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Design, synthesis, and structure–activity relationship of new isobenzofuranone ligands of protein kinase C

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Abstract—Protein kinase C (PKC) is a family of enzymes, which play important roles in intracellular signal transduction. We have designed novel PKC ligands having an isobenzofuranone template, based on the proposed interaction of DAG (1,2-diacyl-*sn*-glycerol) with the PKCδ C1B ligand-binding domain. Several isobenzofuranone derivatives were synthesized and their PKCα binding activities were evaluated. The pivaloyl derivative **1f** was found to be a strong PKCα ligand, and the structure–activity relationship is well explained by our proposed binding model.

Protein kinase C (PKC) isozymes play important roles in intracellular signal transduction of a variety of cell events, such as proliferation, differentiation, and apoptosis.[1] The isozymes are divided into three classes, conventional PKCs (α, βI/βII, γ), novel PKCs (δ, ε, η, θ), and atypical PKCs (ζ, ι/λ). DAG (1,2-diacyl-*sn*-glycerol) is a physiological ligand for the former two classes of isozymes, and several complex natural products, such as phorbol esters, bryostatins, aplysiatoxins, and teleocidins, are strong activators of these isozymes.[2] It is now believed that each isozyme participates in different signaling pathways, and, in some cases, the same enzyme triggers different cell responses depending on the stimulus, though the precise molecular mechanisms of activation and the biological roles of each isozyme remain to be clarified. The conventional PKCs have the regulatory domain, which includes the activator-binding domain (C1) and the Ca^{2+}-binding domain (C2). The C1 domain contains a repeat of cysteine-rich domains C1A and C1B, each of which can potentially bind the endogenous ligand, DAG, or an exogenous ligand such as phorbol ester. Although many analogs of these natural products have been synthesized and tested,[3] an isozyme-specific ligand has not been found. Therefore, development of a novel PKC ligand template, which has distinct structural features and can be a platform for the synthesis of variety of analogs, is of particular interest. Here we would like to report the design, synthesis, and evaluation of new DAG-type PKC ligands.

DAG is a nontumor-promoting physiological ligand of PKCs, and shows different isozyme selectivity from that of tumor-promoting phorbol esters.[4] DAG itself, however, is metabolically unstable and conformationally too flexible to be useful for further rational structural modifications. Marquez and co-workers have reported thorough studies on conformationally constrained analogs of DAG.[5] Some of their derivatives showed strong binding affinity to PKC; however, they suggested that their monocyclic templates can bind PKC in two different binding modes and this would make further rational structural modification difficult. As a part of our project on clarifying the molecular mechanisms of PKC activation,[6] we decided to design a new DAG-type template based on the crystal structure of the PKCδ C1B domain in the complex with phorbol 13-acetate (Fig. 1).[7] Hydrogen bondings between the C3-carbonyl

