Design, Synthesis, and Biological Activity of Isophthalic Acid Derivatives Targeted to the C1 Domain of Protein Kinase C

Gustav Boije af Gennäs,[†,‡] Virpi Talman,[†,§] Olli Aitio,[†,||] Elina Ekokoski,[§] Moshe Finel,[⊥] Raimo K. Tuominen,[§] and Jari Yli-Kauhaluoma*[,‡]

Division of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland, Division of Pharmacology and Toxicology, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland, Finnish Biological NMR Center, Institute of Biotechnology, University of Helsinki, FI-00014 Helsinki, Finland, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, FI-00014 Helsinki, Finland

Received February 20, 2009

Protein kinase C (PKC) is a widely studied molecular target for the treatment of cancer and other diseases. We have approached the issue of modifying PKC function by targeting the C1 domain in the regulatory region of the enzyme. Using the X-ray crystal structure of the PKC δ C1b domain, we have discovered conveniently synthesizable derivatives of dialkyl 5-(hydroxymethyl)isophthalate that can act as potential C1 domain ligands. Structure−activity studies confirmed that the important functional groups predicted by modeling were indispensable for binding to the C1 domain and that the modifications of these groups diminished binding. The most promising compounds were able to displace radiolabeled phorbol ester ([^{3}H]PDBu) from PKC α and δ at K_i values in the range of 200−900 nM. Furthermore, the active isophthalate derivatives could modify PKC activation in living cells either by inducing PKC-dependent ERK phosphorylation or by inhibiting phorbol-induced ERK phosphorylation. In conclusion, we report here, for the first time, that derivatives of isophthalic acid represent an attractive novel group of C1 domain ligands that can be used as research tools or further modified for potential drug development.

Introduction

Protein kinase C (PKC[a]) is a family of serine/threonine protein kinases that is involved in the regulation of various aspects of cell functions, including cell growth, differentiation, metabolism, and apoptosis.[1] During the past 30 years, it has become clear that PKC isoenzymes play an important role in the pathology of several diseases such as cancer, diabetes, stroke, heart failure, and Alzheimer's disease.[2−8] Therefore, PKC, particularly in the cancer field, has been a subject of intensive research and drug development.[9]

PKC isoenzymes are activated by lipid-derived second messengers and transmit their signal by phosphorylating specific protein substrates. The PKC family consists of 10 isoenzymes, which can be divided into three subfamilies according to their regulatory domain structure and cofactor requirements.[10,11] All PKCs are dependent on anionic phospholipids for activation, with phosphatidyl-L-serine being the most important. In addition, classical PKCs (α, βI, βII, and γ) require calcium and diacylglycerol (DAG) for activation, whereas novel PKCs (δ, ε, η, and θ) require DAG but not calcium. Atypical PKCs (ζ and ι/λ) require neither calcium nor DAG for activation.

DAG is a second messenger that is generated by the phospholipase C-catalyzed hydrolysis of membrane phosphatidylinositol-4,5-bisphosphate (PIP_2).[12] DAG selectively interacts with proteins containing a C1 domain and induces their translocation to discrete subcellular compartments. For some C1 domain-containing proteins, such a translocation leads to their activation.[13] The C1 domain is a cysteine-rich sequence that forms a zinc finger structure.[10] In the classical and novel PKC isoenzymes, the DAG-sensitive C1 domain is duplicated into a tandem C1 domain consisting of C1a and C1b subdomains. Atypical PKCs contain a single C1 domain that is not able to bind DAG. Along with the PKC family, there are six additional families of proteins that contain a DAG-responsive C1 domain: the protein kinase D (PKD), the chimaerins, the guanyl nucleotide-releasing proteins RasGRPs, the Unc-13 scaffolding proteins, myotonic dystrophy kinase-related Cdc42-binding kinases (MRCK), and the DAG kinases β and γ.[14,15]

Because the catalytic domain and, in particular, the ATP binding site of PKC are substantially homologous among protein kinases[16] and the number of C1 domain containing proteins is considerably smaller than the number of kinases[17] the C1 domain provides an attractive target for designing selective PKC modulators (see the review by Blumberg et al.[18]). While the structure of the binding cleft in the C1 domain is highly conserved, selectivity can be achieved by various strategies.[18] Moreover, several studies have already shown that the regulatory domain may have a biological function independent of the

* To whom correspondence should be addressed. Phone: +358 9 19159170. Fax: +358 9 19159556. E-mail: jari.yli-kauhaluoma@helsinki.fi.
† These authors contributed equally to this work.
‡ Division of Pharmaceutical Chemistry, Faculty of Pharmacy.
§ Division of Pharmacology and Toxicology, Faculty of Pharmacy.
|| Finnish Biological NMR Center, Institute of Biotechnology.
⊥ Centre for Drug Research, Faculty of Pharmacy.

[a] Abbreviations: [^{3}H]PDBu, [20-^{3}H]phorbol-12,13-dibutyrate; ATP, adenosine-5′-triphosphate; CDI, 1,1-carbonyldiimidazole; DAG, diacylglycerol; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCE, 1,2-dichloroethane; DHP, 3,4-dihydro-2*H*-pyran; DIPEA, diisopropylethylamine; DMEM, Dulbecco's modified Eagle's medium; DMF, *N*,*N*-dimethylformamide; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethylsulfoxide; DOG, 1,2-dioctanoyl-*sn*-glycerol; ECL, enhanced chemiluminescence; EDC, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBA, hydrogen bond acceptor; HBD, hydrogen bond donor; HOBt, 1-hydroxybenzotriazole; HRP, horseradish peroxidase; MRCK, myotonic dystrophy kinase-related Cdc42-binding kinase; IgG, immunoglobulin G; PBS, phosphate buffered saline; PDBu, phorbol-12,13-dibutyrate; PIP_2 phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PKD, protein kinase D; PMA, phorbol 12-myristate-13-acetate; PPTS, pyridinium *p*-toluenesulfonate; PS, phosphatidyl-L-serine; THF, tetrahydrofuran; THP, tetrahydropyran-2-yl; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl)aminomethane; TTBS, 0.1% Tween-20 in TBS; SAR, structure−activity relationship.

10.1021/jm900229p CCC: $40.75
Published on Web 05/13/2009

catalytic activity of the enzyme,[19,20] thus confirming the validity of targeting the regulatory domain as a therapeutic strategy.

Several classes of high-affinity ligands that target the DAG-binding C1 domain have been previously described. Naturally occurring tumor promoters, phorbol esters, were the first ligands that were found to bind to the C1 domain of PKC.[21] In addition to phorbol esters, naturally occurring C1 domain ligands include bryostatins,[22] teleocidins,[23] aplysiatoxins,[24] ingenols,[25] and iridals.[26] As most of these C1 domain ligands from natural sources are highly complex in their chemical structure, we and other groups have been interested in designing a simple C1 domain binding template that is easily amenable to chemical modification. Blumberg and Marquez groups have described a large number of conformationally constrained DAG-lactones that act as potent C1 domain ligands,[27,28] while the Irie group has studied indolactam and benzolactam derivatives as selective activators of novel PKC isoenzymes.[29,30] A number of "first generation" C1 domain ligands (including calphostin C, bryostatin 1, PEP005, and phorbol esters) are already in clinical trials for the treatment of different types of cancer.[18]

In this study, we describe the design, synthesis, biological activity, and structure−activity relationship of a novel class of C1 domain ligands: small hydrophobic isophthalic acid derivatives. At low micromolar concentrations, these isophthalic acid derivatives can compete with phorbol ester binding to PKC and modulate ERK1/2 phosphorylation in living cells in a PKC-dependent manner.

Design and Synthesis

We used the crystal structure of the PKC δ C1b domain complexed with phorbol-13-*O*-acetate[31] as a starting point in the design of novel C1 domain ligands that bind to the same site as phorbol esters and DAGs. The X-ray structure of the complex shows that the pivotal polar functional groups of the phorbol ester that participate in the recognition of the C1 domain include the hydroxyl groups attached to carbons C20 and C4 and the carbonyl group on C3 (Figure 1). According to structure−activity studies,[32,33] the C13 carbonyl group is also important for binding of phorbol-13-*O*-acetate to the C1 domain. We selected diethyl 5-(hydroxymethyl)isophthalate as a template for our SAR studies because it is commercially available and easily derivatizable. The C_2 symmetry of diethyl 5-(hydroxymethyl)isophthalate enables two binding orientations, similar to DAG-lactones;[34] it also contains two of the phorbol ester pharmacophores, namely the hydroxyl and the carbonyl functionalities with the same interatomic distance as that found in phorbol.[35] Furthermore, dialkyl 5-(hydroxymethyl)isophthalates contain two carbonyl groups, a prerequisite for high affinity binding of DAG-lactones.[34] We conducted docking experiments to verify that the phorbol-like binding mode, with the correct positioning of the hydrogen bonding network, was feasible for dialkyl 5-(hydroxymethyl)isophthalate structures. Indeed, our docked model (**I**) (Figure 2) showed that dipentyl 5-(hydroxymethyl)isophthalate was anchored to the bottom of the binding site via the same interactions as the phorbol ester, i.e., the ligand's hydroxyl group is hydrogen bonded to the backbone amide proton of Thr 242 and the carbonyls of Thr 242 and Leu 251. The model also showed that one of the carbonyl groups of **I** forms a hydrogen bond with the backbone amide proton of Gly 253 in a similar fashion as the C3 carbonyl of phorbol-13-*O*-acetate. The second carbonyl group of **I** seemed to make no contact with the C1 domain; however, it has been proposed that the complex might be stabilized by either a bridging water molecule between this carbonyl and the backbone carbonyl of Met 239 or by interactions with charged lipid head groups.[36]

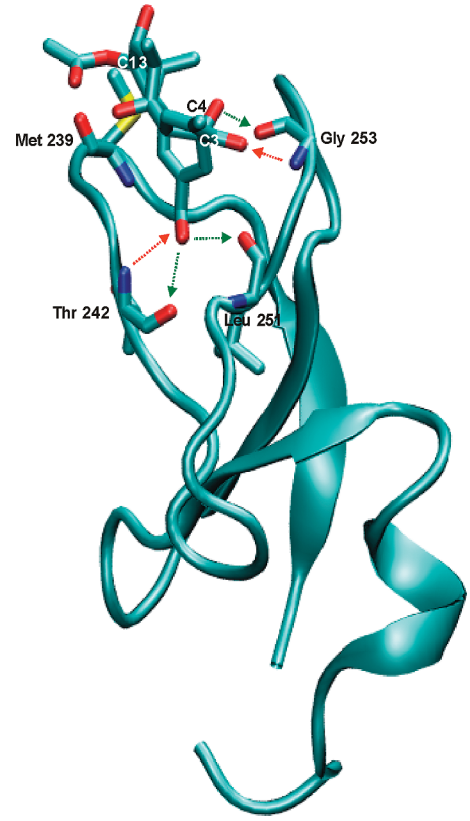


Figure 1. Crystal structure of PKC δ C1b domain complexed with phorbol-13-*O*-acetate.[31] The pivotal polar functional groups of the phorbol ester involved in C1 domain recognition include the hydroxyl groups attached to carbon C4 and C20 and the carbonyl group on C3. The carbonyls of Gly 253 and Leu 251 are HBAs (green arrows), and the amide proton of Gly 253 and Thr 242 are HBDs (red arrows). The figure was created using VMD (v.1.8.6., University of Illinois).

Hydrophobic interactions are crucial for high affinity binding to the C1 domain. The hydrophobic side chains of phorbol esters, DAGs, and their analogues are thought to interact with either the hydrophobic amino acids (Met 239, Pro 241, Phe 243, Leu 250, Trp 252, and Leu 254) surrounding the ligand binding cleft or with the hydrophobic moiety in the lipid bilayer.[31,37] Because it is not known how the C1 domain interacts with the membrane, hydrophobic interactions are difficult to model. We therefore prepared dialkyl 5-(hydroxymethyl)isophthalate derivates with different side chains to analyze their effect on binding affinity and activity.

