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**PAPER**

# Development of diacyltetrol lipids as activators for the C1 domain of protein kinase C†

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The protein kinase C (PKC) family of serine/threonine kinases is an attractive drug target for the treatment of cancer and other diseases. Diacylglycerol (DAG), phorbol esters and others act as ligands for the C1 domain of PKC isoforms. Inspection of the crystal structure of the PKCδ C1b subdomain in complex with phorbol-13-*O*-acetate shows that one carbonyl group and two hydroxyl groups play pivotal roles in recognition of the C1 domain. To understand the importance of two hydroxyl groups of phorbol esters in PKC binding and to develop effective PKC activators, we synthesized DAG like diacyltetrols (DATs) and studied binding affinities with C1b subdomains of PKCδ and PKCθ. DATs, with the stereochemistry of natural DAGs at the *sn*-2 position, were synthesized from (+)-diethyl L-tartrate in four to seven steps as single isomers. The calculated $EC_{50}$ values for the short and long chain DATs varied in the range of 3–6 μM. Furthermore, the fluorescence anisotropy values of the proteins were increased in the presence of DATs in a similar manner to that of DAGs. Molecular docking of DATs (**1b–4b**) with PKCδ C1b showed that the DATs form hydrogen bonds with the polar residues and backbone of the protein, at the same binding site, as that of DAG and phorbol esters. Our findings reveal that DATs represent an attractive group of C1 domain ligands that can be used as research tools or further structurally modified for potential drug development.

## Introduction

Mammalian cells contain many structurally distinct forms of *sn*-1,2-diacyl-glycerols (DAGs) that differ with respect to the type and degree of unsaturation of their fatty acyl groups.[1–3] Under equilibrium conditions, mammalian cells generally contain low concentration of DAGs.[1,4] When cells are stimulated by various stimuli, hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)$P_2$) catalyzed by phosphatidylinositol-specific phospholipase C (PLC) rapidly produces DAGs at the plasma membrane (PM) that act as second messengers to activate protein kinase C (PKC) and other proteins.[5–9] The other product of PtdIns(4,5)$P_2$ hydrolysis, inositol-1,4,5-triphosphate ((1,4,5)$IP_3$) releases $Ca^{2+}$ from ER which also activates various proteins, including $Ca^{2+}$-dependent PKC isoforms.[5–15] PKC is a family of serine/threonine kinases that is a major cellular receptor for DAG/tumor promoting phorbol esters. PKC isoenzymes play an important role in pathology of several diseases such as cancer, diabetes, stroke, heart failure and Alzheimer's disease.[6,11,13,16] Therefore, PKC isoforms particularly in the cancer field have been a subject of intensive research and drug development.[17]

The PKC isoforms are activated by DAGs and transmit their signal by phosphorylating specific proteins. The PKC protein family consists of at least eleven known isoforms, categorized into conventional (calcium, DAG and phospholipid-dependent), novel (calcium-independent, but DAG and phospholipids dependent) and atypical (calcium and DAG-independent) subgroups.[16,18] Other families of signaling proteins like Ras guanyl nucleotide-releasing proteins (RasGRPs), chimerins, protein kinase D (PKD), Unc-13 scaffolding proteins, myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK), and the DAG kinases β and γ also share the C1 domain with PKC isoforms.[9,16,19] The DAG molecules selectively interact with proteins containing a C1 domain. This interaction induces their translocation to the discrete subcellular compartments. For some of the C1 domain containing proteins, such translocation leads to activation.[16,20,21] The sizes of the C1 domains are small, overall structures are highly conserved and there is only one ligand binding site. Also, the number of C1 domain containing proteins is considerably smaller than the number of protein kinases and the catalytic domains of PKC isoforms are substantially homologous. Several studies already reported that the regulatory domain of PKCs may have independent biological functions. Therefore, C1 domains have become an attractive target in designing selective PKC ligands.[15,21–23]

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Several classes of structurally complex and rigid ligands such as phorbol esters,[24] bryostatins,[25] teleocidins,[26] aplysiatoxins,[27] ingenols,[28] and others that target the DAG binding C1 domain have been previously reported. Most of these C1 domain ligands are from nature or derivatives of natural products. Recently developed novel PKC isoforms specific indolactam[29] and benzolactam[30] derivatives are also structurally complex, so modification and large-scale production may be unfeasible. Many of these reported compounds have very strong affinity for C1 domains in comparison with the flexible, natural DAG agonists. The family of conformationally constrained DAG lactones overcomes the spread in potency between the natural product ligands and DAG.[31–35] Therefore, there is a clear and unmet need to design simple surrogates, whose structure could be easily modified to achieve higher specificity and selectivity among the C1 domains of the PKC isoforms. Recently reported simple isophthalic acid derivatives[15,21,23] have shown stronger affinity for the C1 domain of PKC isoforms. Some of the C1 domain ligands such as *N*-benzyladriamycin-14-valerate (AD 198),[36] calphostin C[37] and resveratrol[38] are already in clinical trials for the treatment of different types of cancer.

In this study, we describe the design, synthesis and binding properties of a novel class of tetrol based lipids, diacyltetrols (DATs), to the C1b subdomains of PKCδ and PKCθ. DATs interact with the C1b subdomains of PKC isoforms through the DAG or phorbol ester binding site and forms hydrogen bonds with the residues at the activator binding site. The results show that the positions of two hydroxyl groups play an important role in recognizing C1 domains of PKC isoforms.

## Results and discussion

We selected tetrol based unnatural lipids for the development of PKC modulators. The structures of C1 domain binding natural product ligands revealed the presence of more than one hydroxyl group. For example, the phorbol esters interact with the C1 domain through two hydroxyl groups including the other functionalities.[39,40] The X-ray structure of phorbol-13-*O*-acetate bound PKCδ C1b shows that the hydroxyl groups attached to C-20 and C-4, including the carbonyl group on C-3, form hydrogen bonds with the protein residues.[40] This leads us to develop diacyltetrols (DATs) containing two of the phorbol ester pharmacophores, the hydroxyl and the carbonyl functionalities within the same molecule. The DATs have very high structural similarity to the natural diacylglycerols (DAGs). The lipids **1**, **3** and **4** contain at least one hydroxymethyl group just as DAGs where as compound **2** have only two hydroxyl groups. The positions of the hydroxyl groups were varied to get more insight on which two hydroxyl groups would be better anchored to the binding pocket of the C1 domain. The presence of an additional hydroxyl group also gives us the opportunities for further development of PKC isoforms specific and selective tetrol based ligands.

The lipids were synthesized from commercially available (+)-diethyl L-tartrate. The $C_2$ symmetric diethyl L-tartrate was used to avoid mixing of enantiomeric products during synthesis. The choice of stereochemistry of lipids is crucial, as the diversity of stereochemistry of these structures complicates the specificity. The (+)-diethyl-L-tartrate provides the stereochemistry

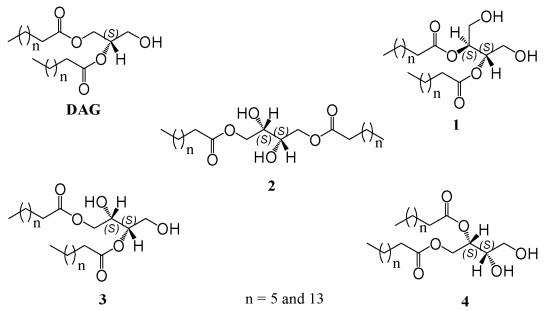
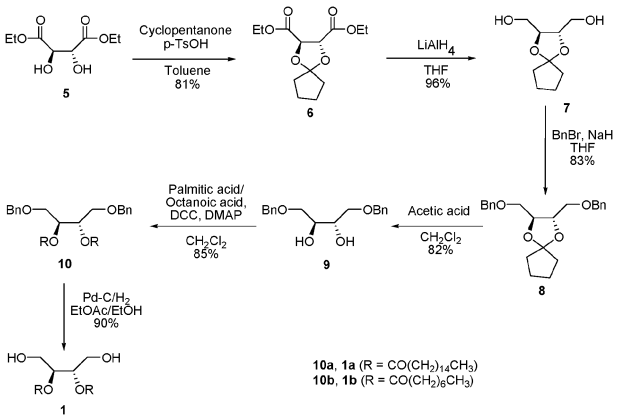



**Fig. 1** Structure of diacylglycerols and diacyltetrols (**1–4**) used for the present study.

as there in natural DAGs at the *sn*-2 position. The tetrol based lipids 1,4-dihydroxy-diacyltetrol (**1**), 2,3-dihydroxy-diacyltetrol (**2**), 1,3-dihydroxy-diacyltetrol (**3**) and 1,2-dihydroxy-diacyltetrol (**4**) were synthesized in four to seven steps from the starting material (Fig. 1). DATs with long (palmitic acid) and short chain (octanoic acid) fatty acids were synthesized in order to study the impact of hydrophobicity on the binding affinity.

