Merck (Germany), DIPEA and *S. chromofuscus* phospholipase D were from Sigma-Aldrich (USA). Other reagents were from Reakhim (Russia). DIPEA was distilled over ninhydrin, then over powdery KOH; chloroform and methylene chloride were distilled over phosphorus pentoxide; other solvents were used after conventional purification. For column chromatography, silica gel Kieselgel 60 (Merck) was used, for TLC -Kieselgel 60 plates (Merck), detection with phosphomolybdic acid, ninhydrin, and UV irradiation. *N*-[15-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-*s*-indacene-8-yl)pentadecanoic acid [23], sphingosine-1-phosphocholine (**I**), *N*-[12-(9-anthryl)-11*E*-dodecenoyl]sphingosine-1-phosphocholine (**IIa**) [19], and *N*-[7-(4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4adiaza-*s*-indacene-8-yl)heptanoyl]sphingosine-1-phosphocholine (**IIb**) [20] were prepared as described. Evaporations *in vacuo* were performed at temperature $<40^\circ\text{C}$.

***N*-[15-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora3a,4a-diaza-*s*-indacene-8-yl)pentadecanoyl]sphingosine-1-phosphocholine (IIc)**

A solution of 15-(Me₄-BODIPY)-pentadecanoic acid (16 mg, 33 μmol) and 12 μL DIDEA (68 μmol) in 1 mL dry CH₂Cl₂ was added to sphingosine-1-phosphocholine (15 mg, 31 μmol) dissolved in 0.5 mL dry DMSO, followed by 30 μL 50% DCC (~ 70 μmol) solution in chloroform and the mixture was stirred for 12 h. Column chromatography in gradient system chloroform against methanol with 5% 7 N NH₄OH (10 \rightarrow 25%) afforded 23 mg (76%) sphingomyelin (**IIc**) as a dark orange gum, R_f 0.48 in chloroform–methanol–7 N NH₄OH, 65 : 35 : 8, system. UV, λ_{max} , nm (ϵ , M⁻¹ cm⁻¹): 495 (7.8×10^4). MS, m/z : calculated for C₅₁H₉₁F₂N₄O₆P [$M+H$]⁺ 935.6737, found 935.672. ^1H NMR (CD₃OD–CDCl₃): 6.06 (2H, s, ar), 5.68 (1 H, dt, J_1 14.8, J_2 7.2, =CHCH₂), 5.42 (1H, dt, J_1 14.8, J_2 6.6, CH(OH)CH=), 3.59 (2H m, CH₂N), 3.20 (9H, s, (9H, s, NMe₃), 2.95 (2H, t, J 7.7, arCH₂), 2.47 and 2.42 (12H, two s, arCH₃), 2.15 (2H, t, J 7.4, CH₂CO), 1.99 (2H, m, =CHCH₂), 1.62 (2H, m, COCH₂CH₂), 1.30–1.20 (40H, br m, CH₂), 0.86 (3H, t, J 7.0, CH₂CH₃). Fluorescence: λ_{ex} 496 nm (λ_{em} 530 nm), λ_{em} 504 nm (λ_{ex} 480 nm).

Enzymatic hydrolysis of sphingomyelin \rightarrow ceramide-1-phosphate (general procedure)

The original preparation of PLD from *S. chromofuscus* (solution in a water–glycerol mixture, 53435 units/mL) was diluted by bidistillate to the ~ 500 units/mL concentration. Initially, sphingomyelin (**II**) (10–12 mg, 10–15 μmol) was added to a medium composed of 2 mL CH₂Cl₂, 1.5 mL 50 mM Na-borate buffer, pH 8.0, containing 0.02% NaN₃, 1.5 mL bidistillate, and 40 μL 0.1 M CaCl₂; the mixture was dispersed by ultrasonic water-bath treatment (1–2 min). Then 40 μL PLD solution (~ 20 units) was added to the mixture, the

free volume of the vessel was filled with argon gas, and the vessel was stoppered and vortexed at 32°C. The hydrolysis course was checked by thin layer chromatography using a chloroform–methanol–7 N NH₄OH, 65 : 35 : 8, solvent system (SM: $R_f \sim 0.5$; C1P: $R_f \sim 0.2$). When the initial SM content in the mixture appeared negligible (usually, 2–4 h) it was treated with 0.5 mL acetic acid, evaporated and dried *in vacuo* (up to 20 Pa). The residue was extracted with chloroform–methanol, 2 : 1, mixture (3 × 15 mL; phase separation by centrifugation), and the extract was filtered through filter cel and evaporated. C1P analogs (**III**) were separated by column chromatography with stepwise gradient system chloroform–methanol–7 N NH₄OH, 90 : 10 : 0.5 → 70 : 27 : 3, followed by gel filtration on Sephadex LH-20 in a chloroform–methanol, 1 : 1, mixture.