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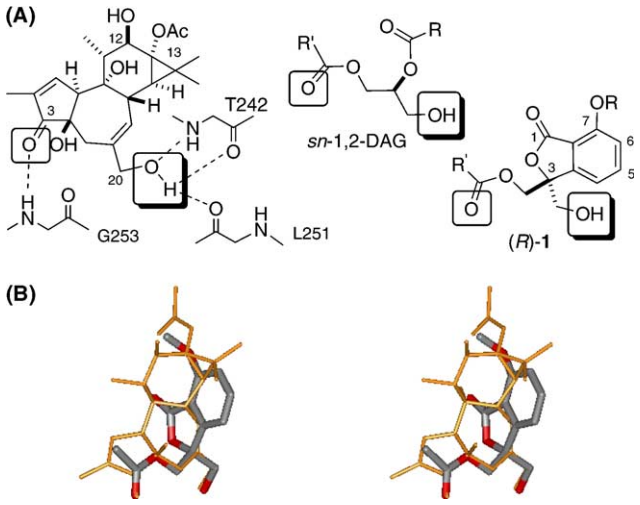
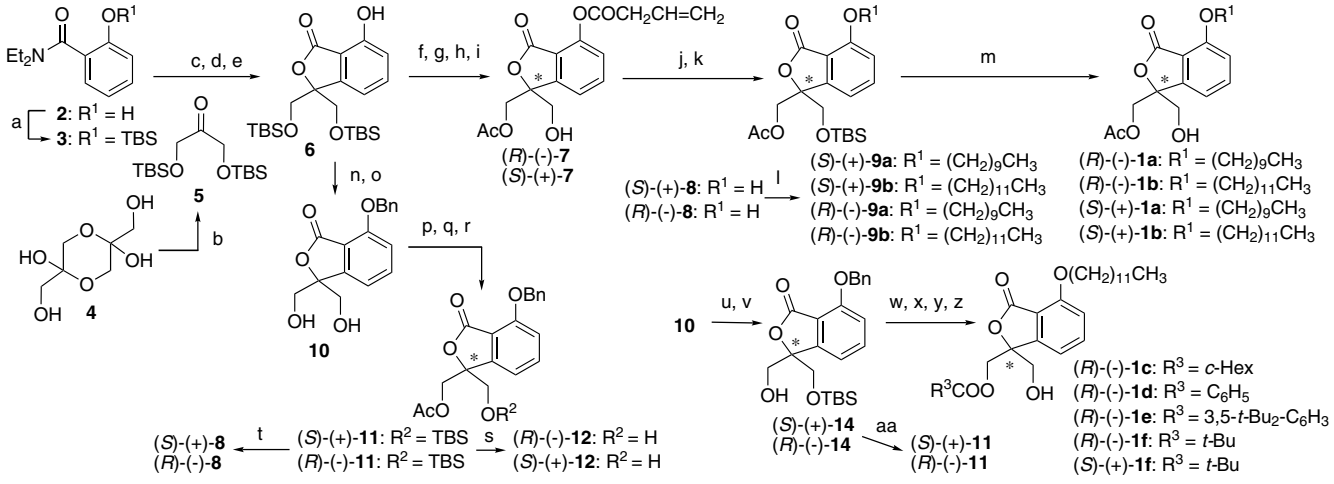


Figure 1. (A) Hydrogen bondings in the PKCδ-phorbol 13-acetate complex, and design of novel isobenzofuranone ligand, based on the DAG structure. (B) Superposition of (*R*)-**1** (R = R′ = Me) on phorbol 13-acetate.

group of phorbol and the NH group of Gly253 and between the C20-hydroxyl group of phorbol and the NH, and carbonyl groups of Thr242 and Leu251 were found to be critical for the ligand binding, and these interactions should be conserved throughout the isozymes. Though DAG has two carbonyl groups, SAR studies indicated that the *sn*-1 carbonyl group is more important than the *sn*-2 carbonyl group.[8] A molecular modeling study also suggested that the *sn*-1 carbonyl group of DAG would preferentially form a hydrogen bond with Gly253.[9] If this is the case, there is much extra room in the binding pocket in the direction of the *sn*-2 side chain. We designed the novel isobenzofuranone template **1**, which is expected to fit into the binding pocket. According to our binding model, the (*R*)-stereochemistry of the quaternary carbon center should be preferable, and the side chain at the C7 position can be oriented to a direction similar to that of the C12 or C13 side chain of phorbol ester. Furthermore, this isobenzofuranone template has considerable scope for modification on the benzene ring, which may be effective for the development of an isozyme-selective analog.

To see if this novel isobenzofuranone template can act as a PKC ligand, we first synthesized optically active isobenzofuranones **1a** and **1b** having a hydrophobic side chain at the C7 position. Scheme 1 shows the synthetic route to both enantiomers of **1a** and **1b**. The *tert*-butyldimethylsilyl (TBS) ether of salicylamide **3** was lithiated and then reacted with the ketone **5** prepared from the dihydroxyacetone dimer **4**. Treatment of the resulting adduct with sodium hydride afforded the five-membered lactone. The TBS group of the aromatic alcohol was selectively removed by treatment with potassium carbonate in methanol to give **6**. After protection of the phenol moiety with an allyloxycarbonyl (alloc) group, two TBS groups were removed, and monoacetylation was carried out using acetyl chloride in the presence of silica gel. Separation of enantiomers of the monoacetate **7** was achieved by preparative HPLC using a chiral phase column. The optically pure (*R*)- and (*S*)-**7** was then converted to (*S*)- and (*R*)-**8**, respectively, via two-step sequences. After introduction of a hydro-