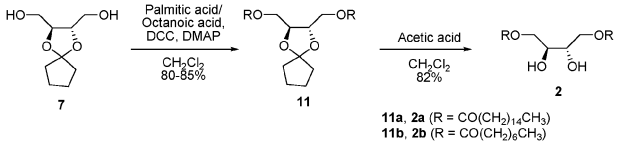
### Synthesis

The synthesis was accomplished *via* a modified reported reaction protocol and the products obtained in moderate to high yields were characterized by NMR, mass, IR and melting temperature analysis. We initially prepared DAT analogs **1a** and **1b** in order to investigate the role of two hydroxymethyl groups within the same molecule in binding with the C1b subdomains of PKC. Lipids **1a** and **1b** were prepared in six steps from (+)-diethyl L-tartrate as shown in Scheme 1. Introduction of cyclopentylidene acetal[41,42] into diol of (+)-diethyl L-tartrate (**5**) and subsequent ester reduction of fully protected tartrate analog **6** by 2.2 equiv. of lithium aluminium hydride[43] resulted in bishydroxymethyl compound **7** in 78% yield (two steps).[42] Next, dibenzylation[44] of compound **7** and careful removal of the cyclopentylidene acetal under mild acidic conditions furnished diol **9** in 68% yield (two steps). The acyl chains were then introduced into **9** using a standard DCC-mediated coupling reaction[45] with readily available palmitic acid and octanoic acid to produce **10a** and **10b**, respectively, in 85–90% yield. Finally, the benzyl groups were removed using 0.1 equiv. of 10% Pd–C under hydrogenation conditions[46] to access DAT analogs **1a** and **1b** in 90% yield.




**Scheme 1** Synthesis route to 1,4-dihydroxy-diacyltetrol (**1**).

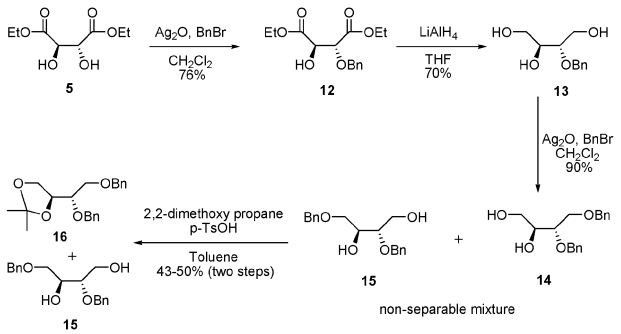
 



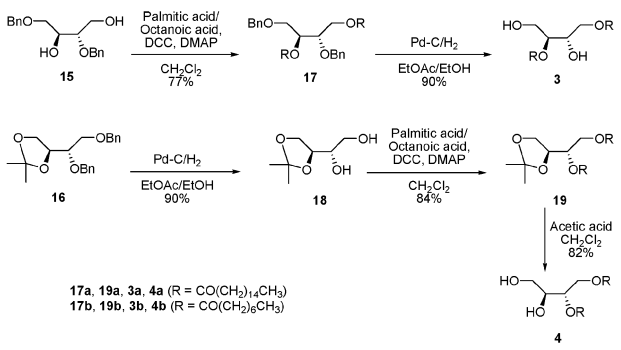

**Scheme 2** Synthesis route to 2,3-dihydroxy-diacyltetrol (**2**).

To understand the importance of two hydroxymethyl groups in binding with the C1b subdomains of PKC, we prepared compound **2a** and **2b** having two hydroxyl groups. The DAT analogs **2a** and **2b** were synthesized from the bishydroxymethyl intermediate **7** in two steps (Scheme 2). The bishydroxymethyl compound **7** was subjected to a DCC-mediated coupling reaction[45] with palmitic acid and octanoic acid to yield **11a** and **11b** respectively, in 80–85% yield. Removal of the cyclopentylidene acetal under mild acidic conditions resulted in compounds **2a** and **2b** in 82% yield.

To gain more information about the importance of both hydroxyl and hydroxymethyl groups in their interactions with the C1b subdomains of PKC, we prepared compounds **3** and **4** containing one hydroxyl and one hydroxymethyl group at 1,3 and 1,2 positions respectively (Schemes 3 and 4). For the synthesis of DAT analogs **3** and **4**, the precursor **5** was first monoprotected with the benzyl group using 1.5 equiv. of silver oxide in anhydrous dichloromethane[47] and further ester reduction by 2.5 equiv. of lithium aluminium hydride produced triol **13** in 63% yield (two steps). Benzylation of triol **13** using 1.5 equiv. of silver oxide in anhydrous dichloromethane[47] resulted in a non-separable mixture of 1,2-diol (**14**) and 1,3-diol (**15**) as




**Scheme 3** Synthesis route to (2*S*,3*S*)-2,4-bis (benzyloxy)butane-1,3-diol (**15**) and (*S*)-4-((*S*)-1,2-bis(benzyloxy)ethyl)-2,2-dimethyl-1,3-dioxolane (**16**).




**Scheme 4** Synthesis route to 1,3-dihydroxy-diacyltetrol (**3**) and 1,2-dihydroxy-diacyltetrol (**4**).

major products. Regioselective protection of the mixture of **14** and **15** *via* acetonide formation[41,43,48] provided fully protected **16** in 44% yield (two steps) and unprotected **15** in 43% yield (two steps). The acyl chains were installed into **15** using the standard DCC-mediated coupling reaction[45] with palmitic acid to yield **17a** and octanoic acid to yield **17b**. Deprotection of benzyl groups of **17a** and **17b** using 0.1 equiv. of 10% Pd–C under hydrogenation conditions[46] yielded DAT analogs **3a** and **3b** in 77–91% yield. After the removal of benzyl groups from **16** under mentioned hydrogenation conditions[46] the acyl chains were installed into **18** using the DCC-mediated coupling reaction[45] to afford **19** in 81–84% yield. Finally, careful deprotection of the acetonide group of **19** using 80% acetic acid (aq) in dichloromethane produced DAT analogs **4a** and **4b** in 75% yield.

## Binding of DATs with PKC C1b

Intrinsic fluorescence is widely used as a tool to monitor changes in protein structures and to draw inferences regarding local structure and dynamics. The intrinsic fluorescence of the PKC C1b subdomains is due to the presence of a single tryptophan (Trp-252 in delta, and Trp-253 in theta) and tyrosine residues (Tyr-236 and Tyr-238 in delta, Tyr-249 and Tyr-251 in theta) and the change in their conformation or microenvironment caused by ligands can be detected by fluorescence spectroscopy. The binding affinity of diacylglycerols (DAGs) and diacyltetrols (DATs) to C1b subdomains of PKC was assessed *in vitro* by a fluorescence quenching method under similar experimental conditions and the $EC_{50}$ value was measured using the Hill equation for each of the compounds. Fig. 2 shows the representative plot of fluorescence quenching data for PKCδ C1b in the presence of different concentrations (0.09–35 μM) of 1,2-dioctanoyl-*sn*-glycerol ($diC_8$), **1b**, **2b**, **3b** and **4b**. The measured $EC_{50}$ values (Table 1) revealed that compound **4** with different chain lengths bind to the C1b subdomains with highest affinity (3.08–4.85 μM) and other compounds had comparable binding affinity for both the proteins (Table 1). The $EC_{50}$ values of $diC_8$ and $diC_{16}$ are in accordance with the reported results.[49] Compound **4b** shows 3.1 fold and 1.7 fold stronger binding affinity

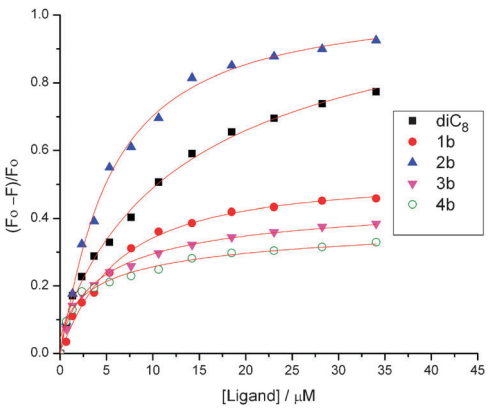



**Fig. 2** Binding of ligands with PKCδ C1b. Representative plot of fluorescence intensity of PKCδ C1b (2 μM) in buffer (50 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4) in the presence of varying concentration of **$diC_8$** (■), **1b** (●), **2b** (▲), **3b** (▼) and **4b** (○), where *F* and $F_0$ are fluorescence intensities in the presence and absence of the ligand, respectively. Solid lines indicate the fit using Hill equation.

**Table 1** $EC_{50}$ values for the binding of DAGs and DATs with the PKCδ C1b and PKCθ C1b proteins[a] at room temperature

| | $EC_{50}$/μM | | |
|---|---|---|---|
| Compound | PKCδ C1b | PKCθ C1b | XLOGP3 |
| $DAG_{16}$ | 6.68 | 7.59 | 14.04 |
| **1a** | 4.29 | 3.70 | 13.42 |
| **2a** | 4.92 | 4.63 | 13.42 |
| **3a** | 3.72 | 3.21 | 13.42 |
| **4a** | 3.34 | 3.08 | 13.42 |
| $DAG_8$ | 15.39 | 6.12 | 5.11 |
| **1b** | 5.89 | 4.15 | 4.49 |
| **2b** | 5.29 | 4.98 | 4.49 |
| **3b** | 5.07 | 4.03 | 4.49 |
| **4b** | 4.85 | 3.59 | 4.49 |

[a] Protein, 2 μM in buffer (50 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4).

than $diC_8$ for PKCδ-C1b and PKCθ-C1b protein, respectively. The binding results also indicate that DATs have higher binding affinity for PKCθ C1b as compared to the PKCδ C1b, possibly due to the additional hydrogen bonds. Overall, the DATs have better binding affinity for the C1b subdomains than DAGs. This could be due to the presence of an additional hydroxyl group. The molecular models of ligand bound protein showed that there were three possible hydrogen bonds among DAG and PKCδ C1b, whereas four hydrogen bonds were possible among DATs and PKCδ C1b.