We initially prepared simple derivatives of the template (**I**) that differed in the composition of the ester groups in order to investigate the length and shape of the alkyl chains required for activity. As starting material for the esters, we chose compounds containing primary and secondary alkyl chains as well as compounds containing aromatic groups. (see Table 1 for the structures). To investigate the hydrophobicity of the compounds that was necessary for activity, we also prepared three additional hydrophilic derivatives.

The dialkyl 5-(hydroxymethyl)isophthalates (**1**) were conveniently synthesized in only four steps using the commercially available diethyl 5-(hydroxymethyl)isophthalate as the starting material (**2**) (Scheme 1). Protection of the hydroxyl group of **2** as a THP ether (**3**), which was followed by hydrolysis of the ester groups, gave the dicarboxylic acid (**4**). This was subsequently treated with an alcohol, CDI, DMAP, and DBU, or with an alkyl halide, KI, and K_2CO_3, to give the diesters (**5a1**−**3** and **5b1**−**19**). Some of the alcohols used in these acylation and

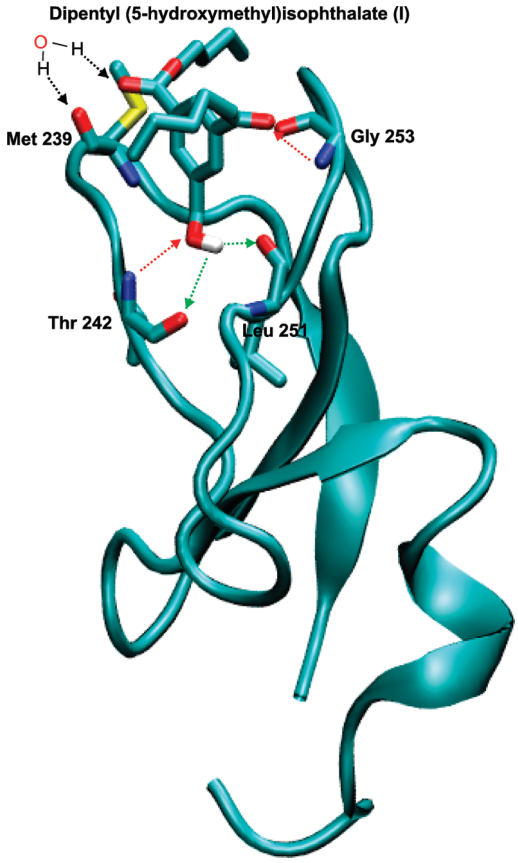


Figure 2. Dipentyl (5-hydroxymethyl)isophthalate (**I**) docked in the PKC δ C1b domain. The hydroxyl group of the ligand is hydrogen bonded to the backbone amide proton of Thr 242 (red arrow) and the carbonyls of Thr 242 and Leu 251 (green arrows). The model also shows that one of the carbonyl groups of the template (**I**) forms a hydrogen bond with the backbone amide proton of Gly 253 (red arrow). A bridging water molecule between the backbone carbonyl of Met 239 and the other carbonyl (black arrows) could stabilize the complex. The figure was created using VMD (v.1.8.6., University of Illinois).

substitution reactions were not commercially available and were synthesized in our laboratory (see Supporting Information). The hydroxymethyl groups of **5a1−3** and **5b1−19** were deprotected to give the targeted diesters (**1a1−3** and **1b1−19**) (Table 1). This procedure was used to prepare all the required diesters with the need for only a single chromatographic purification step. In addition to the diesters, the dicarboxylic acid (**1b20**) was prepared by the hydrolysis of (**2**) (Table 1, see Supporting Information).

The ester groups of **1a3** and **1b2** were selected to further SAR studies. We focused on the hydroxymethyl group of the template (**I**) and synthesized a group of compounds (**15a−e**) that lacked this group (Scheme 2, Table 2). These were prepared from the corresponding carboxylic acids, and one unsymmetrical diester (**15c**) was synthesized among these esters.

To investigate the significance of having two ester groups in the template molecule (**I**), we prepared three monoesters (**22a−c**) (Scheme 3 and Table 2). Treatment of **16** with NaOH gave monomethyl isophthalate (**17**). Reduction of the carboxylic acid was performed with borane dimethyl sulfide complex ($BH_3 \cdot SMe_2$),[38] and the resultant alcohol (**18**) was protected as a THP-ether (**19**). Hydrolysis of the methyl ester (**19**) gave the carboxylic acid (**20**), which was converted to esters (**21a−c**). Finally, the deprotection of the THP group by PPTS yielded the ester products (**22a−c**).

To increase the hydrophilicity of the compounds, we prepared several amide derivatives of the diesters (Schemes 4 and 5; Table 2). Synthesis of the 5-(hydroxymethyl)isophthalamides (**24a−b**) was initiated by coupling the amines with **4**. Subsequent deprotection of the THP groups in **23a−b** produced compounds **24a−b**. The reverse diamide (**27**) was prepared from the 3,5-diaminobenzyl alcohol (**25**) by acylation and the subsequent chemoselective hydrolysis of **26**. The second reverse diamide was synthesized by catalytic hydrogenation of the nitro groups in **28**, followed by acylation of the resultant phenylenediamine (**29**) to give the diamide (**30**), which was used as a negative control compound for binding to the C1 domain of PKC α and δ.

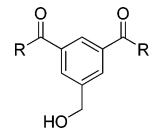
In the following set of synthesis reactions, we wanted to investigate the effect of having one ester and one reverse amide group in the compound. Synthesis of the 3-(hydroxymethyl)-5-(alkanoylamino)benzoates (**36a−e**) was initiated from the commercially available 5-nitroisophthalic acid monomethyl ester (**31**) (Scheme 6 and Table 2). The hydroxymethyl group, which is important for binding, was obtained in the first step by chemoselective reduction of the carboxylic acid (**31**) using borane dimethyl sulfide complex. This gave a better yield (84%) than using the borane tetrahydrofuran complex (70%).[39] The alcohol (**32**) was protected as a THP ether (**33**), and the subsequent catalytic reduction of the nitro group gave the amine product (**34**). The subsequent N-acylation with a set of carboxylic acids yielded the corresponding amides (**35a−e**), while the final deprotection of the THP-groups by PPTS gave the products **36a−e**.

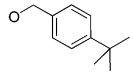
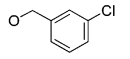
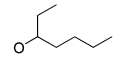
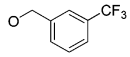
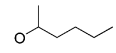
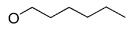
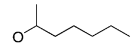
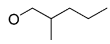
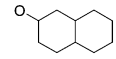
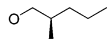
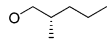
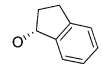
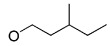
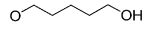
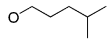
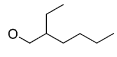
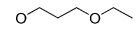
We also prepared one monoester that contained an amide group (**39**). This synthesis commenced with monohydrolysis of the THP-protected diester (**3**) (Scheme 7) and the subsequent N-acylation of 3-(trifluoromethyl)benzylamine with **37** to give **38**. Deprotection of the THP ether using PPTS and purification by SiO_2 column chromatography yielded the amide product (**39**).

Biological Data

Screening and Structure−Activity Analysis for Binding to PKC C1 Domain. We first screened the synthesized compounds for binding to the C1 domain of recombinant human PKC α and δ using a filtration method on 96-well plate format.[40] We used crude cell lysates from baculovirus-infected *Sf*9 cells as the source for PKC in the assay; the lysate from uninfected *Sf*9 cells did not exhibit any phorbol ester binding (not shown). The compounds were used at a concentration range of 0.1−20 μM, with the highest concentration being based on the estimated maximal solubility in aqueous buffer. The results revealed that the first set of compounds with different ester side chains, with the exception of compounds **1b17−1b21** (Table 1), displaced [20-^{3}H]phorbol-12,13-dibutyrate ([^{3}H]PDBu) from PKC α and δ in a concentration-dependent manner. Compounds **1b17**, **1b18**, and **1b19** have more hydrophilic side chains and were unable to displace [^{3}H]PDBu at the concentration range used. In addition, diethyl 5-(hydroxymethyl)isophthalate (**1b21**, Table 1), which contains significantly shorter side chains, and 5-(hydroxymethyl)isophthalic acid (**1b20**, Table 1), which lacks the alkyl side chains, were unable to bind to the PKC C1 domain under the conditions employed in this study. Compounds **1b10**, **1b11**, and **1b13**, all with aliphatic carbon side chains and high hydrophobicity, appeared to be the most effective in displacing [^{3}H]PDBu from PKC α and δ. Total binding was the only factor

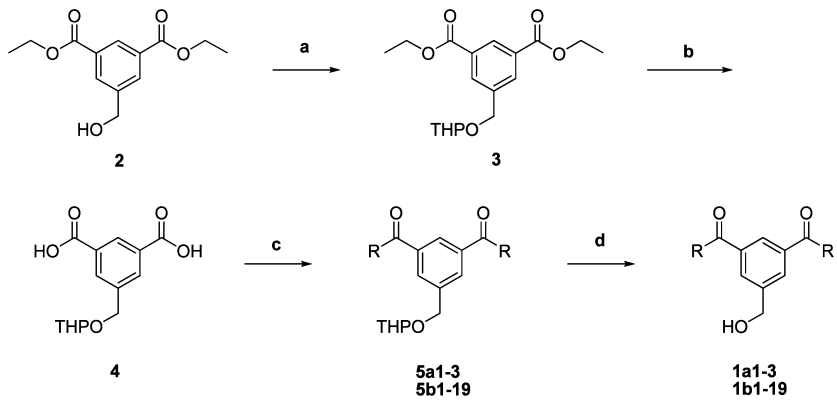
Table 1. 5-(Hydroxymethyl)isophthalates **1a1−3** and **1b1−21**, Their clog*P* Values, and Binding Affinity to PKC α and δ^a



Compound	R	clog*P*	Residual [^{3}H]PDBu binding to PKCα (% of control)	Residual [^{3}H]PDBu binding to PKCδ (% of control)	Compound	R	clog*P*	Residual [^{3}H]PDBu binding to PKCα (% of control)	Residual [^{3}H]PDBu binding to PKCδ (% of control)
1a1		8.35	58.0 ± 2.9	47.2 ± 5.6	**1b10**		5.89	45.0 ± 2.7	30.7 ± 1.7
1a2		6.12	54.7 ± 3.2	44.0 ± 3.6	**1b11**		6.95	33.2 ± 1.7	23.1 ± 3.3
1a3		6.46	56.5 ± 2.9	46.9 ± 2.6	**1b12**		5.89	56.0 ± 3.5	41.1 ± 2.9
1b1		6.33	59.4 ± 3.7	43.4 ± 1.6	**1b13**		6.95	39.3 ± 3.0	28.3 ± 3.2
1b2		6.07	59.9 ± 4.6	45.7 ± 2.8	**1b14**		7.97	73.3 ± 3.7	58.8 ± 0.4
1b3		6.07	53.3 ± 0.9	42.0 ± 2.0	**1b15**		7.97	67.4 ± 3.9	49.6 ± 1.4
1b4		6.07	56.1 ± 2.9	43.3 ± 2.0	**1b16**		5.53	65.7 ± 3.3	44.2 ± 0.6
1b5		6.07	49.6 ± 3.3	41.2 ± 4.1	**1b17**		1.30	116.7 ± 4.6	128.2 ± 5.3
1b6		6.07	52.7 ± 7.3	44.6 ± 1.3	**1b18**		0.69	114.2 ± 7.4	126.1 ± 10.9
1b7		8.19	64.4 ± 4.0	49.5 ± 5.4	**1b19**		2.35	90.0 ± 4.9	89.6 ± 3.1
1b8		6.89	59.5 ± 5.0	38.0 ± 2.0	**1b20**		0.82	97.9 ± 1.4	91.4 ± 4.3
1b9		6.34	68.0 ± 2.9	52.5 ± 0.9	**1b21**		2.10	92.4 ± 2.0	87.1 ± 4.6

[a] The binding affinity was screened as described in the experimental section and is expressed as mean ± sem (n = 3−8) of residual [^{3}H]PDBu binding (% of control) with 20 μM ligand concentration.