***N*-[12-(9-Anthryl)-11*E*-dodecenoyl]sphingosine-1-phosphate (**IIIa**)**

Yellowish gum, R_f 0.25, UV, λ_{\max} , nm (ϵ , M⁻¹ cm⁻¹): 255 (1.2×10^5), 349 ($5\ 900$), 367 (7800) and 385 (7000). MS, m/z : calculated for C₄₄H₆₇NO₆P [$M + H$]⁺ 736.4706, found 736.4712. ¹H NMR: 8.35 (1 H, s, ar H10), 8.27–8.31 (2 H, m, ar), 8.13 (2H, m, ar), 7.96 (2H, m, ar), 7.41 (2 H, m, ar), 6.92 (1H, d, J 11.3, arCH=CH), 6.20 (1H, dd, J_1 11.3, J_2 7.3, arCH=CH), 5.65 and 5.41 (2H, two m, CH=CH), 4.11 (1H, m, CHOH), 3.85 (1H, m, CHNH), 2.28 (2H, t, J 7.3, CH₂CO), 2.25 (2H, dt, J_1 7.3, J_2 6.9, arCH=CHCH₂), 2.09 (2H, t, J 7.2, CH₂CO) 1.96 (2H, m, =CHCH₂) 1.62 (2H, m, CH₂CH₂CO), 1.33–1.15 (34H, br. m, CH₂), 0.84 (3H, t, J 6.5, CH₃). Fluorescence: λ_{ex} 256, 350, 368 and 387 nm (λ_{em} 430 nm), λ_{em} 420 nm (λ_{ex} 370 nm).

***N*-[7-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)heptanoyl]sphingosine-1-phosphate (**IIIb**)**

Red gum, R_f 0.22. UV/visible λ_{\max} , nm (ϵ , M⁻¹ cm⁻¹): 495 (7.8×10^4). MS, m/z : calculated for C₃₈H₆₃BFN₃O₆P [$M - F$]⁺ 718.4532, found 718.4525. ¹H NMR: 6.04 (2H, s, ar), 5.68 (1H, dt, J_1 13.5, J_2 6.5 =CHCH₂), 5.42 (1H, dd, J_1 13.5, J_2 6.2, CH(OH)CH=), 2.93 (2H, t, J 7.8, arCH₂), 2.46 and 2.39 (12H, two c, arCH₃), 2.15 (2H, t, J 7.3, CH₂CO) 1.97 (2H, m, =CHCH₂), 1.60 (2H, m, COCH₂CH₂), 1.28–1.17 (24H, br m, CH₂), 0.85 (3H, t, J 7.1, CH₃). Fluorescence: λ_{ex} 496 nm (λ_{em} 530 nm), λ_{em} 503 nm (λ_{ex} 480 nm).

***N*-[15-(4,4-Difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene-8-yl)pentadecanoyl]sphingosine-1-phosphate (**IIIc**)**

Red gum, R_f 0.24. UV/visible λ_{\max} , nm (ϵ , M⁻¹ cm⁻¹): 495 (7.7×10^4). MS, m/z : calculated for C₄₆H₇₉BFN₃O₆P [$M - F$]⁺ 830.5784, found 830.5804. ¹H NMR: 6.01 (2H, s, ar), 5.67 (1H, dt, J_1 13.9, J_2 6.6 =CHCH₂), 5.43 (1H, dd, J_1 13.9, J_2 6.6, CH(OH)CH=), 2.89 (2H, m, arCH₂), 2.45 and 2.36 (12H, two s, arCH₃), 2.13 (2H, t, J 7.4, CH₂CO), 1.98 (2H, m, =CHCH₂), 1.58 (2H, m, COCH₂CH₂), 1.30–1.19 (40H, br m, CH₂), 0.85 (3H, t, J 7.3, CH₃). Fluorescence: λ_{ex} 496 nm (λ_{em} 530 nm), λ_{em} 505 nm (λ_{ex} 480 nm).

