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Membrane Interaction and Protein Kinase C-C1 Domain Binding Properties of 4-Hydroxy-3-(hydroxymethyl) Phenyl Ester Analogues

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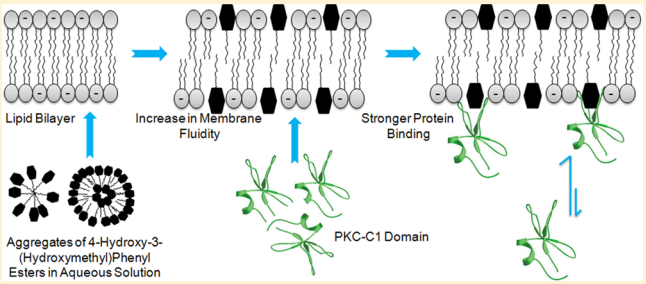
Supporting Information

ABSTRACT: Protein kinase C (PKC)-C1 domain targeted regulator development is considered as a potential therapeutic strategy for the treatment of cancer and immunological and other diseases. Efforts are underway to synthesize small molecules to achieve higher specificity for the C1-domain than the natural activator, diacylglycerols (DAGs). In this regard, we conveniently synthesized 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues and measured in vitro C1-domain binding properties. We also investigated different physicochemical properties of the synthesized molecules, including aggregation behavior in aqueous solution and interaction with lipid bilayers, and others with an aim for better understanding of their C1-domain binding properties. The results showed that the membrane-active compounds aggregate in aqueous solution at a reasonably lower concentration and strongly interact with the lipid bilayer. The hydrophilic part of the compounds localize at the bilayer/water interface and accessible for C1-domain binding. Biophysical studies revealed that the hydroxyl, hydroxymethyl, and carbonyl groups and acyl chain length are important for their interaction with the C1-domain. The potent compound showed more than 10-fold stronger binding affinity for the C1-domains than DAG under similar experimental conditions. Therefore, our findings reveal that these ester analogues represent an attractive group of C1-domain ligands that can be further structurally modified to improve their binding and activity.



INTRODUCTION

There are 518 protein kinases present in the human genome that transfers the phosphate group from adenosine-5-triphosphate to other substrate proteins.[1] These protein kinases specifically phosphorylate serine, threonine, and tyrosine amino acids of the target proteins. The phosphorylation process leads to conformational change of the target proteins that trigger signaling cascades, which in turn regulate cell proliferation, differentiation, migration, survival, and apoptosis.[2,3] The protein kinase C (PKC) enzymes belong to the serine/threonine kinases family. PKC enzymes play an important role in the pathology of several diseases including cancer and neurological, immunological, cardiovascular, and Alzheimer's diseases. In consequence, PKC enzymes are being actively pursued as the subject of intense research and drug development.[4−7]

The PKC enzymes consist of a highly conserved C-terminal catalytic domain and a less conserved N-terminal regulatory domain, which comprises an autoinhibitory pseudosubstrate sequence and one or two membrane-targeting domains (DAG/phorbol ester-binding C1-domain and Ca^{2+}-binding C2-domain). On the basis of their enzymatic properties and activation mechanism, the mammalian PKC enzymes have been categorized into classical (Ca^{2+}-, DAG-, and phospholipid-dependent), novel (Ca^{2+}-independent, but DAG- and phospholipid-dependent), and atypical (Ca^{2+}- and DAG-independent) subgroups.[4,8,9] In eukaryotic cells, the primary source of DAGs at the plasma membrane are the phosphatidylinositol-specific phospholipase C (PI-PLC) catalyzed hydrolytic product of phosphatidylinositol-4,5 bisphosphate [PtdIns(4,5)-P_2].[10,11] The DAGs are also produced at the internal membranes by a concerted action of phospholipase D (PLD) and phosphatidic acid phosphohydrolase on phosphatidylcholine (PC). The cellular translocation of classical PKC enzymes to the plasma membrane is initially mediated by Ca^{2+} binding through C2-domain, followed by C1-domain−DAG interactions in the presence of anionic phospholipids. In contrast, only DAG binding to the C1-domain in the presence of anionic phospholipids activates novel PKC enzymes. DAG binding allows PKC-C1 domain to penetrate into the cellular membrane and folding-out an N-terminal pseudosubstrate region, which allows access of a myriad substrates to the catalytic site of the PKC enzymes.[1,7,12,13]

The presence of a homologous catalytic domain in all protein kinases, including PKCs, compelled researchers to look for an alternate substrate binding sites to regulate PKC-dependent cellular functions.[1,4,9,12,14] In the meantime, detailed mechanistic studies demonstrated that DAG−C1-domain interactions in the presence of anionic phospholipids can regulate PKC activity. The DAG-responsive proteins are considerably smaller

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in number than protein kinases, and C1-domain is the most reliable target for specific substrate binding to activate PKC and control other enzyme activities. For these reasons, the PKC-C1 domains have become an attractive target in designing selective PKC regulators.[9,15] Structure−activity relationship studies of the high-affinity C1-domain ligands including phorbol ester, bryostatin, and others elucidate the ligand binding orientation within the C1-domain.[16,17] Unfortunately, most of these natural compounds are rigid, structurally complex, and also tumor promoters.[12,15,18] Structural modifications and large-scale productions are also difficult and laborious. In this regard, several research groups including us are trying to develop simple surrogates of admissible C1-domain-dependent PKC activity. Reported simple DAG lactone, isophthalic acid, protocatechualdehyde, and curcumin derivatives have shown stronger affinity for PKC-C1 domains and described the need of developing simple molecules for the mentioned purpose.[8,19−25]

In this study, we describe the synthesis, aggregation behavior in aqueous solution, interaction properties with lipid bilayers, and in vitro C1-domain binding properties of 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues. The long-chain ligands increase the fluidity and permeability of the bilayers, which assist their binding abilities with C1-domains. The potent ligands can compete with the DAG binding to the C1-domains. The hydroxymethyl group and hydroxyl group and ester groups of the compounds play a crucial role in distinguishing the PKC-C1 domains. We also presume that these membrane-active potent compounds would be able to activate C1-domain containing PKC isoenzymes under cellular environment. These results give us strong encouragement for this C1-domain directed drug discovery strategy.

EXPERIMENTAL METHODS

General Information. All chemicals and reagents were purchased from Sigma (St. Louis, MO), SRL (Mumbai, India), and Merck (Mumbai, India) and used for the synthesis without further purification. Dry solvent was obtained as per the reported procedure. Reactions were performed under nitrogen atmosphere and monitored using thin-layer chromatographic (TLC) plates prepared from silica gel 60 F254 (0.25 mm). Compounds have been purified through column chromatography using 60−120 mesh silica gel. NMR spectra were recorded using $CDCl_3$ (δ = 7.24 for ^{1}H and δ = 77.23 for ^{13}C NMR) in Varian 400 MHz and Bruker 600 MHz spectrometers. Coupling constants (J values) are reported in hertz, and chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane using residual chloroform (δ = 7.24 for ^{1}H NMR, δ = 77.23 for ^{13}C NMR) as internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). Mass spectra were recorded using a Waters Q-TOF Premier mass spectrometry system, and data were analyzed using the built-in software. 1,2-Dipalmitoyl-*sn*-glycerol (DAG_{16}), 1,2-dioctanoyl-*sn*-glycerol (DAG_8), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine (DPPS), 1,2- dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(5-dimethylamino-1-naphthalenesulfonyl) (NBD-PE) were purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.

Synthesis of Ester Derivatives. *Procedure for Formylation of 4-Hydroxybenzoic Acid (**4**).* To a stirred solution of 4-hydroxybenzoic acid (1 equiv) in trifluoroacetic acid (TFA, 2 mL), a hexamethyltetraamine (2 equiv) solution in TFA was added dropwise over a period of 15−20 min.[26] The reaction mixture was then refluxed around 90 °C overnight. After cooling down to room temperature, 30 mL of water was added to the reaction mixture and was acidified with 4 N HCl solutions. Precipitation occurred gradually with acidification. The resulting mixture was stirred at room temperature until the completion of precipitation. The yellow precipitate was then filtered off and dried. Recrystallization from ethanol gives the desired product as yellow solid (yield 59%).

General Procedure for Esterification. To a solution of carboxylic acid (1 equiv) in dichloromethane (10 mL), *N,N*-dicyclohexylcarbodiimide (1.5 equiv) and 4-dimethylaminopyridine (0.2 equiv) were added at room temperature.[22] The solution was stirred for 15 min and then the respective alcohol (1 equiv) was added. After the completion of reaction, the *N,N*-dicyclohexylurea derivative was filtered off and the remaining solution was concentrated under reduced pressure. The reaction mixture was extracted with ethyl acetate (3 × 15 mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The purification by silica gel column chromatography and a gradient solvent system of 0−20% ethyl acetate to hexanes yielded the target compound.