Scheme 1. Synthesis of the optically active isobenzofuranone derivatives. Reagents and conditions: (a) TBSCl, Et_3N, DMAP, CH_2Cl_2, rt, 15 h (quant); (b) TBSCl, DMAP, pyridine, rt, 5 d (92%); (c) *s*-BuLi, TMEDA, THF, −40 °C, 1.5 h, then **5**, −40—−30 °C, 17 h; (d) NaH, THF, 70 °C, 3 h; (e) K_2CO_3, MeOH, 70 °C, 3 h (46% from **5**, three steps); (f) CH_2=$CHCH_2OCOCl$, Et_3N, DMAP, THF, rt, 3 h (92%); (g) HF·Py, pyridine–CH_2Cl_2, rt, 12 h (95%); (h) AcCl, SiO_2, THF–Et_2O, 0 °C, 21 h (79%); (i) separation by HPLC (CHIRALPAK AD, hexane:*i*-PrOH = 3:1); (j) TBSCl, imidazole, DMF, 0 °C, 3 h (72%); (k) $Pd(PPh_3)_4$, PPh_3, HCOOH, $BnNH_2$, THF, rt, 15 h (96%); (l) R^1OH, DEAD, PPh_3, THF, rt, 10 h (R^1 = $(CH_2)_9CH_3$: **9a**, 92%; R^1 = $(CH_2)_{11}CH_3$: **9b**, 79%); (m) HF·Py, pyridine–CH_2Cl_2, rt, 8 h (R^1 = $(CH_2)_9CH_3$: **1a**, 90%; R^1 = $(CH_2)_{11}CH_3$: **1b**, 89%); (n) BnBr, K_2CO_3, acetone, rt, 19 h (85%); (o) HF·Py, pyridine–CH_2Cl_2, rt (94%); (p) AcCl, SiO_2, THF, 0 °C–rt, 5 d (69%); (q) TBSCl, imidazole, DMF, rt, 3 h (94%); (r) separation by HPLC (CHIRALPAK AD, hexane:*i*-PrOH = 9:1); (s) HF·Py, pyridine–CH_2Cl_2, rt, 113 h (98%); (t) H_2, Pd/C, EtOH, rt (97%); (u) TBSCl, imidazole, DMF, 0 °C, 2 h (51%); (v) separation by HPLC (CHIRALPAK AD-H, hexane:*i*-PrOH = 3:1); (w) PvCl, Et_3N, THF, rt, 5.5 h or R^2COCl, pyridine, rt; (x) H_2, Pd/C, EtOH or MeOH, rt (R^2 = *c*-Hex: 84%, R^2 = C_6H_5: 30%, R^2 = 3,5-*t*-Bu_2-C_6H_3: 75%, R^2 = *t*-Bu: 60%, two steps); (y) $CH_3(CH_2)_{11}OH$, DEAD, PPh_3, THF, rt; (z) HF·Py, pyridine–CH_2Cl_2, 0 °C–rt (R^2 = *c*-Hex: **1c**, 96%, R^2 = C_6H_5: **1d**, quant, R^2 = 3,5-*t*-Bu_2–C_6H_3: **1e**, 96%, R^2 = *t*-Bu: **1f**, 98%, two steps); (aa) AcCl, Et_3N, DMAP,CH_2Cl_2 (64%).

phobic side chain (C_{10} or C_{12}) by means of the Mitsunobu reaction, the TBS group was removed using HF·pyridine complex to give (*R*)- or (*S*)-**1a**,**1b**. Optical purity of these final products was confirmed by HPLC analysis. When tetrabutylammonium fluoride was used for deprotection, significant racemization was observed. Both enantiomers of the 7-benzyloxy derivative **12** were prepared from **6** in six steps, including the separation of the enantiomers of **11** with preparative HPLC. The absolute stereochemistry of (+)-**12** was unequivocally determined to be (*S*) by X-ray analysis of its (1*S*)-camphanic acid ester **13** (Fig. 2). Since the stereochemistry of (−)-**11** was automatically determined to be (*R*) by the correlation with (*S*)-(+)-**12**, the stereochemistry of all the other compounds, **7**, **8**, **9a**,**b**, and **1a**,**b**, was determined by the conversion of (*R*)-(−)-**11** to (*R*)-(−)-**8**.[10]