Acknowledgments

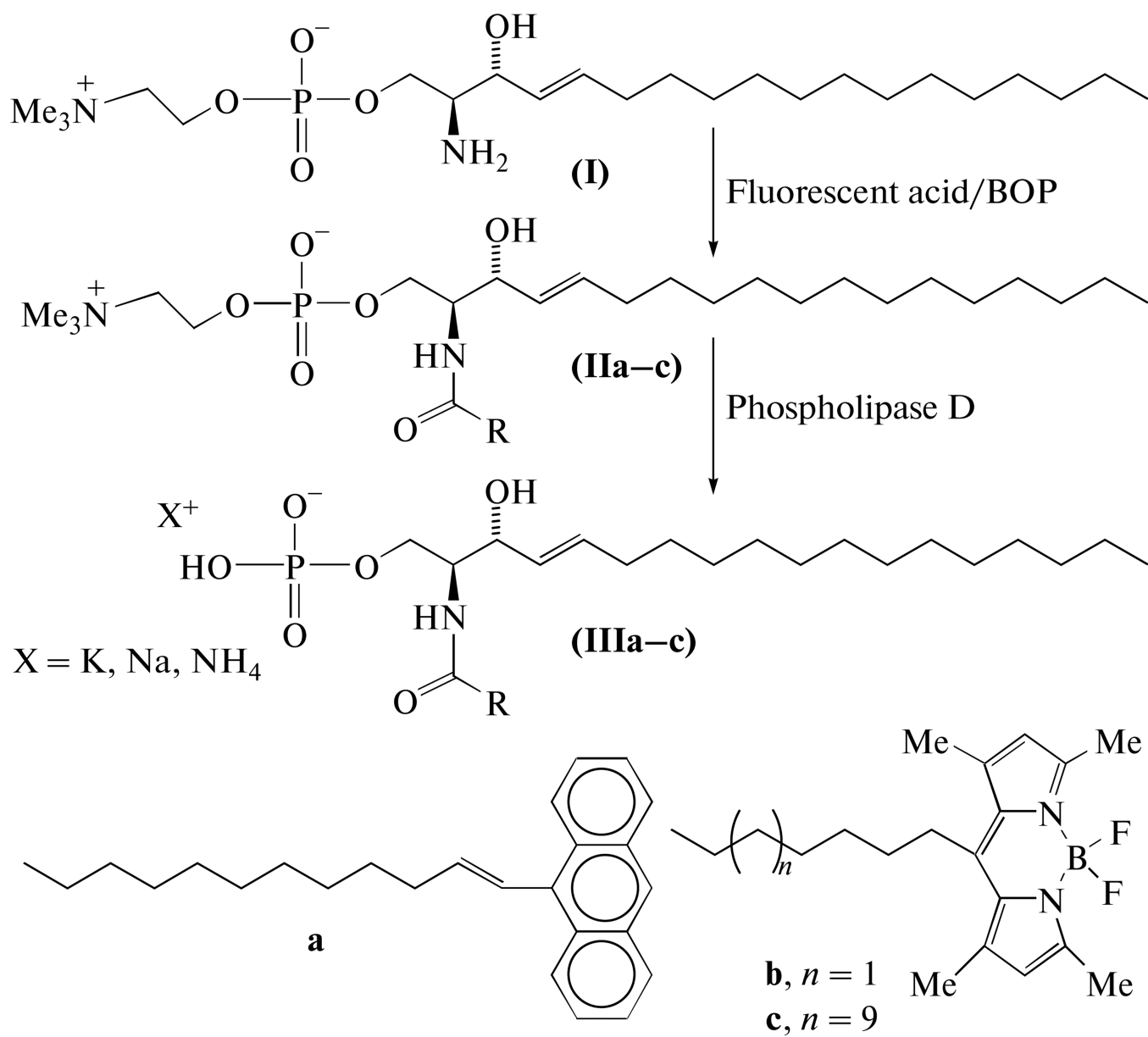
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Abbreviations

AV	9-anthrylvinyl
C1P	ceramide-1-phosphate
DIPEA	diisopropylethylamine
Me₄BODIPY	1,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza- <i>s</i> -indacen-8-yl
PLD	phospholipase D
SM	sphingomyelin

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Scheme.