General Procedure for Reduction of Aldehydes. To a stirred solution of formylphenyl ester analogues (1.0 equiv) in methanol, $NaBH_4$ (2.0 equiv) was added portionwise at 0 °C over a period of 5 min and then stirring was continued for another 1 h at room temperature.[8] After completion of the reaction (monitored by TLC), methanol was removed under reduced pressure and the crude reaction mixture was washed with water (3 mL). The compounds were extracted with ethyl acetate (3 × 10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of 10−30% ethyl acetate to hexane yielded the corresponding compound with 90−92% yield.

Aggregation Studies. Aggregation studies of the compounds in aqueous solution were performed by fluorescence measurements using Fluoromax-4 spectrofluorometer at room temperature.[27] To measure the critical aggregation concentration (CAC) of the compounds in the presence of pyrene, the stock solutions of compounds were freshly prepared by first dissolving compounds in spectroscopic-grade dimethyl sulfoxide (DMSO) and then diluted with water. The amount of DMSO was kept less than 1% (by volume) for each set of experiment and had no effect on any experimental results. For fluorescence measurements, a saturated ethanolic solution of pyrene (2 μM) and varying concentrations of ligands were incubated in water at room temperature. Pyrene was excited at 335 nm, and emission spectra were recorded from 345 to 550 nm. Pyrene gives five intense peaks, but only I_1 (373 nm) and I_3 (383 nm) were considered for the measurement of CAC values.

Liposome Preparation. Vesicles for the determination of partition coefficient and phase transition temperature were prepared according to the reported procedure.[28] Briefly, lipids were dried out from chloroform solutions to form a thin film. The films were hydrated with phosphate-buffered saline (PBS), pH 7. The preparations were then sonicated at 50 °C for 2 min to prepare vesicles (final concentration 1 mM). The small

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unilamellar vesicles were prepared by extruding through a polycarbonate membrane (100 nm) using a hand-held mini-extruder at room temperature. For labeling experiment, NpOH in PBS buffer at PH 7 was added to the solution containing vesicles. The lipid solution was incubated with NpOH solution at 55 °C for 30 min with lipid/probe ratio at 100.

Determination of Partition Coefficient of 1-Naphthol in DPPC Bilayer. The partition coefficient (K_p) of 1-naphthol (NpOH) in aqueous solution of DPPC vesicle was measured by monitoring the NpOH* emission peak as reported earlier.[29−31] A series of solutions with fixed concentration of NpOH (4 μM) and varying concentrations of DPPC vesicle (0.01−0.1 mM) were prepared for the measurement. The other series of lipid solutions were also prepared accordingly in the presence of compounds **1a**, **2a**, and **3a**. Fluorescence spectra of NpOH were recorded in both SG (30 °C) and LC phases (53 °C) of the DPPC vesicles. The K_p of NpOH was calculated from the slope of the double reciprocal plot of $1/F$ vs $1/L$ using eq 1, where F_0 and F represent the fluorescence intensity of NpOH* (λ_{em} = 370 nm) in the absence or presence of DPPC liposomes, respectively.[29−31]

$$\frac{1}{F} = \frac{55.6}{(K_p F_0 L)} + \frac{1}{F_0} \tag{1}$$

The membrane-bound fraction (X_L) of NpOH was calculated using eq 2. The molar concentration of lipid (L) used was 0.1 mM.[29−31]

$$X_L = \frac{K_p L}{(55.6 + K_p L)} \tag{2}$$

Measurement of Phase Transition Temperature of DPPC Liposomes. For the measurement of phase transition temperature (T_m) of DPPC and DPPC/DPPE/DPPS (60:20:20) liposomes test solutions were prepared with a fixed concentration of compounds **1a**, **2a**, and **3a** (200 μM).[29−31] The T_m value of DPPC and DPPC/DPPE/DPPS liposome was determined by monitoring the fluorescence intensity of NpOH* (λ_{em} = 370 nm) at different temperatures.

Extent of Membrane Localization. The extent of localization of the ligands at the liposome interface was studied by the NBD fluorescence quenching method, using PC/Ligand$_{16}$/NBD-PE liposomes (89/10/1) in 50 mM Tris buffer, pH 8.2, containing 150 mM NaCl, according to the reported procedure.[8,22,23] The quenching reaction was initiated by adding sodium dithionite from a stock solution of 0.6 M in 50 mM Tris buffer, pH 11, containing 150 mM NaCl, to give a final concentration of 1 mM. The change in NBD fluorescence emission intensity at 530 nm (λ_{ex} = 469 nm) was recorded for 3 min at room temperature.

Protein Purification. The PKCδ and PKCθ-C1b subdomains were expressed in *E. coli* cells and purified using methods similar to those reported earlier.[8,22,23]

Fluorescence Measurements. To calculate the binding parameters under a membrane-free system, ligand-induced Trp fluorescence quenching measurements were performed on a Fluoromax-4 spectrofluorometer at room temperature.[8,22,23] The stock solutions of compounds were freshly prepared by first dissolving the complexes in spectroscopic-grade dimethyl sulfoxide (DMSO) and then diluted with buffer. The amount of DMSO was kept less than 3% (by volume) for each set of experiments and had no effect on any experimental results. For fluorescence titration, protein (1 μM) and varying concentrations of ligands were incubated in a buffer solution (20 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4) at room temperature. Protein was excited at 284 nm, and emission spectra were recorded from 300 to 550 nm. Proper background corrections were made to avoid the contribution of buffer and dilution effect. The resulting plot of Trp fluorescence as a function of ligand concentration was subject to nonlinear least-squares best-fit analysis to calculate apparent dissociation constant for the monomeric ligands (K_D(ML)), using eq 3, which describes binding to a single independent site.

$$(F_0 - F) = \Delta F_{max}\left(\frac{[x]}{[x] + K_D(\mathrm{ML})}\right) + C \tag{3}$$

where F and F_0 represented the fluorescence intensity at 339 nm in the presence and the absence of ligand, respectively. The ΔF_{max} represents the calculated maximal fluorescence change; $[x]$ represents the total monomeric ligand concentration.

Fluorescence anisotropy measurements were also performed on the same fluorimeter using similar methods described earlier.[8] All anisotropy values of the proteins in the absence or presence of compounds are the mean values of three individual determinations. The degree (r) of anisotropy in the tryptophan fluorescence of the proteins was calculated using eq 4 at the peak of the protein fluorescence spectrum, where I_{VV} and I_{VH} are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and $G = I_{VH}/I_{HH}$ is the instrumental grating factor.

$$r = \frac{(I_{VV} - GI_{VH})}{(I_{VV} + 2GI_{VH})} \tag{4}$$

Analysis of protein-to-membrane Förster resonance energy transfer (FRET) based binding assay was used to measure the binding affinity and specificity of the selected ligands under a liposomal environment.[8] In this assay, the membrane-bound C1 domain was displaced from liposomes by the addition of the DAG_8. The vesicles composed of PC/PE/PS/dPE (60/15/20/5) and PC/PE/PS/dPE/ligand (55/15/20/5/5) was used as control and for ligands, respectively. The stock solution of DAG_8 was titrated into the sample containing C1 domain (1 μM) and excess liposome (100 μM total lipid) in a buffer solution (20 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4) at room temperature. The competitive displacement of protein from the membrane was quantitated using protein-to-membrane FRET signal (λ_{ex} = 280 nm and λ_{em} = 505 nm). Control experiments were performed to measure the dilution effect under similar experimental condition and the increasing background emission arising from direct dPE excitation. Protein-to-membrane FRET signal values as a function of DAG_8 concentration were subjected to nonlinear least-squares-fit analysis using eq 5 to calculate apparent equilibrium inhibition constants ($K_I(DAG_8)_{app}$) for DAG_8.[8]

$$F = \Delta F_{max}\left(1 - \frac{[x]}{[x] + K_I(\mathrm{DAG_8})_{app}}\right) + C \tag{5}$$

where $[x]$ represents the total DAG_8 concentration and ΔF_{max} represents the calculated maximal fluorescence change.

The equilibrium dissociation constant (K_D(L)) for the binding of the C1 domains to the ligand-associated liposomes was calculated from eq 6 using K_D(ML) and $K_I(DAG_8)_{app}$ values. During calculation, the ligand concentration in the liposome interior was ignored, because of their inaccessibility

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for the protein. Thus, the protein accesses about half of lipids in the liposomes. The ligand concentration was used excess relative to the protein. The free ligand concentration was calculated by assuming that most of the protein would bind to the liposome and equimolar amount of ligand can be subtracted from the accessible ligand.

$$K_I(DAG_8)_{app} = K_D(ML)\left(1 + \frac{[L]_{free}}{K_D(L)}\right) \quad (6)$$

where $[L]_{free}$ is the free ligand concentration ($2.63 \pm 0.04\ \mu M$).