Next, the binding affinity of isobenzofuranone derivatives to PKCα was preliminarily examined by measuring competitive inhibition of the binding of tritium-labeled phorbol dibutyrate ([^{3}H]PDBu) to PKCα.[11] As expected, compounds (*R*)-**1a** and **1b** exhibited significant competitive binding to PKCα (Fig. 3). The (*S*)-enantiomers of these compounds were less potent than the (*R*)-enantiomers, supporting our design concept. Compounds having a less hydrophobic side chain, such as alloc ((*R*)-**7**) or benzyl ((*R*)-**12**), were less effective, and at least a C_{12} methylene chain seemed to be required for strong binding to PKC (Fig. 3).

To further improve the affinity of the isobenzofuranone derivatives to PKC, we next synthesized several acyl chain analogs (*R*)-**1c**–**f** according to the reaction sequences shown in Scheme 1. Dose-dependent inhibition curves of these compounds for [^{3}H]PDBu binding to PKCα are shown in Figure 4. As expected, when the acetyl group was changed to a cyclohexanoyl group ((*R*)-**1c**), 10-fold improvement of the binding affinity was observed.[12] The benzoyl derivative (*R*)-**1d** was not as potent as (*R*)-**1c**. Further introduction of a hydrophobic substituent on the benzene ring rather decreased the binding to PKCα. Finally, the pivaloyl derivative (*R*)-**1f** showed the strongest binding to PKCα among these compounds. The K_i value of (*R*)-**1f** was 122 nM, which is much smaller than that of 1-oleoyl-2-acetyl-*sn*-glycerol (OAG, K_i = 540 nM). The (*S*)-enantiomer of **1f** was, again, less effective than the (*R*)-enantiomer.

Figure 5 shows the results of molecular modeling of the complex of the PKCδ C1B domain with the pivaloyl derivative. Calculation was carried out for the compound with the shorter C_3 methylene chain at the C7 position instead of the C_{12} methylene chain of (*R*)-**1f**.[13] The model suggests that positive hydrophobic interaction between the *tert*-butyl group and Leu250 and Leu254 contributes to the increase of the affinity.

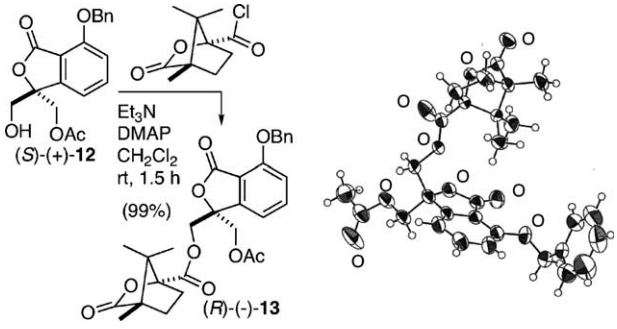


Figure 2. Determination of absolute stereochemistry. The crystal structural information has been deposited in the Cambridge Crystallographic Data Center (deposition no CCDC 223959).

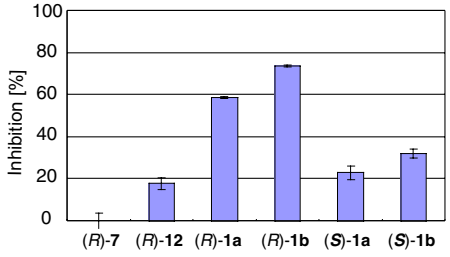


Figure 3. Inhibition of binding of [^{3}H]PDBu to PKCα by the isobenzofuranone derivatives at 100 μM.

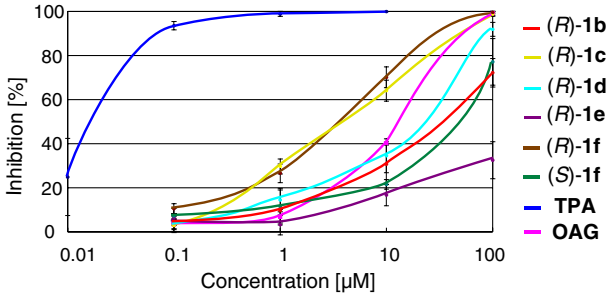


Figure 4. Dose-dependent inhibition curves of **1b**–**f** for [^{3}H]PDBu binding to PKCα.