Scheme 1[a]

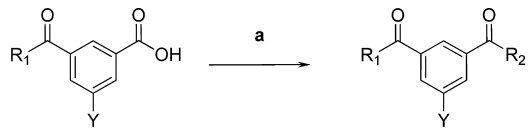


[a] Conditions: (a) DHP, PPTS, DCE, rt, 6 h, 97%; (b) KOH (10%, aq), MeOH, reflux, 1 h, 85%; (c) alkyl halide, K_2CO_3, KI, DMF, 110 °C, 2 h (**5a1−3**) *or* alcohol, CDI, DMAP, DBU, DMF, rt → 40 °C, 22 h (**5b1−19**); (d) Dowex 50W × 8, MeOH, 40 °C, overnight, 10−95% (two steps).

that was measured in these initial assays, and therefore no binding constants or IC_{50} values were calculated based on these results.

We next examined the contribution of the hydroxymethyl group and the ester groups to the binding affinity of isophthalic acid derivatives. All compounds lacking the hydroxymethyl

Scheme 2[a]

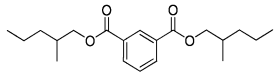
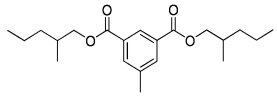
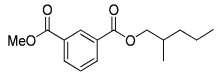
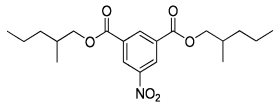
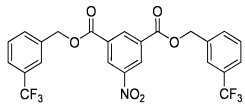
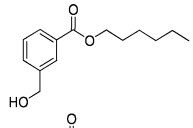
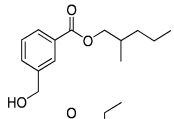
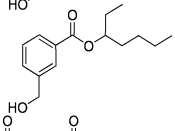
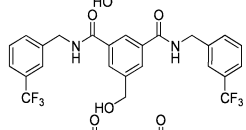
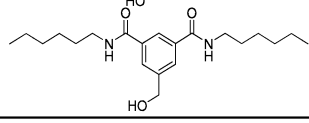
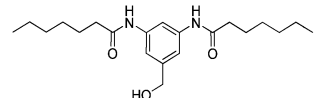
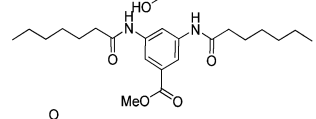
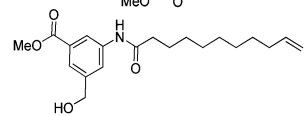
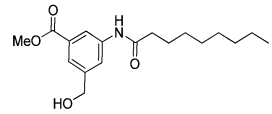
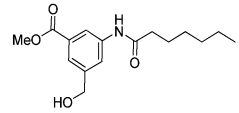
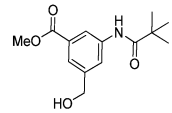
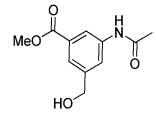
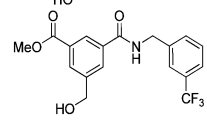


14a (R_1 = OH, Y = H)
14b (R_1 = OH, Y = Me)
14c (R_1 = OMe, Y = H)
14d (R_1 = OH, Y = NO_2)
14e (R_1 = OH, Y = NO_2)

15a (R_1, R_2 = $OCH_2CH(CH_3)CH_2CH_2CH_3$, Y = H)
15b (R_1, R_2 = $OCH_2CH(CH_3)CH_2CH_2CH_3$, Y = Me)
15c (R_1 = OMe, R_2 = $OCH_2CH(CH_3)CH_2CH_2CH_3$, Y = H)
15d (R_1, R_2 = $OCH_2CH(CH_3)CH_2CH_2CH_3$, Y = NO_2)
15e (R_1, R_2 = OCH_2(3-CF_3-Ph), Y = NO_2)

[a] Conditions: (a) 2-methyl-1-pentanol, CDI, DMAP, DBU, DMF, rt → 40 °C, 19−21 h (**14a**−**d**) *or* 3-(trifluoromethyl)benzyl chloride, K_2CO_3, KI, DMF, 80 °C, 2 h, (**14e**), 28−76%.

Table 2. Synthesized Derivatives, Their clog*P* Values, and Binding Affinity to PKC α and δ[a]

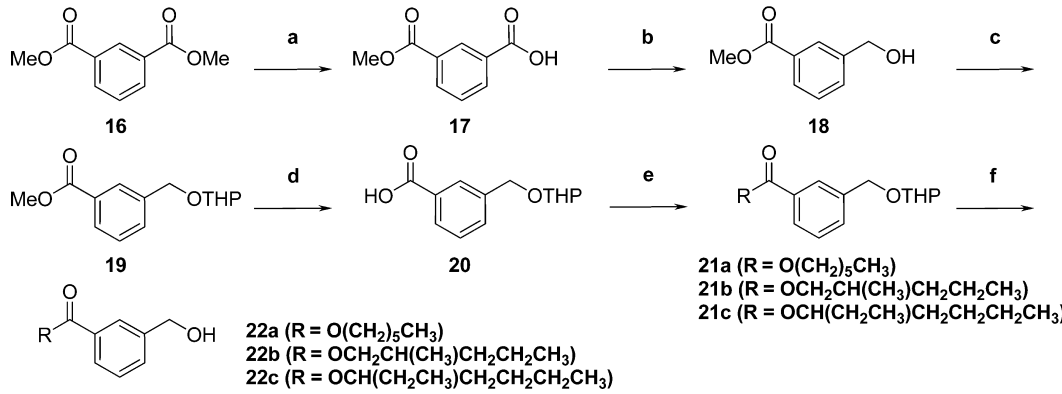
Compound	clog*P*	Residual [3H]PDBu binding to PKCα (% of control)	Residual [3H]PDBu binding to PKCδ (% of control)
15a 	7.11	93.7 ± 5.9	104.9 ± 9.4
15b 	7.61	96.7 ± 3.6	90.0 ± 1.9
15c 	4.6	92.4 ± 4.3	78.1 ± 6.7
15d 	6.85	101.3 ± 3.9	98.8 ± 5.4
15e 	7.25	97.6 ± 5.2	97.7 ± 16.6
22a 	3.72	91.2 ± 3.3	88.4 ± 3.0
22b 	4.12	98.1 ± 4.5	94.0 ± 5.2
22c 	4.03	100.3 ± 8.3	110.1 ± 6.7
24a 	4.70	80.6 ± 1.4	84.7 ± 6.1
24b 	4.29	90.4 ± 5.9	83.0 ± 7.8
27 	4.43	93.2 ± 4.1	83.8 ± 7.0
30 	6.05	102.7 ± 7.2	100.6 ± 10.2
36a 	4.84	66.3 ± 7.1	73.9 ± 4.2
36b 	4.26	83.0 ± 10.3	102.6 ± 7.1
36c 	3.21	96.5 ± 9.2	114 ± 7.1
36d 	1.80	103.6 ± 4.7	102.3 ± 9.6
36e 	0.56	102.9 ± 5.3	118.3 ± 9.8
39 	3.01	91.6 ± 9.2	95.3 ± 6.8

[a] The binding affinity was screened as described in the Experimental Section and is expressed as the mean ± SEM (n = 3−8) of residual [^{3}H]PDBu binding (% of control) with 20 μM ligand concentration.

group (replaced with hydrogen, or a methyl, or a nitro group) exhibited a significantly diminished binding at the concentration range used in this study (Table 2, compounds **15a**−**e**). Additionally, the unsymmetrical ligands that lacked one of the ester groups (Table 2, compounds **22a**−**c**) were not able to displace [^{3}H]PDBu from PKC (Table 2). Thus, the hydroxymethyl group and both of the ester groups are essential for creating sufficient binding affinity to the C1 domain.

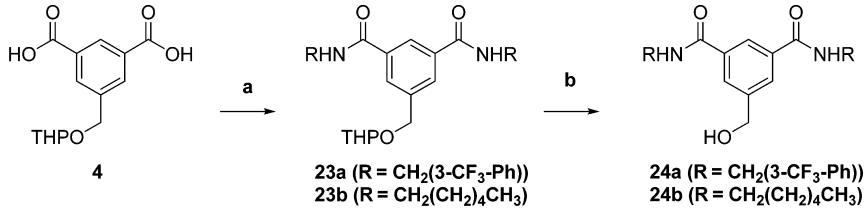
We also investigated the effect of replacing one or both of the ester bonds with amide bonds, both to test derivatives with

Scheme 3[a]



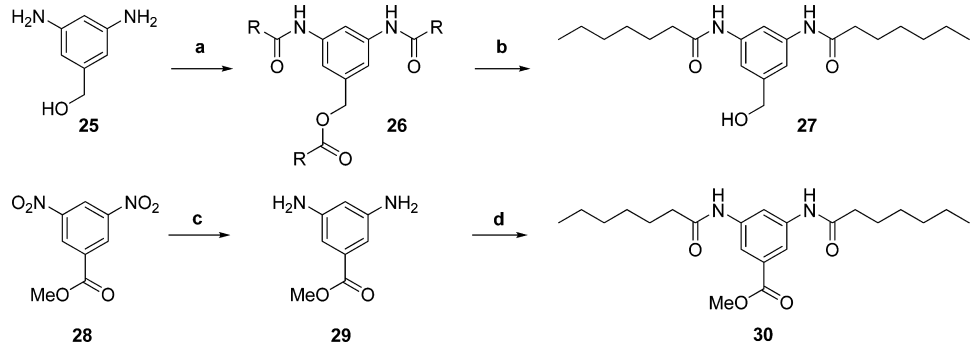
[a] Conditions: (a) NaOH (1.05 equiv), MeOH, acetone, rt, 21 h then NaOH (0.1 equiv), rt, 4 h, 98%; (b) $BH_3 \cdot SMe_2$, THF, 0 °C → rt, 5 h, 29%; (c) DHP, PPTS, DCE, rt, 16 h, 99%; (d) KOH (10%, aq), MeOH, 90 °C, 17 h, 67%; (e) alcohol, CDI, DMAP, DBU, DMF, rt → 40 °C, 21 h; (f) Dowex 50W × 8, MeOH, 40 °C, 17 h, 65−75% (two steps).

Scheme 4[a]



[a] Conditions: (a) DIPEA, EDC, HOBt, amine, rt → 40 °C, 4−23 h; (b) Dowex 50W × 8, MeOH, 40 °C, 23−24 h, 28−39% (two steps).