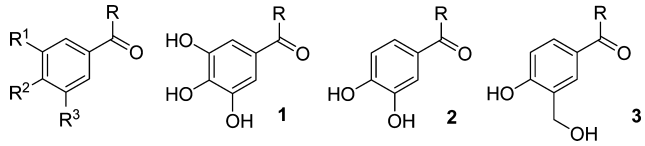
RESULTS AND DISCUSSION

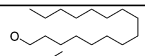
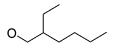
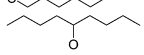
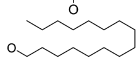
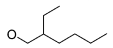
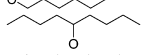
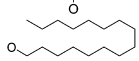
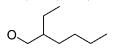
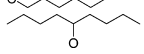
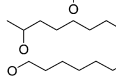
Design and Synthesis. The reported crystal structure of the PKCδ-C1b subdomain in complex with phorbol-13 acetate (1PTR) provides a detailed insight into the mode of ligand interactions within the hydrophobic binding pocket.[15,32] Structural analysis and structure−activity studies show that hydroxyl groups attached to C20, C13, C4, and the carbonyl group on C9, C3 of phorbol ester interact with the backbone amide proton and carbonyls of the C1b subdomain through hydrogen bond formations. Biophysical/biochemical studies of bryostatin and other compounds also described the importance of spatial orientation of these pharmacophores in binding with the PKC-C1 domain through its backbone amide proton and carbonyls.[16,20,32] Recently reported structure−activity relationship studies and/or biological activities suggest that phenyl-ring-based hydrophobic derivatives of isophthalic acid and protocatechualdehyde act as potential C1-domain ligands.[8,15] These compounds contain both the phorbol ester pharmacophores. Molecular models of these compounds docked into the ligand binding site of PKCδ-C1b subdomain show a similar interaction pattern as of phorbol ester and DAGs. The PKC-C1 domain also contains several hydrophobic residues (Met-239, Phe-243, Leu-250, Trp-252, and Leu-254 for the PKCδ-C1b subdomain) surrounding the ligand binding cleft and is known to interact with the membranes through these hybrophobic residues. The hydrophobic moieties of the C1-domain ligands are also known to interact either with these hydrophobic amino acids or with the hydrophobic moiety in the membranes.[11,14]

We selected 4-hydroxy-3-(hydroxymethyl) phenyl esters for the development of PKC-C1 domain modulators (Table 1). These compounds can be synthesized from 4-hydroxybenzoic acid and are easily amenable to structural modification. This natural product has many pharmacological effect including antioxidant and antitumor activities.[33−35] The acid group of the compounds provides access to incorporate different hydrophobic side chains. It is already described that the presence of the hydroxymethyl group is very important in anchoring the ligand into the binding cleft of the PKC-C1 domains. The second hydroxyl group is available for additional hydrogen bonding for optimization of the protein binding. The presence of the acyl group also can assist the compound in C1-domain binding. The hydrophobic interactions are complicated to model. Consequently, we synthesized a series of 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues with different side chains to study their impact on the binding affinity.

We first synthesized 4-hydroxy-3-(hydroxymethyl) phenyl esters (**3**) in three steps using commercially available 4-hydroxybenzoic acid (Scheme 1). To investigate the importance of the hydroxy and hydroxymethyl group of compound **3** in C1-domain binding, we prepared hydroxyphenyl ester analogues **1** and **2** with the required phorbol ester

Table 1. Structures of the Synthesized Compounds Used in the Present Study



Compound	R	R^1	R^2	R^3	LOGP
1a		OH	OH	OH	7.72
1b		OH	OH	OH	4.09
1c		OH	OH	OH	4.66
2a		H	OH	OH	8.03
2b		H	OH	OH	4.93
2c		H	OH	OH	4.97
3a		H	OH	CH_2OH	7.56
3b		H	OH	CH_2OH	3.93
3c		H	OH	CH_2OH	4.50
3d		H	OH	CH_2OH	3.98
3e		H	OH	CH_2OH	4.01

pharmacophores. We presumed that variation in the nature and number of hydroxyl groups would be informative in understanding their C1-domain binding potencies. The esters of gallic acid (**1**) and protocatechuic acid (**2**) contain two and three hydroxyl groups, respectively. It is already documented that amendments of the hydrophobicity of the C1-domain ligands alter their C1-domain binding affinities to a certain extent. In this regard, a series of compounds with different side chains were synthesized. To understand the importance of branched side chains, we also prepared compounds **1b**, **1c**, **2b**, **2c**, and **3b−d** with 2-ethylhexanol, 5-nonanol, and 2-octanol.

Our molecular docking analysis demonstrated that the 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues are anchored to the C1-domain binding site (PDB code 1PTR) in a similar fashion as phorbol esters and DAGs.[32] The hydroxymethyl group of the ligands (**3b−e**) is hydrogen-bonded to the backbone amide proton of Thr-242, and the carbonyls of Thr-242 and Leu-251 (Figure 1 and Figure S1 in the Supporting Information (SI)), where the hydroxyl group of Thr-242 is hydrogen bonded with the hydroxymethyl group of the ligands. The model structures also showed that the 3-hydroxy group of ligand **3** is also hydrogen bonded to the backbone amide proton of Thr-242 and the carbonyls of Thr-242 and Leu-251 (Figure 2). However, the model structures did not show any hydrogen bonding with the backbone amide proton of Gly-253, an important interaction site for ligand binding to the C1-domain. We presumed that the ester carbonyl might be directly involved in interaction with lipid head groups or with the backbone of the amino acids through a bridging water molecule, which was not considered during the molecular docking analysis.

The esters of gallic acid and protocatechuic acid were directly synthesized using the *N,N′*-dicyclohexylcarbodiimide (DCC)-mediated coupling reaction with alcohols.[36] The 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues were conveniently synthesized according to Scheme 1. First, the formyl group was introduced to 4-hydroxybenzoic acid using hexamine in the

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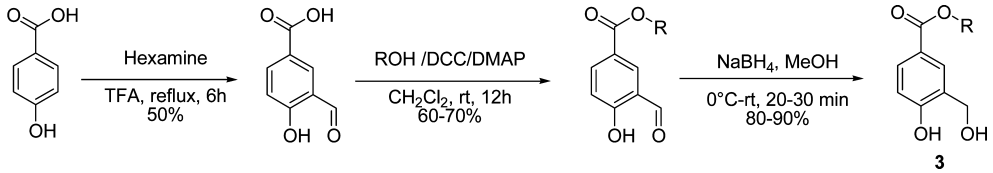
Scheme 1. Synthesis of 4-Hydroxy-3-(hydroxymethyl) Phenyl Ester Analogous to 4-Hydroxybenzoic Acid



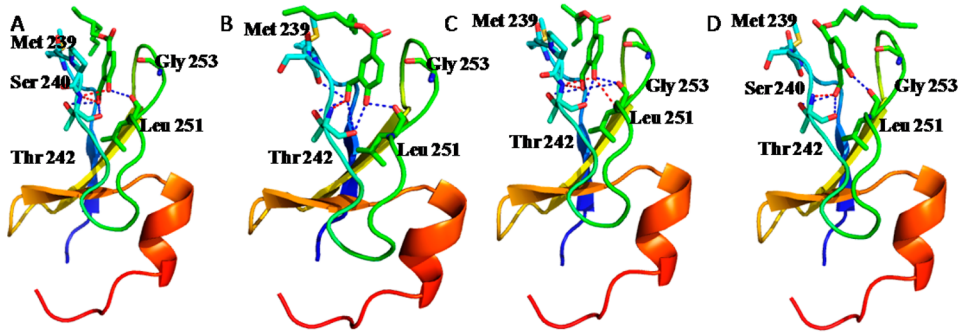


Figure 1. Structures of ligand-bound PKCδ-C1b subdomains. Modeled structure of **3b** (A), **3c** (B), **3d** (C), and **3e** (D) docked into PKCδ-C1b (1PTR) subdomain. The modeled structures were generated using the Molegro Virtual Docker, version 4.3.0. The oxygen atoms and nitrogen atoms are shown in red and blue, respectively. The dotted line indicates possible hydrogen bonds.