Modeling experiments using the crystal coordinates of the C1b subdomain of PKCδ in complex with phorbol-13-*O*-acetate (1PTR)[40] revealed the possible binding orientation when diC8 and DATs were docked into the empty C1b subdomain (Fig. 3 and Fig. S1, ESI†). The model structures suggests that hydroxyl and carbonyl groups of the diC8 are mainly responsible for its interaction with the C1b subdomain as reported earlier.[50] The diC8 hydroxyl group (C-28) was hydrogen bonded to the backbone amide proton of Thr-242 and carbonyl of Leu-251. The C-37 carbonyl group formed a hydrogen bond with the backbone amide proton of Gly-253 (Fig. 3B). Our docked models showed that DATs were anchored to the binding site in a similar fashion, as phorbol esters and DAGs (Fig. 3A and B). The hydroxyl and carbonyl groups of DATs were also hydrogen

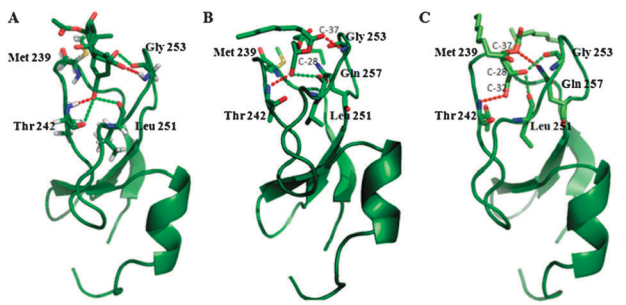



**Fig. 3** Structures of the ligand bound PKCδ C1b subdomain. (A) Crystal structure of phorbol 13-*O*-acetate bound PKCδ C1b; (B) modeled structure of $DAG_8$ docked into PKCδ C1b; (C) modeled structure of a short chain diacyltertol derivative (**4b**) docked into PKCδ C1b. The modeled structures were 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.

bonded to the backbone amide proton and the carbonyls of the C1b subdomain. The DATs could also be involved in additional hydrogen bonding with the side chain of polar amino acid of PKCδ C1b protein. The C-28 and C-35 hydroxyl groups of **1b** were hydrogen bonded to the backbone amide proton of Gly-253, Thr-242 and the carbonyl of Ser-240. The C-33 ester group of **1b** formed a hydrogen bond with the side chain amine proton of Gln-257 (Fig. S1A, ESI†). In the case of **2b** the C-29 and C-33 hydroxyl groups were hydrogen bonded to the backbone carbonyl groups of Ser-240, Thr-242 and Leu-251. Another hydrogen bond was observed for the C-26 ester group of **2b** with the backbone amide proton of Gly-253 (Fig. S1B, ESI†). The C-28 hydroxyl group of **3b** was hydrogen bonded to the backbone amide proton of Thr-242 and carbonyl of Leu-251. The C-33 hydroxyl group of **3b** was hydrogen bonded to the side chain amine proton of Gln-257 and backbone carbonyl of Tyr-238 (Fig. S1C, ESI†). The C-28 hydroxyl group of **4b** was hydrogen bonded to the backbone carbonyls of Leu-251 and Gly-253. The C-32 hydroxyl group and C-37 ester group of **4b** were hydrogen bonded to the backbone amide proton of Thr-242 and the side chain amine proton of Gln-257, respectively (Fig. 3C). The experimental binding results and docking score values obtained from the models do not always corroborate (Table S1, ESI†). This difference indicates that both proteins and compounds can undergo conformational changes under experimental conditions.

To understand the importance of hydrophobicity of DATs in their binding with C1b subdomains similar binding analyses were performed with the long chain DATs. Both proteins showed a similar pattern of dependence on the hydrophobicity of the compounds **1–4** (Table 1). In each case, the affinity increased with the hydrophobicity, with compound **4a** (XLOGP3 = 13.42) having strongest affinity (3.08 μM). For compound **2a** (XLOGP3 = 13.42) and **2b** (XLOGP3 = 4.49), although there is a distinct difference in hydrophobicity, the difference in binding affinity among them is very small for both the proteins. This could be due to the binding orientations with the C1b subdomains through the hydroxyl groups. The octanol–water partition coefficients (log *P*) for all DATs were calculated according to the program XLOGP3. The molecular docking analysis indicates that the overall high binding affinity of compounds **3** and **4** is probably associated with the position of hydroxyl and hydroxymethyl groups, helping them in anchoring to the bottom of the binding site, which may not be possible for compounds **1** and **2**. Thus, the binding affinity values of DATs highlight the importance of ligand hydrophobicity and binding orientation, in a similar manner to those reported for C1 domain ligands.[51] It is also proposed that the hydrophobic amino acids such as Met-239, Phe-243, Leu-250, and Leu-254 for PKCδ C1b, surrounding the ligand binding site of the C1 domain, interact with the hydrophobic moiety of the C1 domain ligands. The hydrophobic moiety of compounds may interact with the membrane lipid bilayer, because the PKC isoforms interact with the membrane through the C1 domain for activation and regulation of other cellular pathways.

In order to obtain more information about ligand–protein interactions, we performed steady-state fluorescence anisotropy measurements of the proteins in the absence or presence of DATs and DAGs at room temperature. The degree of anisotropy of

**Table 2** Anisotropy[a] values of DATs and DAGs in the presence and absence of the PKCδ and PKCθ C1b proteins at room temperature

| Compound | PKCδ C1b | PKCθ C1b |
|---|---|---|
| Buffer[b] | 0.05793 (0.0056) | 0.05529 (0.0032) |
| $DAG_{16}$[c] | 0.10282 (0.0061) | 0.07268 (0.0037) |
| **1a**[c] | 0.10434 (0.0064) | 0.08161 (0.0049) |
| **2a**[c] | 0.10971 (0.0058) | 0.07754 (0.0035) |
| **3a**[c] | 0.11389 (0.0049) | 0.08333 (0.0043) |
| **4a**[c] | 0.12106 (0.0054) | 0.09531 (0.0058) |
| $DAG_8$[c] | 0.09777 (0.0063) | 0.10693 (0.0051) |
| **1b**[c] | 0.10339 (0.0053) | 0.10959 (0.0072) |
| **2b**[c] | 0.10014 (0.0055) | 0.11008 (0.0089) |
| **3b**[c] | 0.10027 (0.0052) | 0.11620 (0.0078) |
| **4b**[c] | 0.10459 (0.0049) | 0.12634 (0.0089) |

[a] Values in the parentheses indicate standard deviations. [b] Protein, 2 μM in buffer (20 mM Tris, 160 mM NaCl, 50 μM $ZnSO_4$, pH 7.4). [c] DAG, **1**, **2**, **3** and **4**, 20 μM; protein, 2 μM in buffer (20 mM Tris, 160 mM NaCl, 50 μM $ZnSO_4$, pH 7.4).

pure PKCθ C1b protein increases from 0.05529 in buffer to 0.10959, 0.11008, 0.11620 and 0.12634 upon interactions with 10-fold excess of ligands **1b**, **2b**, **3b** and **4b** respectively (Table 2). Under similar experimental conditions the degree of anisotropy of pure PKCδ C1b protein increases from 0.05793 in buffer to 0.10339, 0.10014, 0.10027 and 0.10459 in the presence of compounds **1b**, **2b**, **3b** and **4b**, respectively (Table 2). Similar increase in anisotropy was observed for proteins in the presence of DAGs.[49] Although the changes in anisotropy values were different for the compounds, this experiment suggested that the presence of DATs increases the rigidity of the surrounding environment of the protein in a similar manner to that of DAGs.

To investigate the binding properties of the C1b subdomains of PKC isoforms to long chain DAT containing liposomes, we performed tryptophan fluorescence quenching assay (Fig. S2, ESI†). The results indicate that **4a** has good binding affinity for the C1b subdomain of PKCδ and other compounds have comparable binding affinity (Table 3) under the similar experimental conditions. The liposome binding assay for compound **4a** also shows 3.6 fold and 2.4 fold stronger binding affinity than $diC_{16}$ for PKCδ-C1b and PKCθ-C1b, respectively. The liposome binding results suggest that the long chain DATs interact with the C1b subdomain of PKC isoforms in a similar manner to that of DAG. The competitive binding assay showed that DATs interact with the C1b subdomains through the DAG/phorbol ester binding site (Fig. S3, ESI†).

**Table 3** $EC_{50}$ values for the binding of DATs (**1a–4a**) and $DAG_{16}$ containing liposomes[a] with the PKCδ C1b and PKCθ C1b proteins[b] at room temperature

| | $EC_{50}$/nM | |
|---|---|---|
| Compound | PKCδ C1b | PKCθ C1b |
| **1a** | 0.76 | 0.81 |
| **2a** | 0.80 | 0.89 |
| **3a** | 0.44 | 0.58 |
| **4a** | 0.35 | 0.48 |
| $diC_{16}$ | 1.25 | 1.16 |

[a] Liposome composition, PC/PS/$Ligand_{16}$ ($80 - x : 20 : x$, where $x = 0$–10). [b] Protein, 0.5 μM in buffer (50 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4).