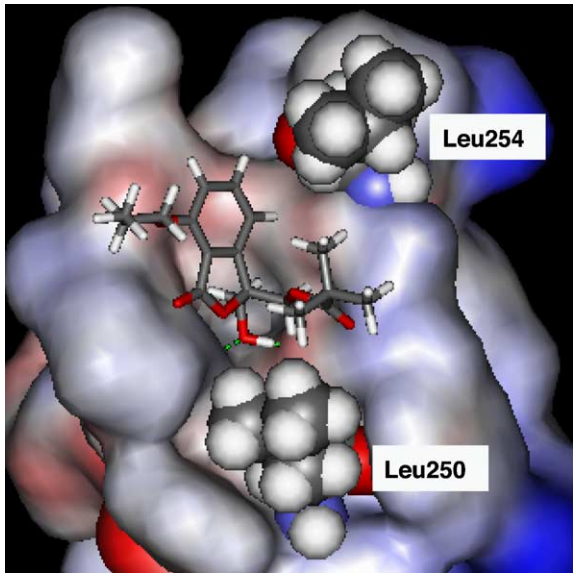


Figure 5. Binding model of (*R*)-**1f** with PKCδ C1B. Modeling was carried out on the compound with the shorter (C_3) side chain.[13]

Although the data shown in Figure 4 were obtained using PKCα, these two leucines are conserved in the PKCα C1B domain (Leu121 and Leu125). As a result, the *tert*-butyl group fills the gap between the two leucines and forms a continuous hydrophobic surface with Leu250 and Leu254, which may be favorable for interaction with the membrane.

In conclusion, we have designed a novel isobenzofuranone template for PKC ligands based on the proposed interaction of DAG with PKC. Several derivatives were synthesized and their PKCα binding activities were evaluated. The pivaloyl derivative (*R*)-**1f** was found to be a strong PKCα ligand, and the structure–activity relationship is well explained by our binding model. Design and synthesis of novel isobenzofuranone analogs based on this binding model should be useful for research to clarify the molecular mechanisms of PKC activation and also for the development of isozyme selective ligands.

Acknowledgements

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10. All compounds were characterized by spectroscopic analyses. Purity of the compounds obtained was determined to be more than 95% based on the ^{1}H NMR spectral analysis. Spectral data of selected compounds are as follows. (***R***)-(−)-**1b**: Colorless oil; $[\alpha]_D^{20}$ −14.6 ($c = 0.640$, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.7$ Hz, 3H), 1.1–1.6 (m, 18H), 1.8–2.0 (m, 2H), 2.03 (s, 3H), 2.20 (br s, 1H), 3.89 (d, $J = 6.6$ Hz, 2H), 4.14 (t, $J = 6.8$ Hz, 2H), 4.42 (d, $J = 11.9$ Hz, 1H), 4.53 (d, $J = 11.9$ Hz, 1H), 6.96 (d, $J = 8.4$ Hz, 1H), 7.02 (d, $J = 7.4$ Hz, 1H), 7.60 (dd,