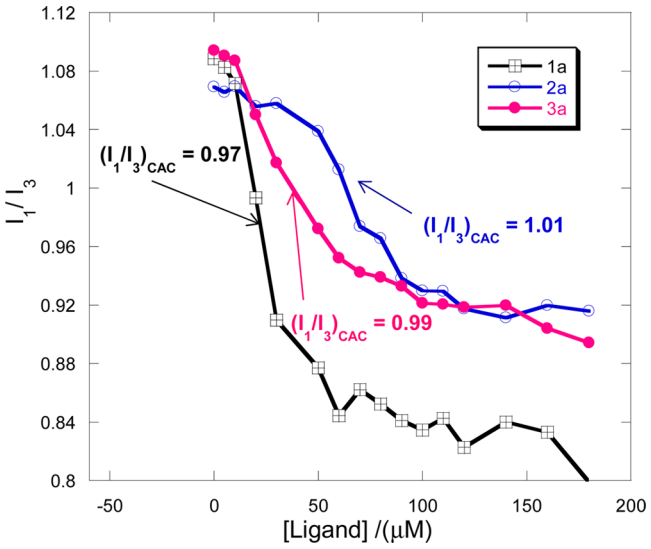


Figure 2. Measurement of critical aggregation concentration of compounds **1a**, **2a**, and **3a** in aqueous solution. Plot of pyrene fluorescence intensity ratio I_1/I_3 against increasing concentration of compounds. [pyrene] = 2 μM, λ_{ex} = 380 nm.

presence of trifluoroacetic acid under reflux condition.[37] The 4-hydroxy-3-formylbenzoic acid was then esterified with alcohols using the DCC-mediated coupling reaction condition.[36] Finally, the reduction of formyl group with $NaBH_4$ provided the target compounds **3a**–**e**.[8] The synthesized compounds were systematically characterized NMR and mass spectral analyses.

Aggregation Studies of the Compounds. The aggregation behavior of the membrane-active compounds was first investigated in aqueous solution by measuring the fluorescence properties of pyrene.[27] Pyrene has been widely used as a polarity indicator to investigate the aggregates formation of the amphiphilic molecules in aqueous solution.[38] We measured the critical aggregation concentration (CAC), an analogy to illustrate critical micelle concentrations for surfactants using pyrene in the presence of compounds in aqueous solution. It is already demonstrated that the shape and intensity of the fluorescence emission spectra of pyrene are very sensitive to its microenvironment.[39] Concentration-dependent aggregation of the compounds and simultaneous incorporation of pyrene molecules into its hydrophobic core is reflected by the increase in fluorescence intensity of the pyrene emission maximum (Figure S2 in the SI). Increase in compound concentrations from 0 to 180 μM resulted in a continuous increase in pyrene emission maxima, supporting a continuous decrease in polarity of the medium. The intensity ratio of I_1/I_3 of pyrene is considered as a measure of the polarity of its microenvironment. Above the CAC values, the higher and lower ratios of I_1/I_3 indicate the polar (loose aggregates) and hydrophobic (compact aggregates) environment. The measured CAC of the compounds **1a**, **2a**, and **3a** were 27, 63, and 40 μM, respectively (Figure 2). The plot of I_1/I_3 with compound concentration also suggests that, above 60–80 μM compound concentrations, the ratio of I_1/I_3 reached a somewhat flat terrain. Interestingly, with further increase in compound concentrations the I_1/I_3 ratio gradually decreases, indicating that these compounds form loosely bound aggregates in aqueous solution with a wide concentration range. We hypothesize that this noncompactness of the compound aggregates could be due to their specific molecular structures (Figure 2). However, further characterization of these aggregates is beyond the scope of this investigation. These compounds with hydrophobic cetyl alcohol and hydrophilic gallic acid, protocatechuic acid, and 4-hydroxy-3-(hydroxymethyl)benzoic acid are expected to form aggregation in aqueous solution. The plot also shows that all these compounds aggregate at lower concentrations in aqueous solution, probably because of the absence of charges in the headgroups. This concentration range of aggregates formation of these amphiphilic molecules is essential in understanding their interaction pattern with lipid bilayers and PKC-C1 domain under monomeric form in aqueous solution.

Interaction with Lipid Bilayers. Membranes protect tissues and cells from foreign molecules and also select the cellular penetration of foreign compounds with certain

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Table 2. Partition Coefficient and Mole Fractions of 1-Naphthol within the DPPC Vesicle in the Absence and Presence of Compounds

liposomes	partition coefficient (K_p) and mole fractions of NpOH		
	30 °C, SG phase	41 °C at T_m	53 °C, LC phase
DPPC only	(0.77 ± 0.04) × 10^5; 12.66 ± 2%	2.95 ± 0.12 × 10^5; 35.79 ± 1%s	(0.88 ± 0.11) × 10^5 ; 14.21 ± 2%
DPPC + **1a** (100 μM)	(4.29 ± 0.20) × 10^5; 85.53 ± 1%		(1.76 ± 0.16) × 10^5; 24.90 ± 2%
DPPC + **2a** (100 μM)	(2.64 ± 0.15) × 10^5; 51.85 ± 1%		(1.25 ± 0.11) × 10^5; 19.05 ± 1%
DPPC + **3a** (100 μM)	(3.91 ± 0.19) × 10^5; 57.88 ± 1%		(1.78 ± 0.12) × 10^5; 25.20 ± 1%

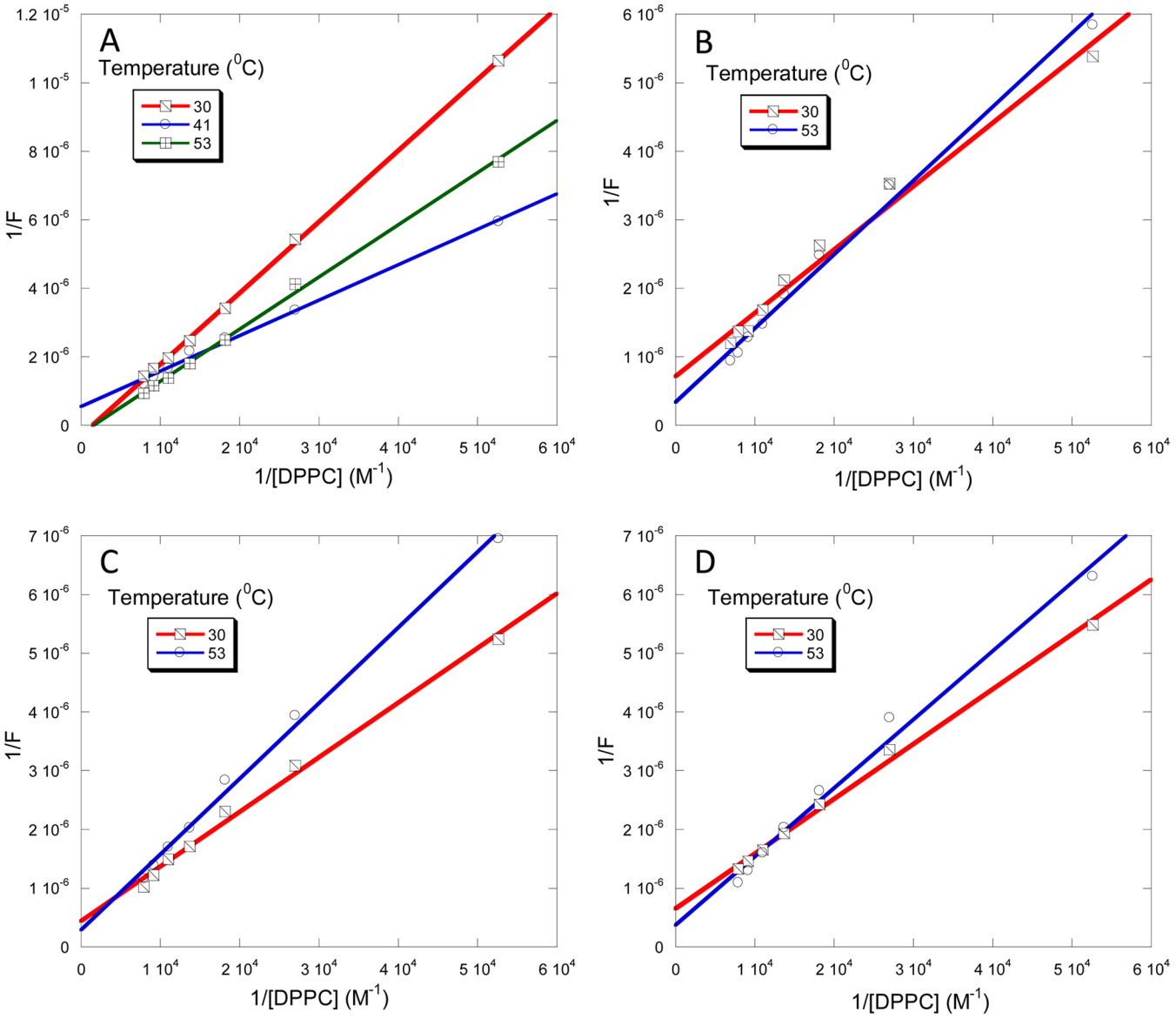

Figure 3. Double reciprocal plot of NpOH* fluorescence intensity vs DPPC concentrations in the absence of compound (A), and in the presence of different concentrations of compounds **1a** (B), **2a** (C), and **3a** (D) in SG (30 °C) and LC (53 °C) phases; λ_{ex} = 290 nm, λ_{em} = 370 nm, [1-naphthol] = 4 μM.