Scheme 5[a]



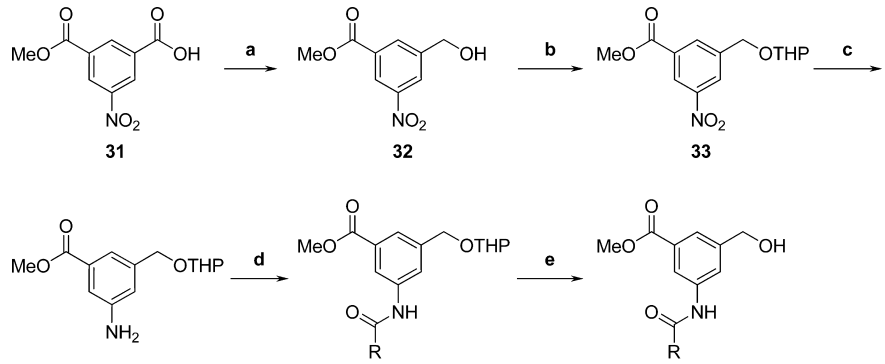
[a] Conditions: (a) heptanoyl chloride (R = $(CH_2)_5CH_3$), pyridine, DCM, rt, 20 h; (b) KOH (10%, aq), MeOH, rt, 4 h, 95%; (c) H_2, Pd/ C (10%), EtOH: EtOAc (2:1), rt, 22 h, 99%; (d) Heptanoyl chloride, DIPEA, DCM, rt, 21 h, 48%.

different groups around the aromatic backbone and to increase the hydrophilicity of the compounds. The C_2 symmetric amides, where both of the ester groups of the template **I** were replaced with amide groups (Table 2, compounds **24a**−**b**, **27**, and the negative control **30**), were unable to bind to PKC with a comparable affinity compared to the original compounds. This was true irrespective of the orientation of the amide group (compound **24b** versus the reverse amide **27**). Additionally, most of the monoamide derivatives (Table 2, compounds **36a**−**e** and **39**) were unable to displace [^{3}H]PDBu from the PKC C1 domain as effectively as the original compounds. Only compound **36a**, which had a long hydrophobic side chain, showed a slight concentration-dependent binding affinity to PKC α and δ.

In summary, our results show that the hydroxymethyl group and the ester groups of the template (**I**) are indispensable for binding to the PKC C1b domain. Modifying these regions of the molecule leads to diminished binding. In addition to these functional groups, the amount of hydrophobicity of the side chains appears to play an important role in achieving binding affinity in the micromolar range.

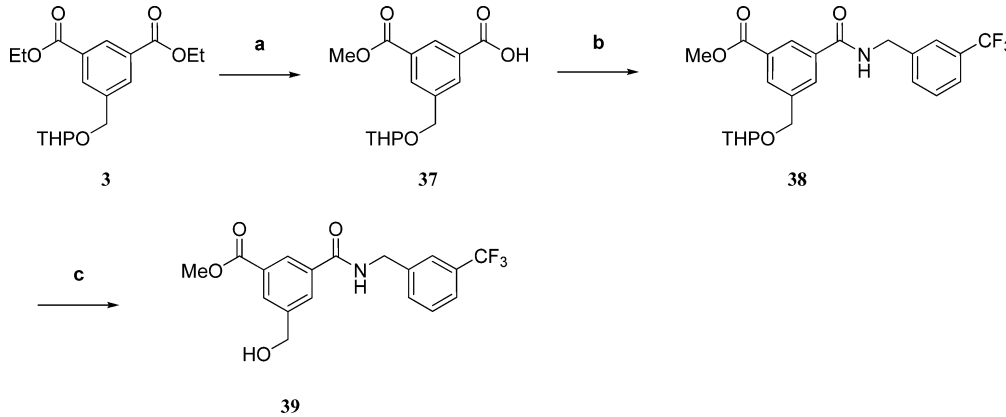
Binding Affinity Constants of Selected Compounds to Purified PKC α and δ. Following the initial screening for binding against PKC α and δ, we determined the binding constants for three diesters (**1a3, 1b10**, and **1b11**). Because the screening data suggested that the affinities of most of the ligands were within the same concentration range, we did not determine binding constants for all of the ligands. The K_d values determined with [^{3}H]PDBu saturation binding and Scatchard analysis to purified commercial recombinant human PKC α and δ were 1.29 and 2.35 nM, respectively (data not shown). We therefore used a concentration of 10 nM for the radioligand in the subsequent competition binding experiments. To validate the assay, we determined the K_i values for 1,2-dioctanoyl-*sn*-glycerol (DOG) and bryostatin 1. DOG inhibited the binding of [^{3}H]PDBu to PKC α with a K_i of 40.1 (±6.0) nM, and bryostatin 1 inhibited the binding of [^{3}H]PDBu to PKC δ with a K_i of 0.44 (±0.08) nM (Table 3). These values are in accordance with published data.[41,42] The inhibitory binding constants for compounds **1a3**, **1b10**, and **1b11** are shown in Table 3. All three ligands were

Scheme 6[a]



[a] Conditions: (a) $BH_3 \cdot SMe_2$, THF, 0 °C → 60 °C, 21 h, 86%; (b) DHP, PPTS, DCE, rt, 25 h, 99%; (c) H_2, Pd/C (10%), EtOH:THF (2:1), rt, 23 h, 100%; (d) RCO_2H, DIPEA, EDC, HOBt, DCM, 40 °C, 21−23 h, 13−58%; (e) Dowex 50W × 8, MeOH, 40 °C, 19−24 h, 46−89%.

Scheme 7[a]



[a] Conditions: (a) KOH (1 equiv), MeOH, 40 °C, 20 h, 65%; (b) DIPEA, EDC, HOBt, amine, rt → 40 °C, 4 h; (c) Dowex 50W × 8, MeOH, 40 °C, 23 h, 41% (two steps).

Table 3. Binding Affinities of Selected Isophthalate Derivatives[a]

	K_i (nM) PKC α	K_i (nM) PKC δ
1a3	205 ± 14 (4)	590 ± 195 (4)
1b10	661 ± 90 (3)	915 ± 132 (3)
1b11	319 ± 12 (3)	529 ± 61 (3)
DOG	40.1 ± 6.0 (4)	nd
bryostatin 1	nd	0.44 ± 0.08 (5)

[a] Data represent the means ± SEM. The number of independent experiments is shown in parentheses (nd = not determined).

capable of displacing the radioactively labeled PDBu with a comparable submicromolar affinity. The ligands bound to both PKC α and δ at nearly identical affinities and with no significant selectivity toward either of the isoenzymes. In general, the K_i values were somewhat lower for PKC α than for δ. However, compound **1a3** was able to displace a substantially smaller proportion of the radioligand from PKC δ than from α, while **1b10** and **1b11** exhibited similar efficacy toward both isoenzymes (Figure 3).

The Effect of Isophthalic Acid Derivatives on ERK1/2 Phosphorylation in HeLa Cells. Because the compounds could compete with PDBu in vitro, we wanted to examine whether they were able to penetrate the cell membrane and modulate PKC activity within the cellular environment. To investigate this issue, we studied their ability to modulate ERK1/2 phosphorylation in living cells. ERK1/2 is member of the mitogen activated protein (MAP) kinase signaling cascade that controls a broad range of cellular activities.[43] It has been shown that multiple PKC isoforms can induce ERK1/2 phosphorylation via the Raf-MEK pathway; additionally, PMA-induced ERK1/2 phosphorylation is mediated by PKC.[44]

Human cervical cancer (HeLa) cells were treated with a 20 μM concentration of compounds **1a3**, **1b10**, and **1b11** for 1−20 min. Compounds **1a3** and **1b10**, but not **1b11**, induced ERK1/2 phosphorylation within 3 min (Figure 4A). The phosphorylation induced by **1a3** and **1b10** was blocked by pretreatment with the MEK inhibitor U0126 (Figure 4A). The PKC inhibitor Gö6983 (final conc 1 μM) inhibited the **1a3** and the **1b10**-induced ERK1/2 phosphorylation by 38% ± 7% and 36% ± 9%, respectively (Figure 4A), as determined by the quantification of Western blots from 3−4 independent experiments. Because **1b11** was able to displace [^{3}H]PDBu from PKC in vitro but could not induce ERK1/2 phosphorylation by itself, we examined whether it could still compete with phorbol esters binding to PKC in living cells. HeLa cells were treated with various concentrations of **1b11** for 30 min before stimulating the cells for 5 min with 10 nM PMA. PMA alone induced a pronounced phosphorylation of ERK1/2 (Figure 4B). Pretreatment of the cells with **1b11**

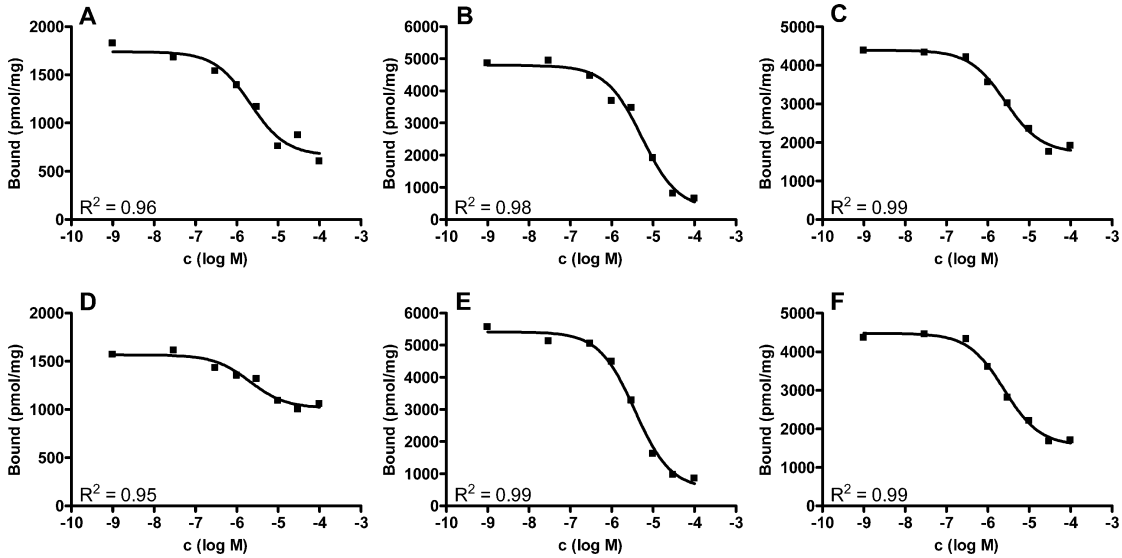


Figure 3. Binding of selected isophthalate derivatives to purified PKC α and δ. Binding of 10 nM [^{3}H]PDBu was measured in the presence of different concentrations of the isophthalate derivatives. Representative competition binding curves for (A) PKC α and **1a3**, (B) PKC α and **1b10**, (C) PKC α and **1b11**, (D) PKC δ and **1a3**, (E) PKC δ and **1b10**, and (F) PKC δ and **1b11**. Data represent means from three parallel samples in a single experiment.

inhibited the PMA-evoked phosphorylation of ERK1/2 in a concentration-dependent manner (Figure 4B).

Discussion

Several laboratories have focused their studies on the C1 domain of PKC in order to discover PKC-modulating compounds for drug development. Bryostatin 1, a marine-derived compound that acts through the C1 domain, is currently in phase II clinical trials for the treatment of cancer. However, naturally occurring C1 domain ligands are highly complex in their structure. Therefore, structural modification with the aim of altering the ligand specificity is difficult.[18] DAG-lactones and indolactam derivatives, the major groups of synthetic C1 domain ligands, are considerably simpler than the natural products,[18] however, they are also laborious to synthesize and modify. We therefore sought to find simpler template for ligand synthesis, whose structure could be easily modified and fine-tuned in order to achieve selectivity.