Thus, the fluorescence binding assay confirms that C1b subdomains interact with DATs in a lipid free system, as well as DAT containing liposomes. The results also indicate that the long chain DATs can differentially influence the activation and membrane translocation properties of PKCθ and PKCδ. The affinity differences between the proteins are solely because of the differences in the residues and surface area of the activator binding pockets. The activating effect of DATs could be lower than the phorbol esters or other natural compounds under similar experimental conditions. The protein binding measurements also suggest that the C1b subdomains interact differentially with the DATs and their interactions can specifically modulate the PKC isoforms.

## Conclusion

We described here the synthesis of a novel group of compounds, diacyltetrols (DATs), and their binding properties with the C1b subdomains of PKCθ and PKCδ. Synthesis of these tetrol lipids from the scratch provided the ability to generate pure samples of a single lipid structure and freedom to explore the role of hydroxyl and hydroxymethyl groups at different positions. The results showed that DATs interact with the C1b subdomain of PKC isoforms through the diacylglycerol (DAG) or phorbol ester binding site and forms hydrogen bonds with the residues at the activator binding site. Compound **4b** with hydroxymethyl and hydroxyl groups at 1 and 2 positions, respectively, showed better binding affinity among the DATs. Therefore, DAT molecules can be further used as research tools or lead compounds in PKC based drug development. Undoubtedly, further studies are needed to understand fully the importance of DATs in activation and regulation of PKC isoenzymes. Nevertheless, the present study demonstrates that a combination of experimental and computational approaches can provide valuable insight into the interaction of DATs with C1b subdomains of PKC isoforms.

## Experimental section

### General methods

All reagents were purchased from Sigma, USA and Merck, India, and used directly without further purification. Dry solvents were obtained according to the reported procedures. Column chromatography was performed using 60–120 mesh silica gel. Reactions were monitored by TLC on silica gel 60 F254 (0.25 mm). $^1$H NMR and $^{13}$C NMR spectra were recorded at 400 MHz and at 100 MHz, respectively, by using a Varian AS400 spectrometer. Coupling constants ($J$ values) were reported in Hertz (Hz). The chemical shifts were shown in parts per million (ppm) downfield from tetramethylsilane, using residual chloroform ($\delta = 7.24$ in $^1$H NMR, $\delta = 77.23$ in $^{13}$C NMR), as an internal standard. Multiplicities were reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet) and br (broadened). Infrared (IR) spectra were recorded in KBr or neat using a Perkin-Elmer Spectrum One FT-IR spectrometer from 4000 to 450 $cm^{-1}$. Melting points were determined using Büchi B-545 melting point apparatus and are uncorrected. Mass spectra were recorded using a WATERS MS system, Q-tof premier, and data analyzed by using the

built-in software. Optical rotations were measured on a Perkin-Elmer instruments model 343-Polarimeter at room temperature. 1,2-Dipalmitoyl-*sn*-glycerol ($diC_{18}$), 1,2-dioctanoyl-*sn*-glycerol ($diC_8$), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl) were purchased from Avanti Polar Lipids. Ultrapure water (Milli-Q system, Millipore, USA) was used for the preparation of buffer.

**General procedure for the preparation of DAT esters (I)[45].** Palmitic acid/octanoic acid (2.2 equiv.), dicyclohexylcarbodiimide (2.2 equiv.) and *N*,*N*-dimethylaminopyridine (0.1 equiv.) were added to a solution of protected diol (1.0 equiv.) in anhydrous dichloromethane (8 mL) under a $N_2$ atmosphere. Stirring was continued for 12 h at room temperature. After completion of the reaction (monitored by TLC), the reaction mixture was filtered and washed (3×) with dichloromethane. The filtrate was concentrated under reduced pressure and the column chromatography was performed with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane to yield corresponding esters. The average yields were 77% to 85%.

**General procedure for the deprotection of benzyl groups (II)[46].** To a solution of benzyl ether containing compounds (1 equiv.) in 10 mL of ethyl acetate/ethanol (3 : 1 ratio) was added 10% Pd–C (0.1 equiv.) and stirring was continued under a $H_2$ (80 psi) atmosphere, at room temperature for 8–9 h. After completion of the reaction (monitored by TLC), the catalyst (Pd–C) was filtered off through a pad of Celite and the solvent was removed under reduced pressure. The crude reaction mixture was purified by column chromatography with silica gel and a gradient solvent system of 5–25% of ethyl acetate to hexane to provide diol derivatives. The average yields were 77% to 98%.

**General procedure for the deprotection of the cyclopentylidene and dimethyl acetal groups (III)[42,43].** To a stirring solution of protected tetrols (1 equiv.) in dichloromethane (10 mL), 3–5 mL of 80% acetic acid was added and stirring was continued at 40 °C for 4–5 h. After completion of the reaction (monitored by TLC), the solvent was removed under reduced pressure to yield a residue. The residue was further dissolved in dichloromethane (10 mL) and washed with saturated solution of sodium bicarbonate (3×). The organic layer was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure. Purification by silica gel column chromatography and a gradient solvent system of 5–20% ethyl acetate to hexane yielded diol derivatives. The average yields were 75% to 82%.

**2,3-Diethyl(2*R*,3*R*)-1,4-dioxaspiro[4.4]nonane-2,3-dicarboxylate (6)[41,42].** To a stirring solution of (+)-diethyl-L-tartrate (5.2 g, 25.22 mmol) in toluene (50 mL) *p*-toluenesulfonic acid (480 mg, 2.52 mmol) and cyclopentanone (5.03 mL, 63.05 mmol) were added respectively. The solution was refluxed for 14 h and the azeotrope was collected in a Dean–Stark trap. The reaction mixture was then cooled to room temperature and reaction mixture was quenched by adding 20% solid sodium bicarbonate. After stirring for another 30 min at room temperature, the reaction mixture was filtered 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 **6** (5.6 g, 81%) as a yellow coloured oil. $R_f$ 0.30 (EtOAc/Hexane, 1 : 4): $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.65 (s, 2H), 4.20 (q, $J$ = 7.2 Hz, 4H), 1.93–1.88 (m, 2H), 1.82–1.75 (m, 2H), 1.67–1.62 (m, 4H), 1.24 (t, $J$ = 7.2 Hz, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 169.1, 122.7, 76.6, 61.2, 36.2, 23.0, 13.6; FT-IR (KBr) $\nu$ 2946, 2867, 1705, 1639, 1450, 1376, 1320, 1059 cm$^{-1}$.

**[(2*S*,3*S*)-3-(hydroxymethyl)-1,4-dioxaspiro[4.4]nonan-2-yl]-methanol (7)[43].** Protected diester **6** (1.59 g, 5.8 mmol) in anhydrous THF (3.5 mL) was added dropwise to a suspension of lithium aluminium hydride (479.5 mg, 12.75 mmol) in anhydrous THF (20 mL) at 0 °C under a $N_2$ atmosphere. Stirring was continued at room temperature for 1 h and then the solution was refluxed for additional 6 h. After completion of the reaction (monitored by TLC), the temperature was lowered to 0 °C and the reaction was cautiously quenched by addition of 1 mL of deionized water, followed by 1 mL of 10% NaOH and 2 mL of deionized water. The mixture was warmed to room temperature, and stirred until the gray color disappeared. The reaction mixture was filtered in a sintered glass crucible using Celite. Resulting solution was dried over anhydrous $Na_2SO_4$ and concentrated under reduced pressure. Column chromatography with silica gel and a gradient solvent system of 10–40% ethyl acetate to hexane yielded **7** (1.05 g, 96%) as a colourless oil. $R_f$ 0.32 (EtOAc/Hexane, 1 : 2); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.24–4.20 (m, 1H), 4.14–4.03 (m, 2H), 3.88–3.77 (m, 2H), 3.67–3.63 (m, 1H), 2.07 (s, 2H), 1.88–1.78 (m, 4H), 1.67–1.64 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 119.1, 78.4, 62.4, 37.1, 23.3; FT-IR (KBr) $\nu$ 3445, 2928, 1634, 1403, 1260, 1084 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_9H_{16}O_4Na^+$ $[M + Na]^+$: 211.0946, found: 211.0951.

**(2*S*,3*S*)-2,3-bis[(benzyloxy)methyl]-1,4-dioxaspiro[4.4]nonane (8).** Sodium hydride (60% in mineral oil, 282.24 mg, 11.76 mmol) was first taken in a flame-dried round-bottom flask under a $N_2$ atmosphere and washed thrice with anhydrous hexane to remove mineral oil. Diol **7** (1 g, 4.89 mmol) in anhydrous THF (5 mL) was added dropwise to a stirred suspension of NaH in anhydrous THF (15 mL) at 0 °C and stirred for 1 h. Then benzyl bromide (1.4 mL, 11.76 mmol) was added dropwise to the reaction mixture and stirred overnight at room temperature.[44] The reaction was quenched by a saturated solution of ammonium chloride and the residue was extracted with diethyl ether (3 × 20 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 0–10% ethyl acetate to hexane yielded **8** (1.5 g, 83%) as a colorless oil. $R_f$ 0.42 (EtOAc/Hexane, 1:6); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.25–7.15 (m, 10H), 4.46 (s, 4H), 3.90–3.89 (m, 2H), 3.52–3.46 (m, 4H), 1.75–1.72 (m, 4H), 1.57–1.56 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 137.9, 128.2, 127.5, 119.4, 77.2, 73.3, 70.6, 37.2, 23.4; FT-IR (KBr) $\nu$ 3031, 2930, 2870, 1637, 1453, 1334, 1259, 1205, 1102, 907, 799, 736, 698, 604 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{23}H_{28}O_4Na^+$ $[M + Na]^+$: 391.1880, found: 391.1881.