biochemical or physiological role. Consequently, the distribution of the foreign compounds in the body is largely affected in terms of time and concentration. For some of these foreign compound membranes, residency is crucial for their optimum biological activities. In consequences, the interaction of drug or druglike molecules with the lipid bilayer structures plays an important role in understanding their pathological processes.[39] The properties of cellular membranes, including fluidity, permeability, surface potential, and others, get affected by the distribution of drug or druglike molecules. Peripheral proteins like PKC and others get activated at the membrane interface primarily due to their interaction with the membrane-localized lipophilic ligands. The membrane binding surface and ligand binding groove of the C1-domains of PKC proteins interact with the membrane-localized lipophilic second messenger, DAGs, and control the cellular activities of PKCs. Fluidity of the lipid bilayer and loose spacing in the polar headgroup region also play a significant role on the PKC kinase activities.

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Ability of the PKC-active compounds to localize at the membrane−water interface is directly linked with its C1-domain binding properties. Therefore, the C1-domain ligands would be expected to interact significantly with the lipid bilayer and alter membrane properties including, fluidity, permeability, and others. In this regard, the interactions of 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues with the liposomes were studied by investigating their effect on permeability and phase transition of lipid bilayer and extent of membrane localization patterns.

Effect of Lipophilic Compounds on Membrane Permeability. Recent studies described that the excited-state proton transfer (ESPT) of 1-naphthol (NpOH) is sensitive to the change in polarity of its microenvironment.[40] The activated NpOH emits fluorescence signal from its neutral form (NpOH*) in nonpolar media and anionic form (NpO^-*) in aqueous media. The change in lipid bilayer organization was investigated by using this fluorescence property of NpOH. The ratio of fluorescence intensities of anionic form (λ_{em} = 470 nm) to neutral form (λ_{em} = 370 nm) is sensitive to the microenvironment of NpOH. Under liposomal environment, the fluorescence signal of NpOH* is due to the membrane-bound fraction of NpOH, while the NpO^-* emission is because of the presence of NpOH in the semipolar membrane interface section and water-soluble fraction of NpOH.

We measured the partition coefficient (K_p) values of NpOH in the absence and presence of the membrane-active compounds containing model liposomes, to understand their effect on membrane permeability. Dipalmitoylphosphatidylcholine (DPPC) liposomes were used as a model lipid bilayer due to their high abundance in cellular membranes. The cellular membrane also contains several other lipids, but addition of other lipids would make it difficult to monitor the membrane permeability and lipid−bilayer interaction properties of the compounds. The fluorescence intensity of NpOH decreases both in solid gel (SG) and liquid crystalline (LC) phases of the DPPC liposomes with increasing concentration of the compounds (Figure S3 in the SI). The changes in K_p values were measured by monitoring the changes in NpOH* fluorescence intensity. The calculated K_p values of NpOH provide information regarding membrane permeability of the DPPC bilayer in the absence or presence of compounds (Table 2). We used compounds **1a**, **2a**, and **3a** with the same chain length for this study as they would reveal the effect of different headgroups of the compound on the permeability of the DPPC lipid bilayer. Membrane permeability of small molecules like NpOH is altered by the nonpolar part of the amphiphilic compounds and nature and chain length of the fatty acid present within the membrane-active compounds. Figure 3 shows the double reciprocal plot of NpOH* fluorescence intensity vs DPPC liposome concentration in the absence or presence of compounds at different temperatures. The calculated K_p values and mole fractions of NpOH (Table 2) in DPPC liposomes in different lipid phases showed that partitioning of NpOH is significantly higher at the phase transition temperature of the DPPC lipids, where the permeability of the lipid bilayer is expected to be higher because of the coexistence of solid and liquid domains. The results also showed that the K_p value and mole fraction of NpOH partitioning into the lipid bilayer increase in the presence of compounds in both SG and LC phases of the liposomes. In the absence of DPPC liposomes, there is no NpOH* emission peak with varying concentrations of the compounds. This indicates that the compounds have no direct interactions with the NpOH molecules. Therefore, the increase in K_p values could be due to the increase in permeability of the compound to the lipid bilayers. However, the increase in K_p values of NpOH in the presence of compounds was more in SG phase than that in LC phase. These could be due to the "synergistic effect" of hydrophobic part of DPPC lipid and the hydrophobic part of the ligands on the lipid bilayer to enhance the membrane permeability in the SG phase.[41] The lateral diffusion of the lipids in the SG-phase membrane is at least 2 orders of magnitude smaller than that in the LC phase, which directs the membrane to reseal at a much faster rate in the LC phase than in the SG phase. This could lead to the difference in NpOH permeability in both the phases.[41] The NpOH* fluorescence intensity decreases with the increase in compound concentrations (Figure S3 in the SI). This could be either due to the higher membrane permeability of NpOH in the presence of compounds or could be due to the presence of water molecule within the lipid bilayer. The results suggest that the amphiphilic nature of the compounds enhances its interaction with the lipid bilayer membrane with an increase in concentration.

Extent of Membrane Localization of the Compounds. The partition coefficient values of NpOH clearly showed that the compounds strongly interact with the lipid bilayer. The decrease in NpOH* fluorescence intensity with the increase in compound concentration also suggests that these synthesized compounds altered the packing of the DPPC headgroups. The hydrophilic part of the amphiphilic compounds are expected to be positioned near the bilayer/water interfacial region while the hydrophobic part gets inserted deep into the hydrophobic environment of the DPPC membrane. However, the extent of localization of the pharmacophores-containing hydrophilic part at the bilayer/water interface is one of essential criteria for their ability to interact with the PKC-C1 domains under the liposomal environment. In this regard, we performed sodium dithionite-induced NBD fluorescence quenching experiments using DPPC/ligand/NBD-PE liposomes. The NBD probe is embedded close to the bilayer/water interface, providing a useful marker for surface interactions of membrane-active C1 domain ligands. The extent of NBD fluorescence quenching provided a measure of membrane localization of the compounds.[8] Figure 4 reveals that ligand-associated fluorescent liposomes showed considerable changes in the rate of dithionite-induced fluorescence quenching of the bilayer-embedded probe. The compounds showed slower quenching rates than the liposomes without any ligand. The results indicate that the NBD probe became more "shielded" from the soluble dithionite quencher, due to the presence of membrane-active compounds in the liposomes. The results also imply that the pharmacophores containing the hydrophilic part of these compounds are more localized at the liposome surface compared to DAG_{16}. Figure 4 also indicates different degrees of localization and perturbation of the bilayer headgroup region by the compounds. Therefore, perturbation of the membrane environment by these compounds may augment the ability of molecules such as NBD and eventually PKC-C1 domain to insert into the membrane bilayer. The results also imply that these ligands are more localized at the bilayer/water interface and more accessible for the PKC-C1 domain binding than DAG. To further investigate the interactions of compounds with lipid bilayers and their effects upon the bilayer properties,

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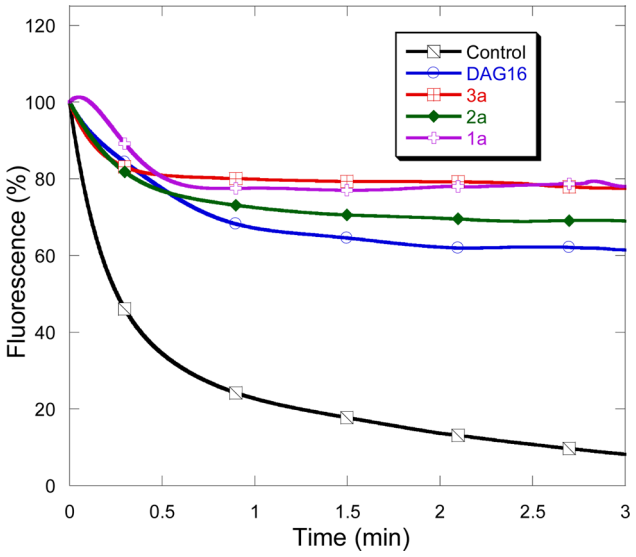

Figure 4. Fluorescence quenching of NBD-PE embedded in PC/ligand$_{16}$/NBD-PE (89:10:1) liposomes. Sodium dithionite = 0.6 μM. Control: no ligand.

we measured the effect of compounds on the phase transition temperature of lipid bilayer.