$J = 8.4$, 7.4 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.10, 20.63, 22.67, 25.81, 28.82, 29.33, 29.51, 29.59, 29.61, 29.65, 31.90, 64.19, 64.49, 69.14, 85.32, 112.73, 113.55, 114.00, 136.41, 149.97, 158.49, 166.89, 170.67; LRMS (EI, m/z) 420 (M^+). (***R***)-(−)-**1c**: Colorless oil; $[\alpha]_D^{22}$ −21.8 ($c = 0.41$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.8$ Hz, 3H), 1.26 (m, 20H), 1.49 (tt, $J = 6.6$, 6.8 Hz, 2H), 1.62–1.82 (m, 6H), 1.89 (tt, $J = 6.6$, 6.8 Hz, 2H), 2.23–2.29 (m, 2H), 3.88 (br s, 2H), 4.14 (t, $J = 6.6$ Hz, 2H), 4.49 (br s, 2H), 6.96 (d, $J = 8.3$ Hz, 1H), 7.02 (d, $J = 7.6$ Hz, 1H), 7.59 (dd, $J = 8.3$, 7.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.11, 22.67, 25.20, 25.24, 25.57, 25.79, 28.76, 29.33, 29.51, 29.58, 29.61, 29.65, 29.69, 31.89, 42.85, 63.71, 64.45, 69.10, 85.73, 112.65, 113.65, 114.09, 136.33, 149.95, 158.40, 166.95, 175.71; MALDI-TOFMS (positive ion, matrix α-cyano-4-hydroxycinnamic acid) calcd for $C_{22}H_{44}O_6Na$ ($M+Na^+$) 511.30; found 511.23. (***R***)-(−)-**1d**: Colorless oil; $[\alpha]_D^{22}$ −15.8 ($c = 0.10$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.8$ Hz, 3H), 1.26 (m, 16H), 1.49 (tt, $J = 7.32$, 6.8 Hz, 2H), 1.90 (tt, $J = 7.3$, 6.8 Hz, 2H), 2.28 (t, $J = 7.1$ Hz, 1H), 3.98 (d, $J = 7.1$ Hz, 2H), 4.15 (t, $J = 6.8$ Hz, 2H), 4.66 (d, $J = 12.0$ Hz, 1H), 4.79 (d, $J = 12.0$ Hz, 1H), 6.97 (d, $J = 8.3$ Hz, 1H), 7.09 (d, $J = 7.3$ Hz, 1H), 7.44 (dd, $J = 7.3$, 7.1, 2H), 7.57 (dd, 7.3, $J = 1.5$ Hz, 1H), 7.59 (dd, 8.3, $J = 7.3$ Hz, 1H), 7.95 (dd, $J = 7.1$, 1.5 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.12, 22.69, 25.80, 28.80, 29.35, 29.53, 29.60, 29.63, 29.66, 29.69, 31.92, 64.47, 64.68, 69.14, 85.51, 112.82, 113.67, 113.96, 128.53, 129.07, 129.79, 133.51, 136.50, 150.00, 158.51, 166.24, 166.92; MALDI-TOFMS (positive ion, α-cyano-4-hydroxycinnamic acid) calcd for $C_{29}H_{38}O_6Na$ ($M+Na^+$) 505.26; found 505.19. (***R***)-(−)-**1e**: Colorless oil; $[\alpha]_D^{23}$ −28.9 ($c = 0.34$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.8$ Hz, 3H), 1.26 (m, 16H), 1.31 (s, 18H), 1.50 (tt, $J = 7.3$, 6.8 Hz, 2H), 1.90 (tt, $J = 7.3$, 6.8 Hz, 2H), 2.35 (br s, 1H), 3.98 (br s, 2H), 4.13 (t, $J = 6.8$ Hz, 2H), 4.66 (d, $J = 12.0$ Hz, 1H), 4.78 (d, $J = 12.0$ Hz, 1H), 6.96 (d, $J = 8.3$ Hz, 1H), 7.09 (d, $J = 7.4$ Hz, 1H), 7.57–7.62 (m, 2H), 7.73 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.12, 22.69, 25.84, 28.84, 29.35, 29.37, 29.53, 29.60, 29.62, 29.66, 31.28, 31.91, 34.87, 64.61, 64.64, 69.04, 85.73, 112.53, 113.56, 114.09, 123.93, 127.66, 128.43, 136.42, 150.16, 151.17, 158.47, 166.79, 166.92; MALDI-TOFMS (positive ion, α-cyano-4-hydroxycinnamic acid) calcd for $C_{37}H_{54}O_6$ Na ($M+Na^+$) 617.38; found 617.30. (***R***)-(−)-**1f**; colorless oil; $[\alpha]_D^{28}$ −29.3 ($c = 0.91$, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, $J = 7.1$ Hz, 3H), 1.08 (s, 9H), 1.26 (m, 16H), 1.48 (m, 2H), 1.89 (tt, $J = 7.1$, 6.8 Hz, 2H), 2.13 (dd, $J = 6.6$, 7.8 Hz, 1H), 3.85 (dd, $J = 6.6$, 12.2 Hz, 1H), 3.89 (dd, $J = 7.8$, 12.2 Hz, 1H), 4.15 (t, $J = 6.8$ Hz, 2H), 4.45 (d, $J = 12.0$ Hz, 1H), 4.54 (d, $J = 12.0$ Hz, 1H), 6.96 (d, $J = 8.3$ Hz, 1H), 7.02 (d, $J = 7.6$ Hz, 1H), 7.59 (dd, $J = 8.3$, 7.6 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.10, 22.66, 25.76, 26.94, 28.73, 29.30, 29.32, 29.51, 29.57, 29.60, 29.64, 31.89, 38.83, 63.95, 64.51, 69.10, 85.98, 112.66, 113.59, 114.22, 136.28, 149.93, 158.37, 166.99, 178.04; MALDI-TOFMS (positive ion, α-cyano-4-hydroxycinnamic acid) calcd for $C_{27}H_{42}O_6Na$ ($M+Na^+$) 485.29; found 485.24.