**(2*S*,3*S*)-1,4-bis(benzyloxy)butane-2,3-diol (9).** Using the general procedure (III), starting from compound **8** (600 mg, 1.63 mmol) compound **9** (406.6 mg, 82%) was isolated as colorless oil.

$R_f$ 0.37 (EtoAc/Hexane, 1 : 2); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.29–7.22 (m, 10H), 4.47 (s, 4H), 3.81–3.79 (m, 2H), 3.55–3.50 (m, 4H), 2.09 (s, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 137.7, 128.2, 127.6, 127.5, 73.1, 71.5, 70.4; FT-IR (KBr) $\nu$ 3304, 3029, 2919, 2862, 1638, 1454, 1386, 1362, 1261, 1090, 949, 907, 804, 750, 734, 696, 610 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{18}H_{22}O_4Na^+$ [M + Na]$^+$: 325.1410, found: 325.1420.

**(2*S*,3*S*)-1,4-bis(benzyloxy)-3-(hexadecanoyloxy)butan-2-yl hexadecanoate (10a).** Using the general procedure (I), starting from compound **9** (200 mg, 0.662 mmol) and palmitic acid (373.4 mg, 1.46 mmol) compound **10a** (437.5 mg, 85%) was isolated as a white solid. Mp: 95–97 °C; $R_f$ 0.45 (EtOAc/Hexane, 1 : 9); $[\alpha]_D^{20}$ = −9.2 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.27–7.19 (m, 10H), 5.30 (m, 2H), 4.38 (dd, *J* = 12 Hz, *J* = 12 Hz, 4H), 3.47 (m, 4H), 2.23–2.20 (m, 4H), 1.52–1.50 (m, 4H), 1.18 (m, 48H), 0.81 (t, *J* = 5.8 Hz, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 137.8, 128.5, 127.9, 73.3, 70.7, 62.4, 35.9, 34.2, 33.5, 32.7, 32.1, 31.0, 29.8, 29.6, 29.5, 29.4, 29.2, 26.4, 25.7, 25.6, 25.5, 24.9, 24.9, 22.8, 14.2; FT-IR (KBr) $\nu$ 2926, 2854, 1711, 1639, 1458, 1263, 1160, 1108, 1026, 790, 713, 697 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{50}H_{82}O_6Na^+$ [M + Na]$^+$: 801.6004, found: 801.6005.

**(2*S*,3*S*)-3-(hexadecanoyloxy)-1,4-dihydroxybutan-2-yl hexadecanoate (1a).** Using the general procedure (II) starting from compound **10a** (250 mg, 0.321 mmol) and 10% Pd–C (3.81 mg, 0.0321 mmol), compound **1a** (171 mg, 90%) was isolated as a white solid. Mp: 97–98 °C; $R_f$ 0.36 (EtOAc/Hexane, 1:7); $[\alpha]_D^{20}$ = −8.0 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.29 (m, 2H), 3.77–3.70 (m, 4H), 2.12–2.11 (m, 2H), 1.92–1.86 (m, 2H), 1.71–1.61 (m, 4H), 1.25 (br s, 48H), 0.88 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.4, 73.3, 62.4, 35.9, 34.2, 33.5, 32.7, 32.0, 30.9, 29.8, 29.6, 29.5, 29.4, 29.2, 26.4, 25.7, 25.6, 25.5, 24.9, 24.8, 22.8, 14.2; FT-IR (KBr) $\nu$ 3448, 3301, 2919, 2850, 1733, 1638, 1549, 1463, 1384, 1248, 1121 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{36}H_{70}O_6Na^+$ [M + Na]$^+$: 621.5065, found: 621.5064.

**(2*S*,3*S*)-1,4-bis(benzyloxy)-3-(octanoloxy)butan-2-yl octanoate (10b).** Using the general procedure (I), starting from compound **9** (200 mg, 0.662 mmol) and octanoic acid (211 mg, 1.46 mmol) compound **10b** (313 mg, 85%) was isolated as colorless syrup. $R_f$ 0.37 (EtOAc/Hexane, 1 : 9); $[\alpha]_D^{20}$ = −6.7 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.25–7.18 (m, 10H), 5.29 (m, 2H), 4.38 (dd, *J* = 12, *J* = 12 Hz, 4H), 3.47 (m, 4H), 2.28–2.19 (m, 4H), 1.57–1.49 (m, 4H), 1.20 (m, 16H), 0.80 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 137.8, 128.5, 127.9, 73.3, 70.7, 68.3, 34.4, 34.2, 31.8, 29.2, 29.15, 29.1, 29.0, 25.1, 24.8, 22.7, 14.2; FT-IR (KBr) $\nu$ 2926, 2855, 1717, 1457, 1263, 1160, 1107, 1026, 804, 746, 713 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{34}H_{50}O_6Na^+$ [M + Na]$^+$: 577.3505, found: 577.3501.

**(2*S*,3*S*)-1,4-dihydroxy-3-(octanoloxy)butan-2-yl octanoate (1b).** Using the general procedure (II), starting from compound **10b** (250 mg, 0.451 mmol) and 10% Pd–C (5.34 mg, 0.0451 mmol), compound **1b** (152 mg, 90%) was isolated as a colourless oil. $R_f$ 0.30 (EtOAc/Hexane, 1 : 4); $[\alpha]_D^{20}$ = −11.0 (c 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.36–5.32 (m, 1H), 5.20–5.16 (m, 1H), 3.70–3.56 (m, 2H), 3.51–3.42 (m, 2H), 2.40–2.31 (m, 4H), 1.65–1.61 (m, 4H), 1.28–1.23 (m, 16H), 0.85 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 73.3, 62.4, 34.4, 34.2, 31.8, 29.2, 29.15, 29.1, 29.0, 25.1, 24.8, 22.7, 14.2; FT-IR (KBr) $\nu$ 3434, 2961, 2926, 2854, 1733, 1638, 1461, 1261, 1095, 1024 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{20}H_{38}O_6Na^+$ [M + Na]$^+$: 397.2561, found: 397.2562.

**[(2*S*,3*S*)-3-[(hexadecanoyloxy)methyl]-1,4-dioxapiro[4.4]nonan-2-yl]methyl hexadecanoate (11a).** Using the general procedure (I), starting from compound **7** (300 mg, 1.6 mmol) and palmitic acid (900 mg, 3.51 mmol) compound **11a** (857 mg, 80%) was isolated as a white solid. Mp: 98–99 °C; $R_f$ 0.35 (EtOAc/Hexane, 1 : 6); $[\alpha]_D^{20}$ = −17.3 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.20–4.08 (m, 2H), 3.93–3.78 (m, 2H), 3.62–3.59 (m, 2H), 2.35–2.26 (m, 4H), 1.89–1.83 (m, 4H), 1.76–1.56 (m, 8H), 1.18 (br s, 48H), 0.82–0.78 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 120.4, 76.0, 63.9, 49.8, 37.4, 36.1, 34.2, 32.9, 32.1, 31.0, 29.84, 29.8, 29.6, 29.56, 29.5, 29.4, 29.3, 26.5, 25.7, 25.5, 25.0, 24.9, 23.7, 22.8, 21.0, 14.3; FT-IR (KBr) $\nu$ 2918, 2849, 1702, 1627, 1463, 1310, 1295, 1088, 940 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{41}H_{76}O_6Na^+$ [M + Na]$^+$: 687.5534, found: 687.5535.

**(2*S*,3*S*)-4-hexadecanoloxy-2,3-dihydroxybutyl hexadecanoate (2a).** Using the general procedure (III), starting from compound **11a** (500 mg, 0.7523 mmol) compound **2a** (370 mg, 82%) was isolated as a white solid. Mp: 95–96 °C; $R_f$ 0.36 (EtOAc/Hexane, 1 : 7); $[\alpha]_D^{20}$ = −10.0 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.24–4.13 (m, 4H), 3.80 (m, 2H), 2.68 (br s, 2H), 2.36–2.30 (m, 4H), 1.60–1.58 (m, 4H), 1.23 (br s, 48H), 0.87–0.84 (m, 6H); $^{13}$C NMR (100 MHz, $CDC_{l3}$): $\delta_{ppm}$ 174.4, 69.8, 65.5, 34.5, 34.4, 32.1, 29.9, 29.9, 29.7, 29.6, 29.5, 29.3, 25.1, 22.9, 14.3; FT-IR (KBr) $\nu$ 3435, 2955, 2917, 2849, 1736, 1638, 1463, 1383, 1178, 1094 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{36}H_{70}O_6Na^+$ [M + Na]$^+$: 621.5065, found: 621.5065.