Effect of Compounds on the Phase Transition Temperature of Lipid Bilayer. Phase transition temperature (T_m) of the bilayers depends on its lipid compositions and it provides information regarding the fluidity of the lipids bilayer.[42] The lipid bilayer organization is affected by the properties and chain length of fatty acids and the headgroup of the lipids. To further probe the interaction of the synthesized membrane-active compounds with lipid bilayers and their effect on the fluidity of the bilayer, we measured T_m values of DPPC bilayers in the absence and presence of compounds. We used membrane-active compounds **1a**, **2a**, and **3a** for this study as it would also reveal the effect of hydrophobic and hydrophilic part of the compounds on the T_m value of the DPPC lipid bilayer. The DPPC was used as a model lipid for bilayer formation. The polarity probe, NpOH, was successfully used to study the thermotropic phase behavior of the lipid bilayer by monitoring changes in the fluorescence intensity of NpOH*. Coexistence of SG and LC phase at the transition temperature makes the bilayer less resistant, allowing local maximum in the fluorescence intensity of NpOH*. Therefore, we monitored the NpOH* fluorescence intensity change to measure the T_m values. The variation in NpOH* fluorescence intensity with temperature demonstrates that phase transition of only DPPC lipid bilayer occurs at 40 °C (Figure 5A) which is in agreement with the reported value measured by differential scanning calorimetry (DSC).[43] The results showed that the T_m values of DPPC bilayer increase from 40 to 43 °C in the presence of compounds. The nature of thermotropic phase behavior also shows that the DPPC bilayer organization remains intact under these experimental conditions (Figure 5A). The increase in the T_m value of the DPPC bilayer in the presence of compounds can be attributed to the increase in van der Waals interactions between hydrophobic moieties and alteration of lipid packing by defluidizing the region. The smaller changes in T_m values are in accordance with the NBD fluorescence quenching data, which indicate prevalent bilayer-surface localization of the compounds. The localization of the headgroup of the compounds hardly affects the thermotropic phase behavior of the lipid bilayers; it primarily depends upon the "tails" of the compounds. Interestingly, the K_p values of NpOH showed that permeability of NpOH increases more in the SG-phase than LC phase for the compound containing liposomes and the measured T_m values also suggest that the membrane-active compounds prefer the SG phase over the LC phase. We also measured the phase transition temperature of PC/PE/PS (60:20:20) liposome in the absence and presence of compounds (Figure 5B). The measured T_m values clearly showed that the liposomes with such complex composition are more fluidic in the presence of compounds. This change in T_m values could be due to the direct effect of the anionic phospholipids on the membrane fluidity or could be because of the lipid packing and solvation effects.

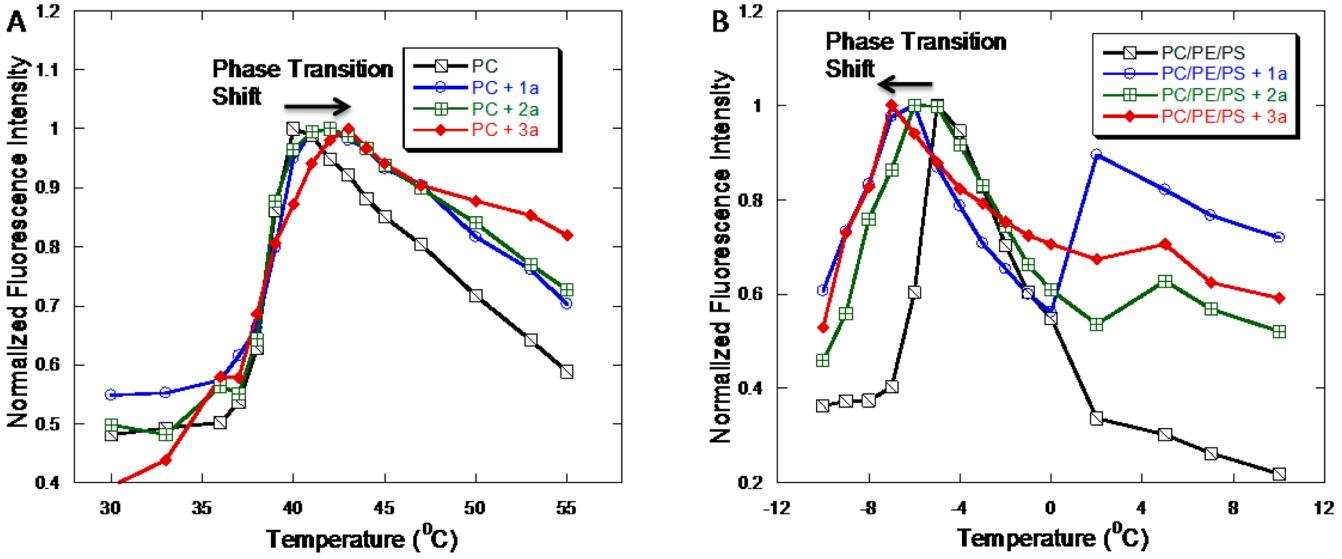

Figure 5. Effect of compounds on the phase transition temperature of only PC (A) and PC/PE/PS (B) vesicles. λ_{ex} = 290 nm, λ_{em} = 370 nm, [1-napthol] = 4 μM, [compound] = 200 μM. PC/PE/PS liposome composition 60:20:20, [DPPC] = 0.2 mM, [PC/PE/PS] = 0.2 mM. The maximum fluorescence intensity was normalized to 1 for better understanding of the phase transition shift by the compounds.

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Protein-Binding Studies. We used the PKCθ- and PKCδ-C1b subdomains to measure the in vitro binding properties of the (hydroxyl) phenyl ester and 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues. The DAG-responsive C1-domain of PKCθ and PKCδ isoenzymes is duplicated into a tandem C1 domain consisting of C1a and C1b subdomains. These C1b subdomains are reported to have adequately strong DAG binding affinities and easy to purify from bacterial cells. The intrinsic fluorescence properties of the PKCθ- and PKCδ-C1b subdomains is due to the presence of single tryptophan (Trp-31 in theta, Trp-252 in delta) and tyrosine residues (Tyr-15 and Tyr-17 in theta, Tyr-236 and Tyr-238 in delta), respectively. The protein-binding properties of the 4-hydroxy-3-(hydroxymethyl) phenyl esters were measured by Trp-fluorescence quenching methods, steady-state fluorescence anisotropy, and Förster resonance energy transfer (FRET)-based competitive binding assay.

Interaction with Soluble Ligands. The intrinsic fluorescence properties of the proteins are widely used to examine the changes in microenvironment or protein conformation caused by the ligand binding. The PKCθ- and PKCδ-C1b subdomains contain single Trp residue close to the ligand binding site, which provides an ideal system to monitor ligand binding properties by monitoring intrinsic fluorescence properties. The protein-binding properties of the compounds in aqueous solution was first measured by in vitro Trp-fluorescence quenching method. The compound concentrations used during measurements were well below their CAC values. This clearly indicates that the compounds were mostly in monomeric form under the experimental conditions. Figure S4 in the SI shows a representative plot of Trp-fluorescence quenching data for PKCθ-C1b subdomain in the presence of ligands in Tris buffer pH 7.4. The binding isotherms showed that the synthesized compounds quenched the Trp-fluorescence (340 nm) in a concentration-dependent manner, and a plateau was reached at around 15−20 μM (Figure 6). The binding parameters clearly showed that the compounds with different hydrophobic moieties strongly interact with the PKCθ and PKCδ-C1b subdomains. The compound **3c** showed the highest affinity (0.60−0.74 μM) and other compounds have comparable binding affinities for both the proteins. The compound **3c** showed more than 9- and 12-fold stronger binding affinity than DAG_{16} for PKCθ-C1b and PKCδ-C1b subdomain, respectively (Table 3), whereas compound **3a** with long-chain alcohol also showed more than 7- and 9-fold stronger binding affinity than DAG_{16} for the PKCδ-C1b and PKCθ-C1b subdomains, respectively. It is well documented that an aliphatic hydroxyl group is indispensable for PKC ligands such as phorbol esters, teleocidins, aplysiatoxins, bryostatins, and DAGs to bind PKC C1 domains.[44−46] Moreover, there is large difference in acidity (pK_a values) between the phenolic hydroxyl group and the hydroxymethyl group. To understand the role of acidity of the hydroxyl groups in PKC-C1 domain binding, we calculated the pK_a values of the compounds **1c**, **2c**, and **3c** with the same hydrophobic moiety. The pK_a values of the hydroxyl groups were calculated using ChemAxon MarvinSketch v.5.3.3 software. We also calculated the fraction of protonated and deprotonated forms of the compounds at pH 7.4 using standard Henderson−Hasselbalch equation (Table S1 in the SI). The calculated data shows that, in buffer at pH 7.4, the phenolic hydroxyl group of compound **3c** remains in neutral form (96.9%), whereas for compounds **2c** and **1c** the contribution of deprotonated form at pH 7.4 increases to 6.5% and 18.3%, respectively (Table S1). It is also documented that the deprotonated form of the phenolic hydroxyl groups do not form strong hydrogen bonding, but their binding affinity difference is not that significant. We presume that the small binding affinity difference between **3c** with **2c** and **1c** could be due to the electrostatic interaction of compounds **1c** and **2c** with the C1-domains through their anionic lipid binding groove. It is also important to note that the compound **1c** with 18.3% deprotonated form has 3.7-fold stronger binding affinity than compound **2c** with 6.5% deprotonated form under the similar experimental conditions. However, these binding parameters of the compounds in monomeric form do not show a clear PKC-isoform specificity. Higher binding affinities of compounds **3a−e** for PKCθ-C1b than PKCδ-C1b