C_2 symmetric diethyl 5-(hydroxymethyl)isophthalate is a commercially available and easily derivatizable compound. While being structurally simple, our molecular docking experiments showed that its derivative, dipentyl 5-(hydroxymethyl)-isophthalate, could bind to the C1 domain of PKC in a similar fashion as phorbol esters. The same hydrogen bond interactions that have been proposed to occur for other C1 domain ligands (such as phorbol esters[35] and DAG-lactones[27]) could be attained with the isophthalate core structure. We synthesized a series of isophthalic acid derivatives to confirm their binding to the C1 domain of PKC and to study the impact that different side chains had on the binding affinity. Binding studies showed that the compounds were able to displace [^{3}H]PDBu from the C1 domains of PKC α and δ. Modifying the ester side chains had a very modest effect on the binding as long as the side chains provided sufficient hydrophobicity, which appears to be an important determinant for the binding affinity. The best compounds had clog*P* values higher than 5, whereas analogues with lower clog*P* values, e.g., the ether analogue **1b19** of **1b1** (clog*P* values of 2.35 and 6.33, respectively) did not exhibit high binding affinity. In addition, **1b21**, which carries short ester groups and has a clog*P* value of 2.10, could not compete with [^{3}H]PDBu for binding to PKC α or δ either. The results that highlight the importance of hydrophobicity in ligand binding are similar to those reported for other C1 domain ligands,[35] including the DAG-lactones, which have been shown to have an estimated optimal clog*P* value between 5 and 6.[37] However, because the clog*P* value of PDBu (3.43) is significantly lower and PDBu has been shown to have a higher affinity to the C1 domain[45] than the isophthalate derivatives or the DAG-lactones, it might be possible to modify the structure and reduce the lipophilicity while increasing the binding affinity.

We also studied the effects of replacing the structural moieties that, as predicted by the pharmacophore model, were required for C1 domain recognition. As expected, the hydroxymethyl group and both of the carbonyl groups were important for binding. Surprisingly, the diamide derivatives (**24a**−**b** and **27**) were unable to bind to the PKC C1 domain. This might be due to unfavorable interactions of the two amide protons with the C1 domain. The monoamide derivatives **36a**−**e** were also unable to compete with [^{3}H]PDBu for binding to the C1 domain of PKC α and δ in a similar manner as the corresponding diester analogues. However, the compound with the longest and most hydrophobic amide side chain (**36a**) and therefore the highest clog*P* value (4.84, compared to 0.56−4.26 for **36b**−**e**), exhibited some binding toward PKC. One can therefore speculate that both the decreased hydrophobicity and the lack of the other ester group contribute to the diminished binding affinity observed with the monoamide derivatives. A compound with some similarity to these monoamide derivatives (**36a**−**e**) has previously been described to inhibit phorbol ester binding to the PKC regulatory domain.[46]

On the basis of the results from screening for [^{3}H]PDBu displacement, we selected compounds **1a3**, **1b10**, and **1b11** for further testing. Using the assay developed by the Blumberg group,[47] we were able to show that the compounds bind to PKC α and δ with comparable submicromolar affinities. Compounds **1b10** and **1b11** displaced [^{3}H]PDBu from both PKC isoenzymes in a similar manner. However, compound **1a3** was able to displace only a small proportion of the radioligand from PKC δ while exhibiting more efficient displacement of [^{3}H]PDBu from PKC α. The differences in the spatial geometry of the

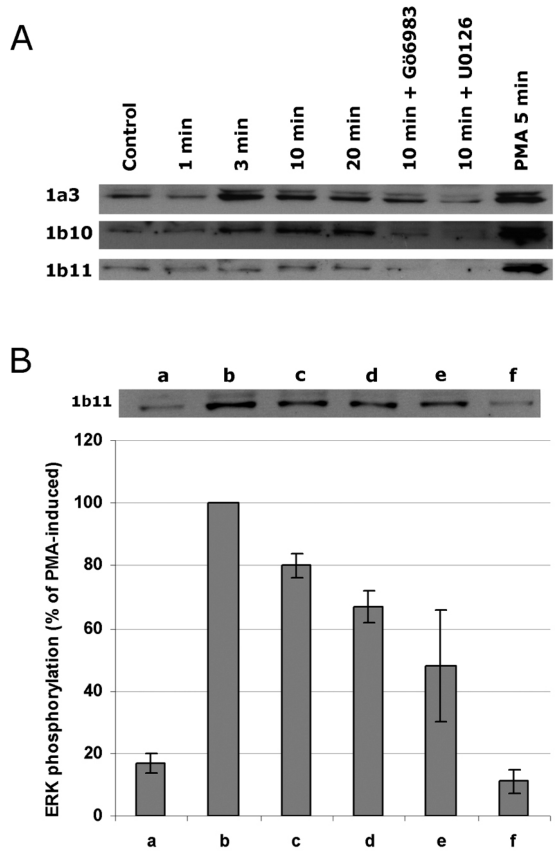


Figure 4. Effects of **1a3**, **1b10**, and **1b11** on ERK1/2 phosphorylation in HeLa cells. (A) Cells were treated with 20 μM concentration of **1a3**, **1b10**, or **1b11** for the indicated times. The PKC inhibitor Gö6983 (1 μM) or the MEK inhibitor U0126 (10 μM) were added to the cells 5 min before the addition of the test compounds. PMA (10 nM for 5 min) was used as a positive control. Cells were harvested and ERK1/2 phosphorylation was detected as described in Experimental Section. (B) Effect of **1b11** on PMA-induced ERK1/2 phosphorylation. A representative blot from a single experiment (upper panel) and quantification results (mean ± SEM) from three independent experiments are shown (lower panel). The cells were treated with different concentrations of **1b11** for 25 min and subsequently stimulated with 10 nM of PMA for 5 min. The cells were then harvested and ERK1/2 phosphorylation was detected as described in the Experimental Section. (a) Untreated cells, (b) PMA alone, (c) 1 μM of **1b11** + PMA, (d) 5 μM of **1b11** + PMA, (e) 20 μM of **1b11** + PMA, (f) 20 μM of **1b11** alone.

side chains could be one possible explanation to the observed phenomenon. The side chains of **1b10** and **1b11** are aliphatic and therefore more flexible than the aromatic side chains of **1a3**, which are more constrained as a result of the planar conformation of the aromatic rings. In addition to the differences in the side chains of the compounds studied, there are also differences between individual C1 domains of different PKC isoenzymes. Particularly, there is a marked difference in DAG binding between the novel and the classical PKC isoenzymes.[48] The novel PKC isoenzymes are more sensitive to DAG binding than the classical isoenzymes, a phenomenon that appears to be dependent on a single amino acid residue within the C1 domain.[49] Because the PKC isoforms studied here include both classical PKCs and novel PKCs (PKC α and δ, respectively), it is possible that the structures of their binding sites would differ sufficiently to contribute to the observed phenomenon. However, differences exist also between the affinity of individual C1a and C1b domains of PKC isoenzymes within the subgroups of PKC isoenzymes.[11] For example, in the case of cPKCs, the C1a domain of PKC α binds DAG with a higher affinity than phorbol esters, whereas the PKC α C1b domain exhibits higher affinity for phorbol esters than for DAG.[41] On the other hand, both the C1a and C1b domains of PKC γ bind DAG and phorbol esters with high affinities.[41] Thus, while it is tempting to speculate about the basis for this potential selectivity of **1a3** toward classical isoenzymes, making definitive conclusions about the specificity mechanism at this stage may be premature. Further studies with different PKC isoenzymes and individual C1 domain constructs are needed to clarify the selectivity profile of these compounds.

The hydrophobicity of the compounds suggests that within a cellular environment the compounds could easily diffuse into the cell membranes and reach PKC in a similar manner as that of phorbol esters and DAGs. To address both the cell membrane penetration and the modulation of PKC activity within cellular environment, we studied the effects of compounds **1a3**, **1b10**, and **1b11** on ERK1/2 phosphorylation in HeLa cells. Compounds **1a3** and **1b10** induced ERK1/2 phosphorylation, which was inhibited by pretreatment with the PKC inhibitor Gö6983, thus confirming that the effect was PKC-dependent. Surprisingly, while the structure of **1b11** differs from **1b10** by only a single carbon in the ester side chains, **1b11** was unable to induce ERK1/2 phosphorylation. However, **1b11** inhibited the PMA-induced ERK1/2 phosphorylation, demonstrating that it can penetrate the cell membrane and bind to the PKC C1 domain in living cells. These results suggest that, under these conditions, compounds **1a3** and **1b10**, but not compound **1b11**, are capable of activating one or more PKC isoforms in HeLa cells. It is possible that **1b11** had a weak activating effect on PKC, which was not detected with the ERK phosphorylation assay. As a weak activator, **1b11** could still antagonize the effect of a much stronger activator (PMA). Such an antagonism has been reported between the PKC activator bryostatin 1 and PMA.[50] The activation and/or inhibition profiles of these compounds need to be studied in more detail.

On the basis of the results presented here, we are currently working toward improving the affinity and selectivity of the isophthalate derivatives. Because there are additional proteins in cells that contain a phorbol-responsive C1 domain, binding of these compounds to non-PKC phorbol ester receptors will be studied. Selectivity, while difficult to attain because of the high degree of conservation within the binding cleft of the C1 domain, is achievable, as demonstrated with the DAG-lactones.[28,51] Furthermore, preliminary studies have shown that a number of compounds described in this report inhibit HeLa cell proliferation (Talman et al. unpublished results); studies to delineate the mechanism of those effects are in progress. Additionally, preliminary pharmacokinetic evaluation of cell membrane permeability and absorption are currently being carried out on these compounds.[52]

Conclusions

In conclusion, we describe here a novel group of compounds, dialkyl 5-(hydroxymethyl)isophthalates, that target the phorbol ester binding site of PKC. The compounds displace [^{3}H]PDBu from the C1 domain of PKC α and δ in vitro at submicromolar concentrations and can regulate PKC-dependent signaling in living cells, as shown by the ERK phosphorylation studies. The compounds are conveniently and effectively synthesized starting from the commercially available diethyl 5-(hydroxymethyl)-isophthalate. This makes isophthalic acid derivatives an at-

tractive candidate for further SAR studies and for further development as research tools or lead compounds in drug development.

Experimental Section

Materials and General Procedures. All reagents were commercially available and were acquired from Fluka (Buchs, Switzerland) and Sigma-Aldrich (Schnelldorf, Germany). THF and Et_2O were distilled from sodium/benzophenone ketyl. $CHCl_3$ was distilled from CaH_2. Anhydrous DMF was from Fluka (Buchs, Switzerland) and was stored over molecular sieves (4 Å) under an inert atmosphere of dry argon. All reactions in anhydrous solvents were performed in flame-dried glassware under an inert atmosphere of dry argon. The progress of chemical reactions was monitored by thin-layer chromatography on silica gel 60-F_{254} plates acquired from Merck (Darmstadt, Germany) using phosphomolybdic acid stain (10% by weight in EtOH) or ninhydrin stain (1.5% by weight in EtOH). Flash SiO_2 column chromatography was performed with a Merck silica gel 60 (230−400 mesh) or with a Biotage high-performance flash chromatography Sp^4-system (Uppsala, Sweden) using a 0.1 mm path length flow cell UV-detector/recorder module (fixed wavelength: 254 nm), 12 mm or 25 mm flash cartridges, and the indicated mobile phase.

^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded on a Varian Mercury 300 MHz or a Varian Unity 500 MHz spectrometer (Varian, Palo Alto, CA) as solutions in $CDCl_3$, DMSO-d_6, CD_3OD, or CD_2Cl_2. Deuterated solvents were purchased from Aldrich. Chemical shifts (δ) are given in parts per million (ppm) relative to the NMR solvent signals ($CDCl_3$ 7.26 and 77.21 ppm, DMSO-d_6 2.50 and 39.52 ppm, CD_3OD 3.31 and 49.00 ppm, CD_2Cl_2 5.32 and 53.80 ppm for ^{1}H and ^{13}C NMR, respectively). Multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), quintet (qn), and m (multiplet). The synthesized compounds were analyzed by HPLC-MS. HPLC-MS analyses were performed to determine the purity of all tested compounds by a HP1100 instrument with UV detector (λ 210 nm) and a Perkin-Elmer Sciex API3000 triple−quadrupole LC/MS/MS mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Canada) with a turbo ESI source. Signal separation was carried out by a XTerra MS RP18 column (4.6 mm × 30 mm, 2.5 μm). High resolution mass spectra (HRMS) were run on a Q-TOF Micro (quadrupole time-of-flight) mass spectrometer (Waters/Micromass, Manchester, UK) with an ESI source in positive ion mode. The instrument was calibrated with an internal standard. The elemental analyses were conducted by Robertson-Microlit Laboratories (Madison, NJ). Melting points were measured with an Electrothermal IA 9100 apparatus. Purity of all tested compounds was >95%. log*P* Values for the compounds were calculated using ChemBioDraw Ultra 11.0 (CambridgeSoft, Cambridge, MA).