**[(2*S*,3*S*)-3-[(octanoyloxy)methyl]-1,4-dioxapiro[4.4]nonan-2-yl]-methyl octanoate (11b).** Using the general procedure (I), starting from compound **7** (300 mg, 1.6 mmol) and octanoic acid (506.18 mg, 3.51 mmol) compound **11b** (600 mg, 85%) was isolated as colourless oil. $R_f$ 0.32 (EtOAc/Hexane, 1 : 7); $[\alpha]_D^{20}$ = −7.3 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.20–4.11 (m, 4H), 3.94–3.93 (m, 2H), 2.30–2.25 (m, 4H), 1.77–1.76 (m, 4H), 1.61–1.55 (m, 8H), 1.22 (br s, 16H), 0.82–0.79 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 174.1, 120.4, 75.9, 63.9, 49.9, 37.4, 35.9, 34.2, 32.7, 31.8, 31.0, 29.8, 29.2, 29.0, 26.4, 25.6, 25.4, 24.9, 32.6, 22.7, 20.9, 14.1; FT-IR (KBr) $\nu$ 2918, 2849, 1703, 1627, 1463, 1310, 1295, 1088, 941 cm$^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{25}H_{44}O_6Na^+$ [M + Na]$^+$: 463.3030, found: 463.3031.

**(2*S*,3*S*)-2,3-dihydroxy-4-(octanoloxy)butyloctanoate (2b).** Using the general procedure (III), starting from compound **11b** (500 mg, 1.135 mmol) compound **2a** (349 mg, 82%) was isolated as a colourless oil. $R_f$ 0.35 (EtOAc/Hexane, 1 : 4); $[\alpha]_D^{20}$ = −9.2 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.21–4.11 (m, 4H), 3.85–3.78 (m, 2H), 2.93 (br s, 2H), 2.36–2.24 (m, 4H), 1.60–1.57 (m, 4H), 1.25 (br s, 16H), 0.86–0.82 (m, 6H); $^{13}$C NMR (100 MHz, $CDC_{l3}$): $\delta_{ppm}$ 174.2, 69.7, 65.4, 34.4, 34.3, 31.8, 29.2, 29.1, 25.0, 22.8, 14.2; FT-IR (KBr) $\nu$ 3449, 3300, 2928,

2854, 1746, 1637, 1547, 1446, 1375, 1229, 1117, 1050 $cm^{-1}$; HR-MS *m/z* (%): calcd. for $C_{20}H_{38}O_6Na^+$ $[M + Na]^+$: 397.2561, found: 397.2561.

**(2*R*,3*R*)-diethyl 2-(benzyloxy)-3-hydroxysuccinate (12)[47].** To a solution of (+)-L-diethyl tartrate (**5**, 1 g, 4.85 mmol) in anhydrous dichloromethane (20 mL), silver oxide ($Ag_2O$, 1.7 g, 7.27 mmol) and benzyl bromide (634 µl, 5.34 mmol) were added and stirred at room temperature for 8 h under a $N_2$ atmosphere. After that the reaction mixture was filtered off through a pad of Celite. The solvent was removed under reduced pressure. The purification by silica gel column chromatography and a gradient solvent system of 5–10% ethyl acetate to hexane afforded **12** (1300 mg, 90%) as a slight yellow colored oil. $R_f$ 0.35 (EtOAc/Hexane, 1 : 4); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.33–7.22 (m, 5H), 4.86 (d, $J$ = 12 Hz, 2H), 4.59 (s, 1H), 4.21 (d, $J$ = 12Hz, 1H), 4.34–4.28 (m, 2H), 4.13–4.02 (m, 2H), 3.39 (bs, 1H), 1.32 (t, $J$ = 7 Hz, 3H), 1.16 (t, $J$ = 7 Hz, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.1, 169.3, 137.8, 128.6, 128.4, 128.2, 128.1, 78.3, 72.9, 72.3, 62.0, 61.5, 14.2, 14.0; FT-IR (KBr) $\nu$ 3450, 2930, 1732, 1634, 1455, 1384, 1265, 1015, 861, 740, 700 $cm^{-1}$; HR-MS *m/z* (%): calcd. for $C_{15}H_{20}O_6Na^+$ $[M + Na]^+$: 319.1152, found: 319.1100.

**(2*S*,3*S*)-3-(benzyloxy)-1,2,4-triol (13)[43].** The suspension of lithium aluminium hydride (413 mg, 10.98 mmol) in anhydrous THF (15 mL) was prepared in a flame-dried two neck round bottom flask under a $N_2$ atmosphere at 0 °C. Freshly prepared solution of compound **12** (1.3 g, 4.39 mmol) in anhydrous THF (10 mL) was added dropwise *via* a syringe over 30 min. The mixture was stirred at the same temperature for 1 h and stirring was continued for an additional 6 h at 65 °C. Then the reaction mixture was cooled to 0 °C and quenched by dropwise addition of 1 mL of deionized water, 1 mL of 10% NaOH and 2 mL of deionized water in turn with constant stirring for 45 min. The reaction mixture was filtered off through a sintered glass crucible using Celite. Resulting solution was dried over anhydrous $Na_2SO_4$ and then concentrated under reduced pressure. The residue was purified by silica gel column chromatography (40–70% of ethyl acetate and hexane) to furnish **13** (656 mg, 70%) as a white solid. Mp: 77–78 °C [ref. 1 75.3–76.6 °C]; $R_f$ 0.20 (EtOAC); $[\alpha]_D^{20}$ = −27.3 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.31–7.22 (m, 5H), 5.17 (br s, 3H), 4.51 (dd, $J$ = 11.6, $J$ = 11.6 Hz, 2H), 3.85–3.38 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 137.9, 128.7, 128.2, 79.3, 72.7, 72.1, 63.4, 60.7; FT-IR (KBr) $\nu$ 3403, 2938, 1638, 1454, 1213, 1050, 911, 742, 700 $cm^{-1}$; HR-MS *m/z* (%): calcd. for $C_{11}H_{16}O_4Na^+$ $[M + Na]^+$: 235.0941, found: 235.0942.

**(2*S*,3*S*)-2,4-bis(benzyloxy)butane-1,3-diol (15) and (*S*)-4-((*S*)-1,2-bis(benzyloxy)ethyl)-2,2-dimethyl-1,3-dioxolane (16)[41,43,47,48].** Silver oxide ($Ag_2O$, 1.479 g, 4.597 mmol) and benzyl bromide (400.5 µl, 3.371 mmol) were added to a solution of (2*S*,3*S*)-3-(benzyloxy)-1,2,4-triol (**13**, 650 mg, 3.065 mmol) in anhydrous dichloromethane under a $N_2$ atmosphere and the stirring was continued for 8 h. After completion of the reaction (monitored by TLC), the reaction mixture was filtered off through a pad of Celite. The solvent was removed under reduced pressure. The purification by silica gel column chromatography and a gradient solvent system of 30–50% ethyl acetate to hexane afforded a mixture (700 mg, 76%) of 3,4-bis(benzyloxy) butane-1,2-diol (**14**) and 2,4-bis(benzyloxy) butane-1,3-diol (**15**) as colourless oil. $R_f$ 0.30 (EtOAc/Hexane, 2 : 1); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.29–7.18 (m, 20H), 4.45 (s, 4H), 5.54 (dd, $J$ = 11.6, $J$ = 11.6 Hz, 4H), 3.91–3.89 (m, 1H), 3.77–3.72 (m, 3H), 3.64–3.57 (m, 4H), 3.55–3.47 (m, 4H), 2.51 (br s, 2H), 1.91 (br s, 2H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 138.0, 137.9, 137.74, 137.7, 128.2, 127.9, 127.8, 127.74, 127.67, 127.62, 127.56, 127.53, 127.5, 78.6, 78.1, 73.2, 73.1, 72.6, 72.5, 72.4, 72.0, 70.8, 70.3, 69.5, 63.3, 61.0; FT-IR (KBr) $\nu$ 3437, 2926, 2855, 1637, 1455, 1261, 1188, 1119, 802, 741, 698 $cm^{-1}$; HR-MS-*m/z* (%): calcd. for $C_{18}H_{22}O_4Na^+$ $[M + Na]^+$: 325.1410, found: 325.1397.

To a solution of mixture **14** and **15** (700 mg, 2.32 mmol) in anhydrous THF (20 mL) were added *p*-toluenesulfonic acid (44 mg, 0.232 mmol) and 2,2-dimethoxy propane (341 µl, 2.784 mmol) under a $N_2$ atmosphere at room temperature. The reaction mixture was stirred at 65 °C for 3 h. The reaction mixture was quenched by addition of anhydrous $K_2CO_3$ (200 mg, 1.5 mmol). The solid residue was removed by filtration and washed with diethyl ether (3 × 5 mL). Solvent was removed under reduced pressure and purified by silica gel column chromatography (elution with ethyl acetate/hexane 5–15%) to furnish **16** (400 mg, 50%) as colourless oil and recover **15** (300 mg, 43%) as colourless oil. (2*S*,3*S*)-2,4-Bis(benzyloxy)butane-1,3-diol (**15**): $R_f$ 0.30 (EtOAc/Hexane, 2 : 1); $[\alpha]_D^{20}$ = −4.1 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.35–7.28 (m, 10H), 5.20 (s, 2H), 4.80 (d, $J$ = 11.2 Hz, 2H), 4.48 (d, $J$ = 11.6 Hz, 2H), 4.12–4.10 (m, 2H), 3.91–3.81 (m, 4H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 137.2, 135.6, 128.8, 128.7, 128.6, 128.4, 128.3, 78.8, 76.9, 73.0, 67.1, 63.5; FT-IR (KBr) $\nu$ 3430, 2925, 2855, 1628, 1455, 1383, 1261, 1188, 1120, 803, 746, 698 $cm^{-1}$; HR-MS *m/z* (%): calcd. for $C_{18}H_{22}O_4Na^+$ $[M + Na]^+$: 325.1410, found: 325.1397. (*S*)-4-((*S*)-1,2-Bis(benzyloxy)ethyl)-2,2-dimethyl-1,3-dioxolane (**16**): $R_f$ 0.34 (EtOAc/Hexane , 1 : 1); $[\alpha]_D^{20}$ = −19.5 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.29–7.19 (m, 10H), 5.23(s, 2H), 4.64 (dd, $J$ = 12 Hz, $J$ = 12 Hz, 2H), 4.23–4.18 (m, 1H), 3.93–3.90 (m, 1H), 3.72–3.68 (m, 1H), 3.64–3.61 (m, 1H), 3.51–3.44 (m, 2H), 1.35 (s, 3H), 1.29 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 138.2, 128.3, 127.8, 127.7, 109.2, 79.5, 76.5, 75.6, 72.7, 68.3, 63.3, 26.3, 25.3; FT-IR (KBr) $\nu$ 2925, 2852, 1637, 1462, 1381, 1262, 1100, 1020, 801, 669 $cm^{-1}$; HR-MS *m/z* (%): calcd. for $C_{21}H_{26}O_4Na^+$ $[M + Na]^+$: 365.1723, found: 365.1722.