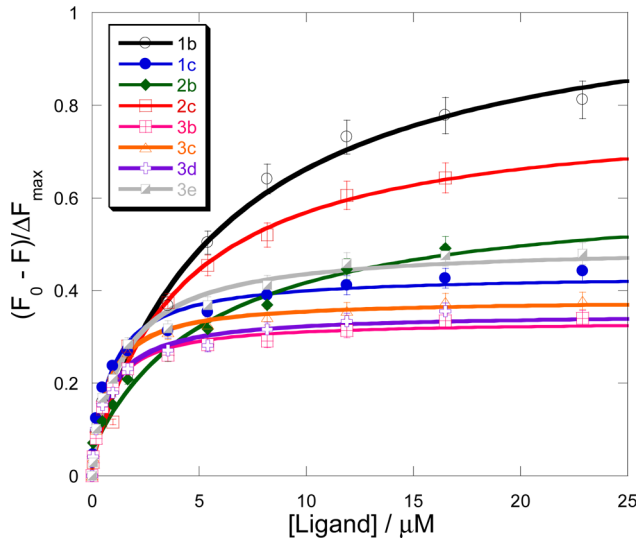

Figure 6. Binding of compounds with the PKCθ-C1b subdomain. Representative plot of Trp-fluorescence intensity of PKCθ-C1b (1 μM) in buffer (20 mM Tris, 160 mM NaCl, 50 μM $ZnSO_4$, pH 7.4) in the presence of varying concentrations of **1−3**, where F and F_0 are fluorescence intensities in the presence and absence of the ligands, respectively. The solid lines are nonlinear least-squares best-fit curves.

Table 3. K_D(ML) Values for the Binding of Ligands with the PKCδ-C1b and PKCθ-C1b Proteins[a] at Room Temperature

	K_D(ML) (μM)	
compd	PKCθ-C1b	PKCδ-C1b
DAG_8	6.74 ± 0.54	12.41 ± 0.59
DAG_{16}	6.35 ± 0.37	7.04 ± 0.43
1a	1.16 ± 0.17	1.35 ± 0.19
1b	2.16 ± 0.17	–
1c	1.08 ± 0.15	–
2a	2.19 ± 0.16	2.41 ± 0.17
2b	5.63 ± 0.25	–
2c	3.98 ± 0.26	–
3a	0.75 ± 0.05	1.07 ± 0.18
3b	0.79 ± 0.09	1.28 ± 0.11
3c	0.74 ± 0.08	0.60 ± 0.06
3d	0.80 ± 0.07	0.84 ± 0.13
3e	1.30 ± 0.08	1.81 ± 0.16

[a]Protein, 1 μM in buffer (20 mM Tris, 160 mM NaCl, 50 μM $ZnSO_4$, pH 7.4). Values represent the mean ± SD from triplicate measurements.

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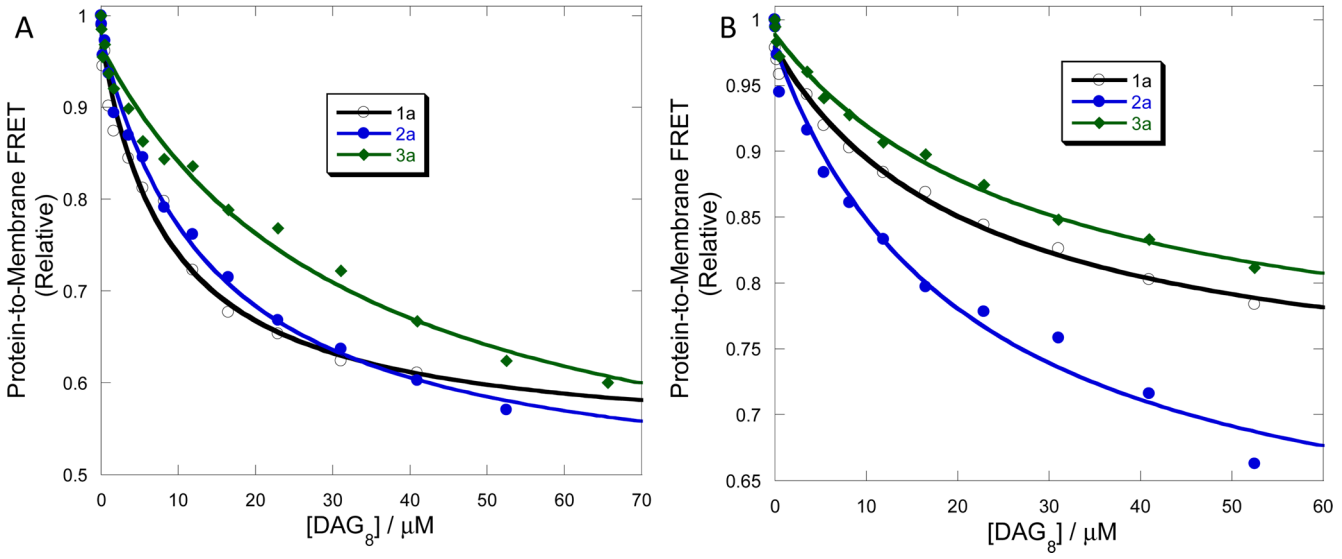

Figure 7. Competitive displacement assay for the PKCδ-C1b (A) and PKCθ-C1b (B) subdomains (1 μM) bound to liposome containing ligands **1a, 2a**, and **3a**. The bound complex was titrated with the DAG_8.

subdomain could be because of the additional hydrogen bond formation with the DAG/phorbol ester binding site. Nevertheless, there is a very small difference in monomeric binding affinity between compounds **3b−3d** with the C1-domains. We hypothesize that the strong binding of the pharmacophores with the C1-domain suppresses the effect of hydrophobicity of the branched alkyl chains.

The molecular docking analysis indicates that the compounds **1c**, **2c**, and **3c** with the same hydrophobic moiety presumably form five to seven hydrogen bonds between DAG and PKCδ-C1b. Compound **1c** with three hydroxyl groups forms seven hydrogen bonds with the PKCδ-C1b subdomain. Compound **3c** with one hydroxymethyl and one hydroxyl groups forms five hydrogen bonds with the PKCδ-C1b subdomain. However, the binding parameters of the monomeric ligands (K_D(ML)) showed that compound **3c** has 1.5- and 5.4-fold strong binding affinity for the PKCθ-C1b subdomain than compounds **2c** and **1c**, respectively. This points out that the presence of a hydroxymethyl group presumably anchored the ligand within the DAG/phorbol ester binding site, allowing the compounds to interact strongly with the C1-domains, whereas the hydroxyl group at the *para*-position allows the compounds **3a−e** to interact more strongly through H-bond formation, as indicated by the molecular model structures (Figure 1).[8,12,15,22,23] However, the measured binding parameters and docking scores obtained from the models do not always agree. This dissimilarity signifies that both protein and ligand can experience conformational adjustments under experimental circumstances and the involvement of bridging water molecules between ligand and protein could produce strong interactions. Therefore, both hydroxymethyl and hydroxyl groups of 4-hydroxy-3-(hydroxymethyl) phenyl ester analogues are important for their interaction with the PKC-C1 domains. We deduce that the stronger C1-domain binding affinity of the synthesized compounds than DAG could be because of their structural rigidity, which decreases the number of probable rotameric forms of the compounds. We also assume that one of the rigid rotamers would mimic the explicit conformation of physiologically active DAG. The stronger binding of compounds to PKC-C1 domain than DAG apparently governed by the structural constrains to reduce the entropic loss due to binding.