11. **Assay protocol**: Inhibition of [^{3}H]PDBu binding was measured essentially according to the reported procedure. See: Tanaka, Y.; Miyake, R.; Kikkawa, U.; Nishizuka, Y. *J. Biochem.* **1986**, *99*, 257–261; Briefly, plastic test tubes of each assay mixture (300 μL) contained 50 mM Tris–HCl (pH 7.5), 4 mM $CaCl_2$, 100 μg/mL 1,2-di-(*cis*-9-octadecenoyl)-*sn*-glycero-3-phospho-L-serine sodium salt (L-PS, from SIGMA), 4 mg/mL BSA, 10 nM [^{3}H]PDBu, 4.3–4.8 nM protein kinase Cα (human, recombinant, *Spodoptera frugiperda* from CALBIOCHEM), and each concentration of the synthetic compound or TPA. L-PS was sonicated in 50 mM Tris–HCl at 0 °C prior to use. After incubation at 0 °C for 2 h, the mixture was diluted with cold 0.5 % DMSO (2.5 mL) then filtered through a glass-fiber filter (Whatman GF/B), which had been pretreated with 0.3% polyethyleneimine for 1 h. The filter was washed four times with 2 mL of cold 0.5% DMSO. The radioactivity of each filter was counted in a scintillation vial with 5 mL of scintillator (Clear-sol I from NACALAI TESQUE) using a liquid scintillation counter. The count for the tube with 10 μM TPA was taken as the background (100% inhibition) and subtracted from the count of each tube. The K_d value of [^{3}H]PDBu under these assay conditions was determined to be 0.19 nM by Scatchard plot analysis. Nonspecific binding was measured in the presence of 10 μM TPA. Free [^{3}H]PDBu was determined by subtraction of the bound [^{3}H]PDBu from the total [^{3}H]PDBu. K_i value was determined according to the reported method. See: Sharkey, N. A.; Blumberg, P. M. *Cancer Res.* **1985**, *45*, 19–24.
12. Increase of PKC binding affinity of branched derivatives of DAG analogs was also reported. See, Refs. 5e,9.
13. **Modeling method**: To construct a binding model of isobenzofuranone and PKCδ C1B domain, a computational docking study was performed based on the reported PKCδ C1B-phorbol 13-acetate complex (pdb code 1PTR). The isobenzofuranone derivative with a shorter side chain (C_3) was used instead of **1f**. The docking model was constructed in the Affinity module of the INSIGHT II molecular program developed by MSI (now succeeded by Accelrys, San Diego). The first binding placement of isobenzofuranone within the PKCδ C1B domain was made by superimposing the molecule on the critical functional groups of phorbol 13-acetate. We constructed a 'grid', which is partitioned into bulk (nonflexible) and movable atoms of the ligand/receptor system; interactions among bulk atoms are approximated by using the accurate and efficient molecular mechanical/grid (MM/Grid) method, while interactions among movable atoms are treated using a full force field representation. The grid was defined by the amino acid residues within 6 Å around the isobenzofuranone. The binding model was made using a Monte Carlo type procedure to search both conformational and Cartesian space.