[20-^{3}H]Phorbol-12,13-dibutyrate ([^{3}H]PDBu) was custom labeled by Amersham Radiolabeling Service (GE Healthcare, Little Chalfont, UK). Unlabeled PDBu, phorbol 12-myristate-13-acetate (PMA), phosphatidyl-L-serine (PS), and bovine immunoglobulin G (IgG) were purchased from Sigma-Aldrich (Steinheim, Germany). Purified recombinant human PKC α and δ were from Invitrogen (Carlsbad, CA). Protease inhibitors (Complete Protease Inhibitor Cocktail Tablets) and phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail Tablets) were from Roche (Mannheim, Germany). Anti-ACTIVE MAPK polyclonal antibody was from Promega (Madison, WI), anti-GAPDH monoclonal antibody, and HRP-conjugated goat antimouse IgG were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and the HRP-conjugated goat antirabbit IgG was from Bio-Rad Laboratories (Hercules, CA). Precision Plus Protein Kaleidoscope standard ladder (Bio-Rad Laboratories, Hercules, CA) was used as the SDS-PAGE marker. ECL reagents (SuperSignal-West-Pico-Chemiluminescent-Substrate-Kit) were from Pierce (Thermo Fisher Scientific Inc., Rockford, IL).

Cell culture solutions and reagents were purchased from Invitrogen (Carlsbad, CA) unless otherwise stated. *Sf*9 cells were purchased from Invitrogen. HeLa cells were acquired from American Type Culture Collection (ATCC, Manassas, VA) and cultured at 37 °C in a humidified, 5% CO_2 atmosphere with DMEM (Sigma-Aldrich, Steinheim, Germany) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Molecular Modeling. Molecular docking modeling was performed using the crystal structure of PKC δ C1b domain complexed with phorbol-13-*O*-acetate (Protein Data Bank code: 1PTR).[31] First, the phorbol-13-*O*-acetate was removed from the structure. The hydrogens, as well as the side chains of Lys 234, Arg 273, and Glu 274 not visible in the X-ray structure, were subsequently added to the protein and the structure was energy minimized in SYBYL 7.0 (Tripos, St Louis, MO) using the TRIPOS force field. A hundred iterations using the conjugate gradient method were performed. Superimposition of the experimental and minimized structures showed no significant deviations. The minimized structure was used for ligand docking. Ligand geometry was optimized using the semiempirical AM1[53] method in Spartan 5.1.3 (Wave function Inc., Irvine CA). The docking experiments were performed using AUTODOCK3.0.[54] Gasteiger−Hückel partial charges were calculated in SYBYL7.0 for both protein and ligand. Grid maps with 60 × 60 × 60 points and a grid-point spacing of 0.375 Å centered in the phorbol binding pocket were calculated. All ligand torsion angles were considered as flexible. Fifty separate docking simulations were performed using the Lamarckian genetic algorithm. A validation of the docking protocol was performed using phorbol-13-*O*-acetate as ligand. The X-ray structure was well reproduced as described previously.[37]

Synthesis. Procedures for the synthesis and analysis of the remaining compounds are available in the Supporting Information.

Diethyl 5-(Tetrahydropyran-2-yloxymethyl)isophthalate (3). A mixture of diethyl (5-hydroxymethyl)isophthalate (**2**) (5.72 g, 22.7 mmol), 3,4-dihydro-2*H*-pyran (5.54 mL, 61.3 mmol, 2.7 equiv), pyridinium *p*-toluenesulfonate (285 mg, 1.14 mmol, 0.05 equiv), and 1,2-dichloroethane (50 mL) was stirred at room temperature for 6 h. The reaction mixture was quenched by the addition of cold water (50 mL), extracted with DCM (50 mL) and washed with a saturated solution of $NaHCO_3$ (3 × 20 mL) and brine (3 × 20 mL). The organic phases were dried over Na_2SO_4, filtered, and evaporated in vacuo to give **3** as a pale-yellow oil (7.41 g, 97% yield). This oil was used without further purification, and full product analysis was carried out for the following product.

5-(Tetrahydropyran-2-yloxymethyl)isophthalic Acid (4). A mixture of **3** (3.46 g, 10.3 mmol), a 10% solution of KOH (46.2 mL, 82.4 mmol, 8 equiv), and MeOH (50 mL) was refluxed for 1 h. The solvents were evaporated in vacuo and pH was adjusted with a 25% solution of $KHSO_4$ to 4.0. The white precipitate was filtered, washed with water (10 mL), dissolved into a solution of EtOAc and THF (1:1, 3 × 50 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to give **4** as a white solid (2.45 g, 85% yield). This solid was used without further purification. ^{1}H NMR (300 MHz, DMSO-d_6): δ_{ppm} 13.3 (bs, 2H), 8.39 (t, 1H, *J* 1.5 Hz), 8.13 (d, 2H, *J* 1.5 Hz), 4.80 (d, 1H, *J* 12.6 Hz), 4.72 (t, 1H, *J* 3.0 Hz), 4.59 (d, 1H, *J* 12.6 Hz), 3.82−3.75 (m, 1H), 3.52−3.45 (m, 1H), 1.80−1.62 (m, 2H), 1.58−1.48 (m, 4H). ^{13}C NMR (75.4 MHz, DMSO-d_6): δ_{ppm} 166.5, 139.8, 132.1, 131.3, 129.0, 97.7, 67.3, 61.4, 30.1, 25.0, 19.1.

Bis(3-trifluoromethylbenzyl) 5-(Tetrahydropyran-2-yloxymethyl)isophthalate (5a3). A mixture of **4** (1.00 g, 3.57 mmol), 3-(trifluoromethyl)benzyl chloride (2.08 mL, 10.7 mmol, 3 equiv), K_2CO_3 (2.47 g, 17.8 mmol, 5 equiv), and KI (650 mg, 3.92 mmol, 1.1 equiv) in dry DMF (18 mL) was heated at 80 °C for 150 min. The reaction mixture was cooled to rt, quenched by the addition of an ice−water mixture (20 mL), extracted with EtOAc (3 × 20 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to give **5a3** as a brown oil. This and the following crude diesters (**5b10**−**11**) were used in the subsequent deprotection step without further purification, and full product analysis was carried out for the products **1a3** and **1b10**−**11**.

Bis(1-ethylbutyl) 5-(Tetrahydropyran-2-yloxymethyl)isophthalate (5b10). A mixture of **4** (250 mg, 0.89 mmol) and 1,1-carbonyldiimidazole (318 mg, 1.96 mmol, 2.2 equiv) in dry DMF

(1 mL) was stirred at room temperature for 1 h. 3-Hexanol (330 μL, 2.68 mmol, 3 equiv), 4-(dimethylamino)pyridine (11 mg, 0.09 mmol, 0.1 equiv), and 1,8-diazabicyclo[5.4.0]undec-7-ene (332 μL, 1.78 mmol, 2 equiv) were added to the reaction mixture that was stirred at 40 °C for 43 h. The reaction was quenched with ice−water (10 mL) and extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with a saturated solution of $NaHCO_3$ (2 × 20 mL), brine (20 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to give the crude **5b10**, which was obtained as a yellow oil after work up.

Bis(1-ethylpentyl) 5-(Tetrahydropyran-2-yloxymethyl)isophthalate (5b11). A mixture of **4** (250 mg, 0.89 mmol) and 1,1-carbonyldiimidazole (318 mg, 1.96 mmol, 2.2 equiv) in dry DMF (1 mL) was stirred at room temperature for 1 h. 3-Heptanol (380 μL, 2.68 mmol, 3 equiv), 4-(dimethylamino)pyridine (11 mg, 0.09 mmol, 0.1 equiv), and 1,8-diazabicyclo[5.4.0]undec-7-ene (332 μL, 1.78 mmol, 2 equiv) were added to the reaction mixture and stirred at 40 °C for 43 h. The reaction was quenched with ice−water (10 mL) and extracted with EtOAc (3 × 20 mL). The combined organic phases were washed with a saturated solution of $NaHCO_3$ (2 × 20 mL), brine (20 mL), dried over Na_2SO_4, filtered, and evaporated in vacuo to give the crude **5b11**, which was obtained as a yellow oil after work up.

A General Procedure III, Deprotection of the THP Ethers: Bis(3-trifluoromethylbenzyl) 5-(Hydroxymethyl)isophthalate (1a3). A mixture of the crude **5a3** and Dowex 50W × 8 (750 mg) in MeOH (8 mL) was stirred at 40 °C for 23 h. The crude product was purified with flash SiO_2 column chromatography (*n*-hexane/EtOAc, 12:1 → 1:2) to give **1a3** as a white solid (765 mg, 42% yield for two reaction steps). R_f 0.21 (*n*-hexane/EtOAc, 2:1); mp 90.1−90.6 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} 8.67 (s, 1H), 8.28 (s, 2H), 7.71 (s, 2H), 7.66−7.62 (m, 4H), 7.53 (t, 2H, *J* 7.8 Hz), 5.44 (s, 4H), 4.83 (d, 2H, *J* 5.5 Hz), 1.87 (t, 1H, *J* 5.5 Hz).^{13}C NMR (75.4 MHz, $CDCl_3$): δ_{ppm} 165.5, 142.3, 136.8, 132.6, 131.9, 131.3 (q, *J* 32.5 Hz), 130.8, 130.3, 129.4, 125.5 (q, *J* 3.74 Hz), 125.3 (q, *J* 3.85 Hz), 124.1 (q, *J* 273 Hz), 66.5, 64.3. Anal. ($C_{25}H_{18}F_6O_5$) C, H: calcd, 3.54; found, 3.31.

Bis(1-ethylbutyl) 5-(Hydroxymethyl)isophthalate (1b10). The general procedure III was followed except that **5b10** was used and the reaction mixture was stirred for 20 h. The crude product was purified with flash SiO_2 column chromatography (*n*-hexane/EtOAc, 2:1) to give **1b10** as a colorless oil (199 mg, 61% yield for two reaction steps). R_f 0.47 (*n*-hexane/EtOAc, 2:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} 8.62 (app t, 1H, *J* 1.5 Hz), 8.22 (app d, 2H, *J* 1.2 Hz), 5.16−5.08 (m, 2H), 4.82 (s, 2H), 1.86 (bs, 1H), 1.76−1.56 (m, 8H), 1.48−1.30 (m, 4H), 0.98−0.91 (m, 12H). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ_{ppm} 165.8, 141.8, 132.0, 131.8, 130.1, 76.8, 64.6, 36.1, 27.3, 18.9, 14.2, 9.9; DEPT 132.0 (CH), 130.1 (CH), 76.8 (CH), 64.6 (CH_2), 36.1 (CH_2), 27.3 (CH_2), 18.9 (CH_2), 14.2 (CH_3), 9.9 (CH_3). HRMS-ESI (*m*/*z*): $[M + HCOO^-]^-$ calcd 409.2226; found 409.2238.