**(2*S*,3*S*)-1,3-bis(benzyloxy)-4-(hexadecanoyloxy)butan-2-yl hexadecanoate (17a).** Using the general procedure (I), starting from compound **15** (200 mg, 0.662 mmol) and palmitic acid (373 mg, 1.456 mmol) compound **17a** (400 mg, 77%) was isolated as a white solid. Mp: 88–90 °C; $R_f$ 0.36 (EtOAc/Hexane, 1 : 9); $[\alpha]_D^{20}$ = −13.1 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.26–7.16 (m, 10H), 5.28–5.24 (m, 1H), 5.19–5.15 (m, 1H), 4.56 (dd, $J$ = 12, $J$ = 11.6 Hz, 2H), 4.43 (s, 2H), 4.27–4.15 (m, 1H), 4.10–4.01 (m, 1H), 3.85–3.67 (m, 1H), 3.59–3.51 (m, 1H), 2.26–2.13 (m, 4H), 1.51 (m, 4H), 1.18 (br s, 48H), 0.78 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.5, 173.4, 138.1, 128.6, 128.1, 128.03, 128.01, 127.9, 75.5, 73.4, 73.2, 69.4, 62.4, 34.4, 34.2, 34.1, 32.1, 29.9, 29.6, 29.5, 29.4, 29.3, 29.2,

26.3, 25.4, 25.1, 24.9, 24.9, 22.8, 14.2; FT-IR (KBr) $\nu$ 2921, 2846, 1732, 1638, 1455, 1109, 905, 801, 733, 696 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{50}H_{82}O_6Na^+$ $[M + Na]^+$: 801.6004, found: 801.6004.

**(2*S*,3*S*)-4-(hexadecanoyloxy)-1,3-dihydroxybutan-2-yl hexadecanoate (3a).** Using the general procedure (II), starting from compound **17a** (300 mg, 0.385 mmol) and 10% Pd–C (4.6 mg, 0.0385 mmol), compound **3a** (210 mg, 91%) was isolated as a white solid. $R_f$ 0.40 (EtOAc/Hexane, 1 : 7); mp: 96–97 °C; $[\alpha]_D^{20} = -5.7$ (*c* 0.98, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.36–5.24 (m, 2H), 4.32–4.22 (m, 1H), 4.18–4.08 (m, 1H), 3.92–3.79 (m, 1H), 3.67–3.59 (m, 1H), 2.35–2.24 (m, 4H), 1.59–1.56 (m, 4H), 1.26 (br s, 48H), 0.87 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.5, 173.3, 76.4, 72.7, 65.5, 61.6, 34.5, 34.4, 34.34, 34.3, 32.1, 29.9, 29.8, 29.6, 29.54, 29.5, 29.3, 25.1, 25.0, 22.9, 14.3; FT-IR (KBr) $\nu$ 3451, 2922, 2849, 1656, 1519, 1461, 1383, 1261, 1224, 1083 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{36}H_{70}O_6Na^+$ $[M + Na]^+$: 621.5065, found: 621.5066.

**(2*S*,3*S*)-1,3-bis(benzyloxy)-4-(octanoyloxy)butan-2-yl octanoate (17b).** Using the general procedure (I), starting from compound **15** (200 mg, 0.6619 mmol) and octanoic acid (210 mg, 1.456 mmol) compound **17b** (300 mg, 82%) was isolated as a colourless oil. $R_f$ 0.40 (EtOAc/Hexane, 1 : 7); $[\alpha]_D^{20} = -26.1$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 7.25–7.18 (m, 10H), 5.27–5.25(m, 1H), 5.17–5.16 (m, 1H), 4.57 (dd, $J$ = 12 Hz, $J$ = 11.6 Hz, 2H), 4.44 (s, 2H), 4.24–4.16 (m, 1H), 4.10–4.02 (m, 1H), 3.84–3.72 (m, 1H), 3.58–3.52 (m, 1H), 2.27–2.13 (m, 4H), 1.53–1.49 (m, 4H), 1.19 (br s, 16H), 0.80 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.5, 173.4, 138.1, 128.6, 128.2, 128.1, 128.0, 127.8, 76.5, 75.6, 73.4, 69.6, 62.4, 34.5, 34.43, 34.4, 34.29, 33.2, 31.8, 29.9, 29.2, 29.1, 25.1, 25.0, 22.8, 14.2; FT-IR (KBr) $\nu$ 2922, 2854, 1732, 1637, 1455, 1383, 1113, 733, 698, 630 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{34}H_{50}O_6Na^+$ $[M + Na]^+$: 577.3500, found: 577.3501.

**(2*S*,3*S*)-1,3-dihydroxy-4-(octanoyloxy)butane-2-yl octanoate (3b).** Using the general procedure (II), starting from compound **17b** (250 mg, 0.451 mmol) and 10% Pd–C (5.3 mg, 0.0451 mmol), compound **3b** (160 mg, 77%) was isolated as colourless oil. $R_f$ 0.34 (EtOAc/Hexane, 1 : 7); $[\alpha]_D^{20} = -15.1$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.26–5.16 (m, 2H), 4.27–4.17 (m, 1H), 4.11–4.03 (m, 1H), 3.87–3.72 (m, 1H), 3.61–3.53 (m, 1H), 2.28–2.13 (m, 4H), 1.53–1.49 (m, 4H), 1.19 (br s, 16H), 0.80 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 173.1, 74.4, 70.5, 65.4, 62.7, 34.2, 34.0, 31.6, 29.0, 28.4, 28.3, 26.1, 25.2, 24.9, 24.7, 22.6, 14.4; FT-IR (KBr) $\nu$ 3449, 2955, 2926, 2851, 1733, 1705, 1640, 1463, 1382, 1235, 1197, 1109, 1096 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{20}H_{38}O_6Na^+$ $[M + Na]^+$: 397.2561, found: 397.2560.

**(*S*)-1-((*S*)-2,2-dimethyl-1,3-dioxolan-4yl)ethane-1,2-diol (18).** Using the general procedure (II), starting from compound **16** (300 mg, 0.876 mmol) and 10% Pd–C (10.37 mg, 0.0876 mmol), compound **18** (139 mg, 98%) was isolated as a colourless oil. $R_f$ 0.35 (3 : 1); $[\alpha]_D^{20} = -27.3$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 4.18–4.11 (m, 1H), 4.06–4.02 (m, 1H), 3.84–3.80 (m, 1H), 3.65–3.58 (m, 3H), 1.42 (s, 3H), 1.38 (s, 3H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 109.2, 76.3, 72.2, 65.5, 63.4, 26.2, 25.1; FT-IR (KBr) $\nu$ 3446, 2950, 2087, 1637, 1409, 1084, 1042 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_7H_{14}O_4Na^+$ $[M + Na]^+$: 185.0784, found: 185.0786

**(1*S*)-1-[(4*S*)-2,2-dimethyl-1,3-dioxan-4-yl]-2-(hexadecanoyloxy)-ethyl hexadecanoate (19a).** Using the general procedure (I), starting from compound **18** (70 mg, 0.4318 mmol) and palmitic acid (243.6 mg, 0.95 mmol) compound **19a** (224 mg, 81%) was isolated as a white solid. $R_f$ 0.37 (EtOAc/Hexane, 1 : 7); mp: 86–88 °C; $[\alpha]_D^{20} = -7.0$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.13–5.09 (m, 1H), 4.26–4.17 (m, 2H), 4.11–4.06 (m, 1H), 3.98–3.94 (m, 1H), 3.73–3.69 (m, 1H), 2.30–2.20 (m, 4H), 1.57–1.51 (m, 4H), 1.36 (s, 3H), 1.28 (s, 3H), 1.19 (m, 48H), 0.81 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.5, 173.4, 110.0, 74.5, 70.7, 65.6, 62.9, 34.4, 34.2, 34.1, 32.1, 29.9, 29.7, 29.54, 29.5, 29.3, 29.2, 26.3, 25.4, 25.1, 25.0, 24.9, 22.7, 14.3; FT-IR (KBr) $\nu$ 2918, 2850, 1737, 1637, 1463, 1384, 1169, 1099, 1073 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{39}H_{74}O_6Na^+$ $[M + Na]^+$: 661.5378, found: 661.5377.