The protein-binding parameters of the monomeric compounds in aqueous solution suggest that, like other C1-domain ligands, the synthesized compounds show a comparable pattern of hydrophobicity dependence. For compounds **3a** (LOGP = 7.56), **3c** (LOGP = 4.50), and **3e** (LOGP = 4.01), although there is a discrete difference in the hydrophobicity (Table 1), the difference in binding affinity is very small for both the proteins. This could be due to the ligand-binding orientations or side-chain specificity, which was already observed for PKC-C1 domains. It is well documented that the hydrophobic residues surrounding the ligand-binding site of the C1-domain also interact with the hydrophobic side chains of the ligands. The overall higher binding affinities of the synthesized compounds in aqueous solution are possibly because of the hydroxymethyl and hydroxyl groups in the phenyl ring, and the binding affinity difference could be due to the presence of the hydrophobic part of the ligands. Thus, the binding affinity values of the compounds highlight the importance of ligand hydrophobicity and binding orientation, in a manner similar to those reported for C1-domain ligands.

The measured steady-state fluorescence anisotropy data also shows that the compounds strongly interact with the C1b subdomains in aqueous solution. The anisotropy values of the proteins increase in the presence of DAGs and synthesized compounds. As an example, the anisotropy value of the pure PKCθ-C1b protein increases from 0.0682 to 0.3221 upon interaction with 10-fold excesses of ligands **3c** (Table S2 in the SI) in buffer. Even though the changes in anisotropy values do not corroborate with the binding affinity values in aqueous solution, these measurements still suggest that the presence of the compounds increases the rigidity of the surrounding environment of the protein in a manner similar to that of DAGs.

Interaction with Ligand-Associated Liposomes. The membrane-binding surface, surrounding the ligand-binding site of the C1 and C2-domains, allows these protein to interact with the cellular membranes. The interaction of the C1-domains with DAGs and other ligands localized at the cellular

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Table 4. Equilibrium Parameters for PKCδ-C1b and PKCθ-C1b Protein[a] Binding to the Ligand-Associated Liposomes[b] at Room Temperature

	$K_I(DAG_8)_{app}$ (μM)		K_D(L) (nM)	
compd	PKCθ-C1b	PKCδ-C1b	PKCθ-C1b	PKCδ-C1b
1a	8.93 ± 0.15	8.75 ± 0.36	394.13 ± 10.21	481.62 ± 9.01
2a	14.23 ± 1.19	13.91 ± 1.61	480.20 ± 11.23	553.25 ± 12.31
3a	33.05 ± 1.58	28.82 ± 1.14	61.30 ± 6.87	101.79 ± 7.67

[a]Protein, 1 μM in buffer (20 mM Tris, 150 mM NaCl, 50 μM ZnSO4, pH 7.4). [b]Active liposome composition, PC/PE/PS/dPE/ligand (55:15:20:5:5).

membrane fully activate the PKC enzymes. Therefore, to understand the C1-domain binding properties of the synthesized compounds under the membrane environment, we used protein to membrane FRET-based competitive binding assay. The Trp-residue of the PKC-C1b subdomains provides as the FRET donor, and a low density of membrane-embedded, dansyl-PE (dPE) lipid serves as the acceptors.[8] Additional PE was used to improve the stability of the vesicles. PS is known induce the DAG-dependent membrane binding of the C1-domain because of its interaction with the cationic groove of the C1-domains. Hence PS was also incorporated into the vesicles. DAG_8 was titrated into the solution containing C1b-subdomain-bound liposomes. The decrease in the protein-to-membrane FRET signal (Figure S5 in the SI) was examined to quantitatively measure the displacement of protein from the bilayer surface to the bulk solution, and apparent inhibitory constant [$K_I(DAG_8)_{app}$] calculation. Figure 7 represents DAG_8-promoted competitive displacement of the PKCδ-C1b subdomain from ligand-associated liposomes (PC/PE/PS/dPE/ligand). The measurements were performed using membrane-active compounds **1a**, **2a**, and **3a** with long chain (cetyl alcohol). The binding parameters showed that only compound **3a** strongly interacts with the C1b-subdomain. Compounds **1a** and **2a** have very weak binding affinity for the C1b-subdomain under the liposomal environment. In aqueous solution, compounds **1a**, **2a**, and **3a** have very strong binding affinities for the C1-domains. These results support that due to the higher acidity of the phenolic hydroxyl group, compounds **1** and **2** preferably interact with the C1b-subdomains through their cationic groove. This competitive binding assay confirmed that the compound **3a** preferably interacts with the PKC-C1b subdomains through its DAG/phorbol ester binding site. Therefore, the in vitro measurements pointed out that the depotonated form of the esters of gallic acid (**1**) and protocatechuic acid (**2**) affect their C1-domain binding capabilities through the DAG/phorbol ester binding site. Higher concentration of DAG_8 was required for the displacement of C1b-subdomains from the compound **3a** associated liposomes. The equilibrium dissociation constant (K_D(L)) for the PKC-C1b subdomains binding to the liposome-associated targeted ligand was calculated using eq 4. Comparison of the equilibrium dissociation constant also revealed that C1b-subdomains have higher binding affinity for the compound **3a** associated liposomes (Table 4).

The esters of gallic acid, protocatechuic acid, and 4-hydroxy-3-(hydroxymethyl)benzoic acid were conveniently synthesized in one to three steps from their starting materials. Some of these esters have several intriguing pharmacological activities including antioxidant, antitumor, and others. In the present study, we observe that the compounds with long aliphatic chain interact with the lipid bilayers and influence the bilayer properties including fluidity, permeability, and the phase transition temperature (Table 5). The hydrophilic moieties of the compounds are localized at the lipid/bilayer interface and

Table 5. Summary of the Physical Properties of the Membrane-Active Compounds

serial no.	physical properties of the compounds	
1	aggregation concentration in aqueous solution	**2a** < **3a** < **1a**
2	effect on membrane permeability with respect to 1-napthol	**1a** > **3a** > **2a**
3	extent of localization of the pharmacophores at the bilayer−water interface	**3a** > **1a** > **2a**
4	effect on the phase transition temperature of the lipid bilayer	**3a** > **1a** > **2a**
5	protein-binding affinity in aqueous solution	**3a** > **1a** > **2a**
6	protein-binding affinity under liposomal environment	**3a** > **2a** > **1a**

the pharmacophores are accessible for PKC-C1 domain binding under liposomal environment. The hydroxymethyl and hydroxyl groups and ester group are required for binding activity of the compounds to the C1-domains. The membrane-active compounds can differentially influence the in vitro membrane interaction properties of PKCδ and PKCθ enzymes. The compounds **3a**−**e** have stronger binding affinity for the C1-domains, but their affinity differences are negligible except for compound **3e**. The higher binding affinity of the compound **3a** over **3e** for the C1-domains could be either due to true selectivity for the acyl group or the effect of compound **3a** on the lipid bilayer organization. We hypothesize that increase in fluidity makes the structure of the bilayer more loosely packed, allowing the hydrophobic surface of the C1 domains to penetrate into the hydrophobic core of the lipid bilayer in a ligand-dependent binding manner, which is essential for the PKC enzyme activation. However, the stronger binding affinities of compounds **3b**−**d** could be because of the presence of branched alcohols, which strongly interact with the hydrophobic residues surrounding the ligand binding site of the C1 domains. Overall stronger binding affinities of compounds **3a**−**e** for the C1-domains could be due to the presence of the additional hydroxyl group at the *meta*-position. The binding affinity differences between PKCδ- and PKCθ-C1b domains could be because of the differences in surface areas and the residues present within the binding site. The PKC activation ability of the compounds can be lower than that of phorbol ester and other natural products under similar experimental conditions.

CONCLUSIONS

Taken together, our results show that 4-hydroxy-3-(hydroxymethyl)phenyl ester analogues strongly interact with

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the model membrane and C1-domain of novel PKC enzymes. Strong interaction between compounds and model membrane increase fluidity, allow partitioning small molecules like 1-naphthol into the hydrophobic core of the membrane, and alter the phase transition temperature of membrane. The increase in fluidity of the membrane structure could allow the C1-domian to interact strongly with the compounds in the presence of anionic phospholipids. The active compounds interact with the C1-domain through its DAG/phorbol ester binding site and also can compete with DAG for binding to the C1-domain under liposomal environment. The results indicate that hydroxyl, hydroxymethyl, and acyl groups of the compounds are important for protein binding. However, the pK_a value of the hydroxyl groups is important in their protein-binding capabilities under the physiological conditions. Our findings suggest that the 4-hydroxy-3-(hydroxymethyl)phenyl ester analogues are potential regulators of PKC isoforms and can be used in PKC-based drug development.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Characterization data of the synthesized compounds. Molecular docking results of the compounds. Monomeric binding assay and FRET-based competitive binding assay of the compounds. Copies of ^{1}H, ^{13}C NMR, and MS spectra of the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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