Bis(1-ethylpentyl) 5-(Hydroxymethyl)isophthalate (1b11). The general procedure III was followed except that **5b11** was used and the reaction mixture was stirred for 20 h. The crude product was purified with flash SiO_2 column chromatography (*n*-hexane/EtOAc, 2:1) to give **1b11** as a colorless oil (213 mg, 61% yield for two reaction steps). R_f 0.41 (*n*-hexane/EtOAc, 4:1). ^{1}H NMR (300 MHz, $CDCl_3$): δ_{ppm} 8.61 (app t, 1H), 8.23 (m, 2H), 5.10 (qn, 2H, C*H*, *J* 6.0 Hz), 4.82 (s, 2H), 1.95 (bs, 1H), 1.76−1.65 (m, 8H), 1.38−1.30 (m, 8H), 0.95 (t, 6H, *J* 7.5 Hz), 0.89 (t, 6H, *J* 7.2 Hz). ^{13}C NMR (75.4 MHz, $CDCl_3$): δ_{ppm} 165.8, 141.8, 132.1, 131.8, 130.1, 77.1, 64.6, 33.6, 27.7, 27.3, 22.8, 14.2, 9.9. HRMS-ESI (*m*/*z*): $[M + HCOO^-]^-$ calcd 437.2539; found 437.2512.

Cloning and Production of Recombinant Human PKC α and δ in Insect Cells. The cloning of human recombinant PKC α and δ and their expression in baculovirus-infected *Sf*9 insect cells was performed as described previously.[55,56] For production of the PKC proteins, *Sf*9 cells were infected with an optimized amount of the recombinant baculovirus, harvested 2 days postinfection, and washed with PBS. The resultant cell pellets were subsequently frozen. Crude cell lysates were prepared by suspending the cells in buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.1% Triton-X 100, and protease inhibitors according to the manufacturer's instructions (see Materials and General Procedures). After a 30 min incubation on ice, the lysate was centrifuged at 16000*g* for 15 min at 4 °C and the supernatant was collected. The protein content was determined according to Bradford,[57] and the supernatant was used for [^{3}H]PDBu binding experiments.

[^{3}H]PDBu Binding Assay for Initial Screening of Binding Affinity. The ability of the compounds to compete with radioactively labeled phorbol ester [^{3}H]PDBu in binding to the regulatory domain of PKC α and δ was determined according to Gopalakrishna et al.[40] First, 20 μg of protein/well from the supernatant (see Cloning and Production of Recombinant Human PKC α and δ in Insect Cells) was incubated with different concentrations of each compound and [^{3}H]PDBu for 10 min at room temperature in a 96-well Durapore filter plate (Millipore, cat. no. MSHVN4B50, Bedford, MA) in a total volume of 125 μL. The final concentrations in the assay were as follows: 20 mM Tris-HCl (pH 7.5), 40 μM $CaCl_2$, 10 mM $MgCl_2$, 400 μg/mL bovine IgG, 25 nM [^{3}H]PDBu, and 0.1 mg/mL phosphatidyl-L-serine. Proteins were precipitated by the adition of 125 μL of cold 20% poly(ethylene glycol) 6000, and after 15 min of incubation on a plate shaker at room temperature, the filters were washed six times using a vacuum manifold with buffer containing 20 mM Tris-HCl (pH 7.5), 100 μM $CaCl_2$, and 5 mM $MgCl_2$. The plates were dried, and 25 μL of Optiphase SuperMix liquid scintillant (PerkinElmer, Groningen, Netherlands) was added to each well. After equilibration period of at least two hours, radioactivity was measured using Wallac Microbeta Trilux microplate liquid scintillation counter (PerkinElmer, Waltham, MA). All compounds tested were diluted in DMSO to give the same final DMSO concentration in the binding assay (4%) in each well. Since preliminary studies showed that the nonspecific binding was always less than 5%, only the total binding was measured, and all results were calculated as a percentage of control (DMSO) from the same plate. DMSO itself decreased [^{3}H]PDBu binding to PKC α by 20% and to PKC δ by 10%.

[^{3}H]PDBu Binding Assay for Determination of Binding Affinity Constants. To determine K_i values for selected ligands, we used the method developed by the Blumberg group.[47] Briefly, 20 ng/tube of purified human recombinant PKC α or δ was incubated with different concentrations of the ligands for 10 min at 37 °C in a reaction mixture consisting of 50 mM Tris-HCl (pH 7.4), 0.1 mg/mL phosphatidyl-L-serine, 1.8 mg/mL bovine IgG, [^{3}H]PDBu, and 0.1 mM $CaCl_2$ (for PKC α) or 1 mM EGTA (for PKC δ). Samples were chilled on ice for 10 min, and 200 μL of 35% poly(ethylene glycol) 6000 in 50 mM Tris-HCl (pH 7.4) was added. The samples were mixed, incubated on ice for 15 min, and centrifuged at 4 °C (15000*g*, 15 min). Radioactivity was determined from 100 μL aliquots of supernatants and from dried pellets by scintillation counting after addition of Optiphase HiSafe 3 liquid scintillant and 16 h equilibration. The results were calculated according to Lewin and Blumberg.[47] The dissociation constants (K_d) for the individual PKC isoenzymes and inhibitory dissociation constants (K_i) for the compounds were calculated with GraphPad Prism4 software (GraphPad Software Inc., La Jolla, CA).

Determination of ERK Phosphorylation by Immunoblotting. To study the effects of the compounds on ERK1/2 phosphorylation, HeLa cells were treated with the test compounds in the presence or absence of PMA or inhibitors. The amount of phosphorylated ERK1/2 was determined by Western blotting. The cells were seeded onto 6-well plates at a density of 4.0 × 10^5 cells/well. Then, 20−24 h after seeding, the medium was changed to serum-free DMEM. After a 24 h serum starvation incubation, the cells were treated with the test compounds for the indicated times, washed twice with ice-cold PBS, and then harvested in ice-cold lysis buffer containing 20 mM Tris-HCl (pH 7.4), 0.1% Triton X-100, 2 mM EDTA, 10 mM EGTA, 2 mM Na_3VO_4, and protease and phosphatase inhibitors according to manufacturer's

instructions (see Materials and General Procedures). After a 30 min incubation on ice, the cell lysates were centrifuged at 16000*g* for 15 min at 4 °C and the protein concentrations of the supernatants were determined using Bradford's method.[57] The samples were diluted in Laemmli sample buffer at equal concentrations and stored at −20 °C. Then 20 μg of protein per lane were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were washed for 5 min with 0.1% Tween 20 in Tris-buffered saline (TTBS) and then blocked for 1 h with 5% nonfat milk powder in TTBS (milk-TTBS). The membranes were then cut just above the 37 kDa marker band and incubated with the primary antibody (Anti-ACTIVE MAPK pAb 1:5000 or anti-GAPDH mAb 1:10 000 in milk-TTBS) overnight at 4 °C. Membranes were then washed for a total of 35 min with TTBS and incubated with horseradish peroxidase-conjugated secondary antibody (goat antirabbit IgG or goat antimouse IgG 1:3000 in milk-TTBS) for one hour at room temperature. After washing (total of 40 min with TTBS), the bands were visualized with ECL. GAPDH bands were used as controls to ensure equal loading of proteins to all wells in the SDS-PAGE gel. Western blots were quantified by measuring the optical density of the immunoreactive bands with Scion Image software (http://scioncorp.com).

Acknowledgment. We thank Matti Wahlsten, Sirkku Jäntti, and Teemu Nissilä for conducting the LC-MS analysis as well as Minna Baarman, Marjo Vaha, and Tarja Välimäki for technical assistance. This work was supported by grants from the European Commission Research (project no. 503467), the Academy of Finland (project no. 108376), the Finnish Cultural Foundation, and the Magnus Ehrnrooth Foundation.