**(2*S*,3*S*)-1-(hexadecanoyloxy)-3,4-dihydroxybutan-2-yl-hexadecanoate (4a).** Using the general procedure (III), starting from compound **19a** (150 mg, 0.235 mmol) compound **4a** (105 mg, 75%) was isolated as a white solid. $R_f$ 0.36 (EtOAc/Hexane, 1 : 9); mp: 97–98 °C; $[\alpha]_D^{20} = -12.7$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.23–5.19 (m, 1H), 4.36–4.27 (m, 2H), 4.21–4.16 (m, 1H), 4.08–4.04 (m, 1H), 3.83–3.79 (m, 1H), 2.40–2.30 (m, 4H), 1.67–1.61 (m, 4H), 1.29 (br s, 48H), 0.91 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.5, 173.4, 74.5, 70.7, 65.6, 62.9, 34.4, 34.2, 34.1, 32.1, 29.9, 29.6, 29.54, 29.5, 29.3, 29.2, 26.3, 25.4, 25.1, 25.0, 24.9, 22.8, 14.3; FT-IR (KBr) $\nu$ 3430, 2918, 2850, 1638, 1550, 1380, 1245, 1116 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{36}H_{70}O_6Na^+$ $[M + Na]^+$: 621.5065, found: 621.5064.

**(1*S*)-1-[(4*S*)-2,2-dimethyl-1,3-dioxolan-4-yl]-2-(octanoyloxy)-ethyl octanoate (19b).** Using the general procedure (I), starting from compound **18** (70 mg, 0.4318 mmol) and octanoic acid (137 mg, 0.95 mmol) compound **19b** (150 mg, 84%) was isolated as a colourless oil. $R_f$ 0.36 (EtOAc/Hexane, 1 : 7); $[\alpha]_D^{20} = -8.3$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.20–5.16 (m, 1H), 4.33–4.24 (m, 2H), 4.18–4.13 (m, 1H), 4.05–4.02 (m, 1H), 3.80–3.76 (m, 1H), 2.38–2.28 (m, 4H), 1.66–1.58 (m, 4H), 1.43 (s, 3H), 1.35 (s, 3H), 1.29 (m, 16 H), 0.88 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 173.2, 173.1, 109.7, 74.4, 70.5, 65.4, 62.7, 34.2, 34.0, 31.6, 29.03, 29.0, 28.9, 26.1, 25.2, 24.9, 24.8, 22.6, 14.0; FT-IR (KBr) $\nu$ 2922, 2856, 1738, 1642, 1455, 1382, 1222, 1161, 1108, 1071, 964 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{23}H_{42}O_6Na^+$ $[M + Na]^+$: 437.2874, found: 437.2885.

**(2*S*,3*S*)-3,4-dihydroxy-1-(octanoyloxy)butane-2-yl octanoate (4b).** Using the general procedure (III), starting from compound **19a** (150 mg, 0.235 mmol) compound **4a** (105 mg, 75%) was isolated as a colourless oil. $R_f$ 0.35 (EtOAc/Hexane, 1 : 4); $[\alpha]_D^{20} = -22.0$ (*c* 0.1, $CH_2Cl_2$); $^1$H NMR (400 MHz, $CDCl_3$): $\delta_{ppm}$ 5.23–5.19 (m, 1H), 4.36–4.28 (m, 2H), 4.20–4.16 (m, 1H), 4.08–4.04 (m, 1H), 3.83–3.80 (m, 1H), 2.40–2.30 (m, 4H), 1.67–1.64 (m, 4H), 1.31 (br s, 16H), 0.90 (m, 6H); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta_{ppm}$ 179.1, 173.1, 74.4, 70.6, 65.4, 62.8,

34.1, 33.9, 31.6, 29.7, 29.01, 29.0, 28.9, 26.0, 25.1, 24.9, 24.8, 24.7, 22.5, 13.9; FT-IR (KBr) $\nu$ 3451, 2926, 2855, 1745, 1633, 1456, 1372, 1229, 1165, 1047 $cm^{-1}$; HR-MS $m/z$ (%): calcd. for $C_{20}H_{38}O_6Na^+$ $[M + Na]^+$: 397.2561, found: 397.2560.

### Purification of the PKCδ C1b and PKCθ C1b subdomains

The PKCδ C1b and PKCθ C1b subdomains fused with glutathione *S*-transferase (GST) were expressed in BL21 cells and purified as reported earlier.[52,53] The plasmids were a generous gift from Prof. Alexandra Newton (University of California, San Diego, USA). The protein expression was induced by the addition of 250 μM of isopropyl 1-thio-β-D-galactopyranoside and cells were harvested by centrifugation (5000×*g* for 10 min at 4 °C) after 14 h of incubation at 24 °C. The resulting pellet was resuspended in 20 mL of 20 mM Tris buffer, pH 8, containing 160 mM NaCl, 50 μM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and 0.1% Triton X-100. After sonication for 10 cycles the solution was centrifuged for 30 min (48 000×*g* at 4 °C). The supernatant was filtered and 500 μl of the glutathione *S*-transferase Tag resin was added. After incubating this mixture on ice for 1 h with mild shaking at 80 rpm, it was poured onto a column pre-rinsed with 20 mL of 20 mM Tris buffer, pH 8, containing 160 mM KCl. The non-specifically bound proteins were washed off with 100 mL of 20 mM Tris buffer, pH 8, containing 160 mM KCl, and 1 unit of thrombin in cleavage buffer was added to cleave the glutathione *S*-transferase tag and the column was stored at 4 °C for 12 h. The protein was then eluted in five fractions using 500 μl of 20 mM Tris buffer, pH 8, containing 160 mM KCl. Proteins were further purified by fast performance liquid chromatography (Akta Purifier) using a Superdex™ 75 column (GE Healthcare Biosciences), a mobile phase of 50 mm Tris, 100 mm NaCl, pH 7.4, and a flow rate of 0.5 mL $min^{-1}$.

### Fluorescence studies

Fluorescence measurements were performed on a Fluoromax-4 spectrofluorometer using 10 mm path length quartz cuvettes with the slit width of 20 nm at room temperature. An excitation wavelength of 295 nm was applied to selectively excite the tryptophan residues in protein. Proper background corrections were made to avoid the contribution of buffer. For fluorescence titration, protein (2 μM) and varying concentration of ligands (0.67–40 μM for DATs of octanoic acid and 0.099–13 μM for DATs of palmitic acid) were incubated in a buffer solution (50 mM Tris, 150 mM NaCl, 50 μM $ZnSO_4$, pH 7.4) at room temperature. Fluorescence intensity data, $(F_0 - F)/F_0$, were plotted against the ligand concentration to generate the binding isotherms, where $F$ and $F_0$ represent the fluorescence intensity at 350 nm in the presence and in the absence of ligand respectively. The $EC_{50}$ values were calculated for all curves by fitting with the Hill equation using OriginLab software. Fluorescence anisotropy measurements were performed in the same fluorimeter using parallel and perpendicular polarizers. The tryptophan fluorescence of the proteins was recorded over 300–500 nm wavelengths by exciting at 295 nm using an excitation slit width of 2 nm. The anisotropy values were averaged over an integration time of 10 s, and a maximum number of five measurements were made for each sample. All anisotropy values of the proteins in the 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 the following equation 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.

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

### Liposome binding assay

The membrane binding of PKC C1b subdomains was analyzed by fluorescence quenching measurements. The protein (500 nM) was added to PC/PS/$Ligand_{16}$ ($80 - x$: 20: $x$, where $x$ = 0–10) liposomes prepared by an extrusion method,[53] and the emission spectra of protein were measured with the excitation wavelength of 295 nm. Fluorescence intensity data, $(F_0 - F)/F_0$, were plotted against the liposome containing ligand concentrations to generate the binding isotherms, where $F$ and $F_0$ represent the fluorescence intensity at 338 nm in the presence and in the absence of ligand containing liposomes, respectively. The $EC_{50}$ values were calculated for all curves by fitting with the Hill equation using OriginLab software.

### Molecular docking

Molecular docking was performed using the crystal structure of PKCδ C1b subdomain complexed with phorbol-13-*O*-acetate (Protein Data Bank code: 1PTR).[40] The hydrogen as well as the side chains of Lys 234, Arg 273 and Glu 274 not visible in the X-ray structure was subsequently added to the protein. The energy minimized three-dimensional structure of ligands was prepared by using the GlycoBioChem PRODRG2 Server (http://davapc1.bioch.dundee.ac.uk/prodrg/). The GROningen MAchine for Chemical Simulations (GROMACS) library of three-atom combination geometries employing a combination of short molecular dynamics simulations and energy minimizations was utilized for the conversion of 2D molecular structures to 3D structures. Ligand-protein docking was performed using the Molegro Virtual Docker software, version 4.3.0 (Molegro ApS, Aarhus, Denmark).[54] The binding site was automatically detected by the docking software and restricted within spheres with radius of 15 Å. During virtual screening, the following parameters were fixed: number of runs 10, population size 50, crossover rate 0.9, scaling factor 0.5, maximum iteration 2000 and grid resolution 0.30. The docked results were evaluated on the basis of moledock and re-rank score. The poses were exported and examined with *PyMOL* software.

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