Supporting Information Available: Synthesis procedures, degree of purity (elemental analysis), spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Battaini, F.; Mochly-Rosen, D. Happy birthday protein kinase C: past, present and future of a superfamily. *Pharmacol. Res.* **2007**, *55*, 461–466.
(2) Koivunen, J.; Aaltonen, V.; Peltonen, J. Protein kinase C (PKC) family in cancer progression. *Cancer Lett.* **2006**, *235*, 1–10.
(3) Griner, E. M.; Kazanietz, M. G. Protein kinase C and other diacylglycerol effectors in cancer. *Nat. Rev. Cancer.* **2007**, *7*, 281–294.
(4) Das Evcimen, N.; King, G. L. The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacol. Res.* **2007**, *55*, 498–510.
(5) Bright, R.; Mochly-Rosen, D. The role of protein kinase C in cerebral ischemic and reperfusion injury. *Stroke* **2005**, *36*, 2781–2790.
(6) Chou, W. H.; Messing, R. O. Protein kinase C isozymes in stroke. *Trends Cardiovasc. Med.* **2005**, *15*, 47–51.
(7) Sabri, A.; Steinberg, S. F. Protein kinase C isoform-selective signals that lead to cardiac hypertrophy and the progression of heart failure. *Mol. Cell. Biochem.* **2003**, *251*, 97–101.
(8) Alkon, D. L.; Sun, M. K.; Nelson, T. J. PKC signaling deficits: a mechanistic hypothesis for the origins of Alzheimer's disease. *Trends Pharmacol. Sci.* **2007**, *28*, 51–60.
(9) Hofmann, J. Protein kinase C isozymes as potential targets for anticancer therapy. *Curr. Cancer. Drug Targets* **2004**, *4*, 125–146.
(10) Newton, A. C. Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. *Chem. Rev.* **2001**, *101*, 2353–2364.
(11) Steinberg, S. F. Structural basis of protein kinase C isoform function. *Physiol. Rev.* **2008**, *88*, 1341–1378.
(12) Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* **1992**, *258*, 607–614.
(13) Kazanietz, M. G. Novel "nonkinase" phorbol ester receptors: the C1 domain connection. *Mol. Pharmacol.* **2002**, *61*, 759–767.
(14) Colon-Gonzalez, F.; Kazanietz, M. G. C1 domains exposed: from diacylglycerol binding to protein−protein interactions. *Biochim. Biophys. Acta* **2006**, *1761*, 827–837.
(15) Yang, C.; Kazanietz, M. G. Divergence and complexities in DAG signaling: looking beyond PKC. *Trends Pharmacol. Sci.* **2003**, *24*, 602–608.
(16) Parker, P. J.; Coussens, L.; Totty, N.; Rhee, L.; Young, S.; Chen, E.; Stabel, S.; Waterfield, M. D.; Ullrich, A. The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* **1986**, *233*, 853–859.
(17) Manning, G.; Whyte, D. B.; Martinez, R.; Hunter, T.; Sudarsanam, S. The protein kinase complement of the human genome. *Science* **2002**, *298*, 1912–1934.
(18) Blumberg, P. M.; Kedei, N.; Lewin, N. E.; Yang, D.; Czifra, G.; Pu, Y.; Peach, M. L.; Marquez, V. E. Wealth of opportunity—the C1 domain as a target for drug development. *Curr. Drug Targets* **2008**, *9*, 641–652.
(19) Cameron, A. J.; Procyk, K. J.; Leitges, M.; Parker, P. J. PKC alpha protein but not kinase activity is critical for glioma cell proliferation and survival. *Int. J. Cancer* **2008**, *123*, 769–779.
(20) Zeidman, R.; Lofgren, B.; Pahlman, S.; Larsson, C. PKCepsilon, via its regulatory domain and independently of its catalytic domain, induces neurite-like processes in neuroblastoma cells. *J. Cell Biol.* **1999**, *145*, 713–726.
(21) Ono, Y.; Fujii, T.; Igarashi, K.; Kuno, T.; Tanaka, C.; Kikkawa, U.; Nishizuka, Y. Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 4868–4871.
(22) Smith, J. B.; Smith, L.; Pettit, G. R. Bryostatins: potent, new mitogens that mimic phorbol ester tumor promoters. *Biochem. Biophys. Res. Commun.* **1985**, *132*, 939–945.
(23) Kozikowski, A. P.; Wang, S.; Ma, D.; Yao, J.; Ahmad, S.; Glazer, R. I.; Bogi, K.; Acs, P.; Modarres, S.; Lewin, N. E.; Blumberg, P. M. Modeling, chemistry, and biology of the benzolactam analogues of indolactam V (ILV). 2. Identification of the binding site of the benzolactams in the CRD2 activator-binding domain of PKCdelta and discovery of an ILV analogue of improved isozyme selectivity. *J. Med. Chem.* **1997**, *40*, 1316–1326.
(24) Kong, F. H.; Kishi, Y.; Perez-Sala, D.; Rando, R. R. The pharmacophore of debromoaplysiatoxin responsible for protein kinase C activation. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 1973–1976.
(25) Kedei, N.; Lundberg, D. J.; Toth, A.; Welburn, P.; Garfield, S. H.; Blumberg, P. M. Characterization of the interaction of ingenol 3-angelate with protein kinase C. *Cancer Res.* **2004**, *64*, 3243–3255.
(26) Shao, L.; Lewin, N. E.; Lorenzo, P. S.; Hu, Z.; Enyedy, I. J.; Garfield, S. H.; Stone, J. C.; Marner, F. J.; Blumberg, P. M.; Wang, S. Iridals are a novel class of ligands for phorbol ester receptors with modest selectivity for the RasGRP receptor subfamily. *J. Med. Chem.* **2001**, *44*, 3872–3880.
(27) Marquez, V. E.; Blumberg, P. M. Synthetic diacylglycerols (DAG) and DAG-lactones as activators of protein kinase C (PK-C). *Acc. Chem. Res.* **2003**, *36*, 434–443.
(28) Duan, D.; Sigano, D. M.; Kelley, J. A.; Lai, C. C.; Lewin, N. E.; Kedei, N.; Peach, M. L.; Lee, J.; Abeyweera, T. P.; Rotenberg, S. A.; Kim, H.; Kim, Y. H.; Kazzouli, S. E.; Chung, J. U.; Young, H. A.; Young, M. R.; Baker, A.; Colburn, N. H.; Haimovitz-Friedman, A.; Truman, J. P.; Parrish, D. A.; Deschamps, J. R.; Perry, N. A.; Surawski, R. J.; Blumberg, P. M.; Marquez, V. E. Conformationally Constrained Analogues of Diacylglycerol. 29. Cells Sort Diacylglycerol-Lactone Chemical Zip Codes to Produce Diverse and Selective Biological Activities. *J. Med. Chem.* **2008**, *51*, 5198–5220.
(29) Irie, K.; Nakagawa, Y.; Ohigashi, H. Toward the development of new medicinal leads with selectivity for protein kinase C isozymes. *Chem. Rec.* **2005**, *5*, 185–195.
(30) Yanagita, R. C.; Nakagawa, Y.; Yamanaka, N.; Kashiwagi, K.; Saito, N.; Irie, K. Synthesis, conformational analysis, and biological evaluation of 1-hexylindolactam-V10 as a selective activator for novel protein kinase C isozymes. *J. Med. Chem.* **2008**, *51*, 46–56.
(31) Zhang, G.; Kazanietz, M. G.; Blumberg, P. M.; Hurley, J. H. Crystal structure of the cys2 activator-binding domain of protein kinase C delta in complex with phorbol ester. *Cell* **1995**, *81*, 917–924.
(32) Krauter, G.; Von der Lieth, C. W.; Schmidt, R.; Hecker, E. Structure/activity relationships of polyfunctional diterpenes of the tigliane type. A pharmacophore model for protein-kinase-C activators based on structure/activity studies and molecular modeling of the tumor promoters 12-*O*-tetradecanoylphorbol 13-acetate and 3-*O*-tetradecanoylingenol. *Eur. J. Biochem.* **1996**, *242*, 417–427.
(33) Sugita, K.; Neville, C. F.; Sodeoka, M.; Sasai, H.; Shibasaki, M. Stereocontrolled syntheses of phorbol analogs and evaluation of their binding affinity to PKC. *Tetrahedron Lett.* **1995**, *36*, 1067–1070.
(34) Benzaria, S.; Bienfait, B.; Nacro, K.; Wang, S.; Lewin, N. E.; Beheshti, M.; Blumberg, P. M.; Marquez, V. E. Conformationally constrained analogues of diacylglycerol (DAG). 15. The indispensable role of the sn-1 and sn-2 carbonyls in the binding of DAG-lactones to protein kinase C (PK-C). *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3403–3408.
(35) Wang, S.; Zaharevitz, D. W.; Sharma, R.; Marquez, V. E.; Lewin, N. E.; Du, L.; Blumberg, P. M.; Milne, G. W. The discovery of novel, structurally diverse protein kinase C agonists through computer 3D-

database pharmacophore search. Molecular modeling studies. *J. Med. Chem.* **1994**, *37*, 4479–4489.
(36) Kang, J. H.; Peach, M. L.; Pu, Y.; Lewin, N. E.; Nicklaus, M. C.; Blumberg, P. M.; Marquez, V. E. Conformationally constrained analogues of diacylglycerol (DAG). 25. Exploration of the sn-1 and sn-2 carbonyl functionality reveals the essential role of the sn-1 carbonyl at the lipid interface in the binding of DAG-lactones to protein kinase C. *J. Med. Chem.* **2005**, *48*, 5738–5748.
(37) Nacro, K.; Bienfait, B.; Lee, J.; Han, K. C.; Kang, J. H.; Benzaria, S.; Lewin, N. E.; Bhattacharyya, D. K.; Blumberg, P. M.; Marquez, V. E. Conformationally constrained analogues of diacylglycerol (DAG). 16. How much structural complexity is necessary for recognition and high binding affinity to protein kinase C? *J. Med. Chem.* **2000**, *43*, 921–944.
(38) Chen, M. H.; Davidson, J. G.; Freisler, J. T.; Iakovleva, E.; Magano, J. An Efficient and Scalable Synthesis of Methyl 3-Hydroxymethylbenzoate. *Org. Prep. Proced. Int.* **2000**, *32*, 381–384.
(39) Parlow, J. J.; Case, B. L.; Dice, T. A.; Fenton, R. L.; Hayes, M. J.; Jones, D. E.; Neumann, W. L.; Wood, R. S.; Lachance, R. M.; Girard, T. J.; Nicholson, N. S.; Clare, M.; Stegeman, R. A.; Stevens, A. M.; Stallings, W. C.; Kurumbail, R. G.; South, M. S. Design, parallel synthesis, and crystal structures of pyrazinone antithrombotics as selective inhibitors of the tissue factor VIIa complex. *J. Med. Chem.* **2003**, *46*, 4050–4062.
(40) Gopalakrishna, R.; Chen, Z. H.; Gundimeda, U.; Wilson, J. C.; Anderson, W. B. Rapid filtration assays for protein kinase C activity and phorbol ester binding using multiwell plates with fitted filtration discs. *Anal. Biochem.* **1992**, *206*, 24–35.
(41) Ananthanarayanan, B.; Stahelin, R. V.; Digman, M. A.; Cho, W. Activation mechanisms of conventional protein kinase C isoforms are determined by the ligand affinity and conformational flexibility of their C1 domains. *J. Biol. Chem.* **2003**, *278*, 46886–46894.
(42) Wender, P. A.; DeBrabander, J.; Harran, P. G.; Jimenez, J. M.; Koehler, M. F.; Lippa, B.; Park, C. M.; Siedenbiedel, C.; Pettit, G. R. The design, computer modeling, solution structure, and biological evaluation of synthetic analogs of bryostatin 1. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 6624–6629.
(43) Chang, L.; Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **2001**, *410*, 37–40.
(44) Schonwasser, D. C.; Marais, R. M.; Marshall, C. J.; Parker, P. J. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. *Mol. Cell. Biol.* **1998**, *18*, 790–798.
(45) Wang, Q. J.; Fang, T. W.; Fenick, D.; Garfield, S.; Bienfait, B.; Marquez, V. E.; Blumberg, P. M. The lipophilicity of phorbol esters as a critical factor in determining the pattern of translocation of protein kinase C delta fused to green fluorescent protein. *J. Biol. Chem.* **2000**, *275*, 12136–12146.
(46) Wender, P. A.; Koehler, K. F.; Sharkey, N. A.; Dell'Aquila, M. L.; Blumberg, P. M. Analysis of the phorbol ester pharmacophore on protein kinase C as a guide to the rational design of new classes of analogs. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 4214–4218.
(47) Lewin, N. E.; Blumberg, P. M. [^{3}H]Phorbol 12,13-dibutyrate binding assay for protein kinase C and related proteins. *Methods Mol. Biol.* **2003**, *233*, 129–156.
(48) Giorgione, J. R.; Lin, J. H.; McCammon, J. A.; Newton, A. C. Increased membrane affinity of the C1 domain of protein kinase Cdelta compensates for the lack of involvement of its C2 domain in membrane recruitment. *J. Biol. Chem.* **2006**, *281*, 1660–1669.
(49) Dries, D. R.; Gallegos, L. L.; Newton, A. C. A single residue in the C1 domain sensitizes novel protein kinase C isoforms to cellular diacylglycerol production. *J. Biol. Chem.* **2007**, *282*, 826–830.
(50) Hess, A. D.; Silanskis, M. K.; Esa, A. H.; Pettit, G. R.; May, W. S. Activation of human T lymphocytes by bryostatin. *J. Immunol.* **1988**, *141*, 3263–3269.
(51) Pu, Y.; Perry, N. A.; Yang, D.; Lewin, N. E.; Kedei, N.; Braun, D. C.; Choi, S. H.; Blumberg, P. M.; Garfield, S. H.; Stone, J. C.; Duan, D.; Marquez, V. E. A novel diacylglycerol-lactone shows marked selectivity in vitro among C1 domains of protein kinase C (PKC) isoforms alpha and delta as well as selectivity for RasGRP compared with PKCalpha. *J. Biol. Chem.* **2005**, *280*, 27329–27338.
(52) Galkin, A.; Pakkanen, J.; Vuorela, P. Development of an automated 7-day 96-well Caco-2 cell culture model. *Pharmazie* **2008**, *63*, 464–469.
(53) Dewar, M. J. S.; Zoelbisch, E. G.; Healy, E. F.; Stewart, J. J. P. Development and use of quantum mechanical molecular models. 76. AM1: A New General Purpose Quantum Mechanical Molecular Model. *J. Am. Chem. Soc.* **1985**, *107*, 3902–3909.
(54) Morris, G. M.; Goodsell, D. S.; Halliday, R. S.; Huey, R.; Hart, W. E.; Belew, R. K.; Olson, A. J. Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. *J. Comput. Chem.* **1998**, *19*, 1639–1662.
(55) Tammela, P.; Ekokoski, E.; García-Horsman, A.; Talman, V.; Finel, M.; Tuominen, R. K.; Vuorela, P. Screening of Natural Compounds and Their Derivatives as Potential Protein Kinase C Inhibitors. *Drug Dev. Res.* **2004**, *63*, 76–87.
(56) Sandler, C.; Ekokoski, E.; Lindstedt, K. A.; Vainio, P. J.; Finel, M.; Sorsa, T.; Kovanen, P. T.; Golub, L. M.; Eklund, K. K. Chemically modified tetracycline (CMT)-3 inhibits histamine release and cytokine production in mast cells: possible involvement of protein kinase C. *Inflamm. Res.* **2005**, *54*, 304–312.
(57) Kruger, N. J. The Bradford method for protein quantitation. *Methods Mol. Biol.* **1994**, *32*, 9–15.

JM900229P