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Identification of Potent, Selective Protein Kinase C Inhibitors Based on a Phorbol Skeleton

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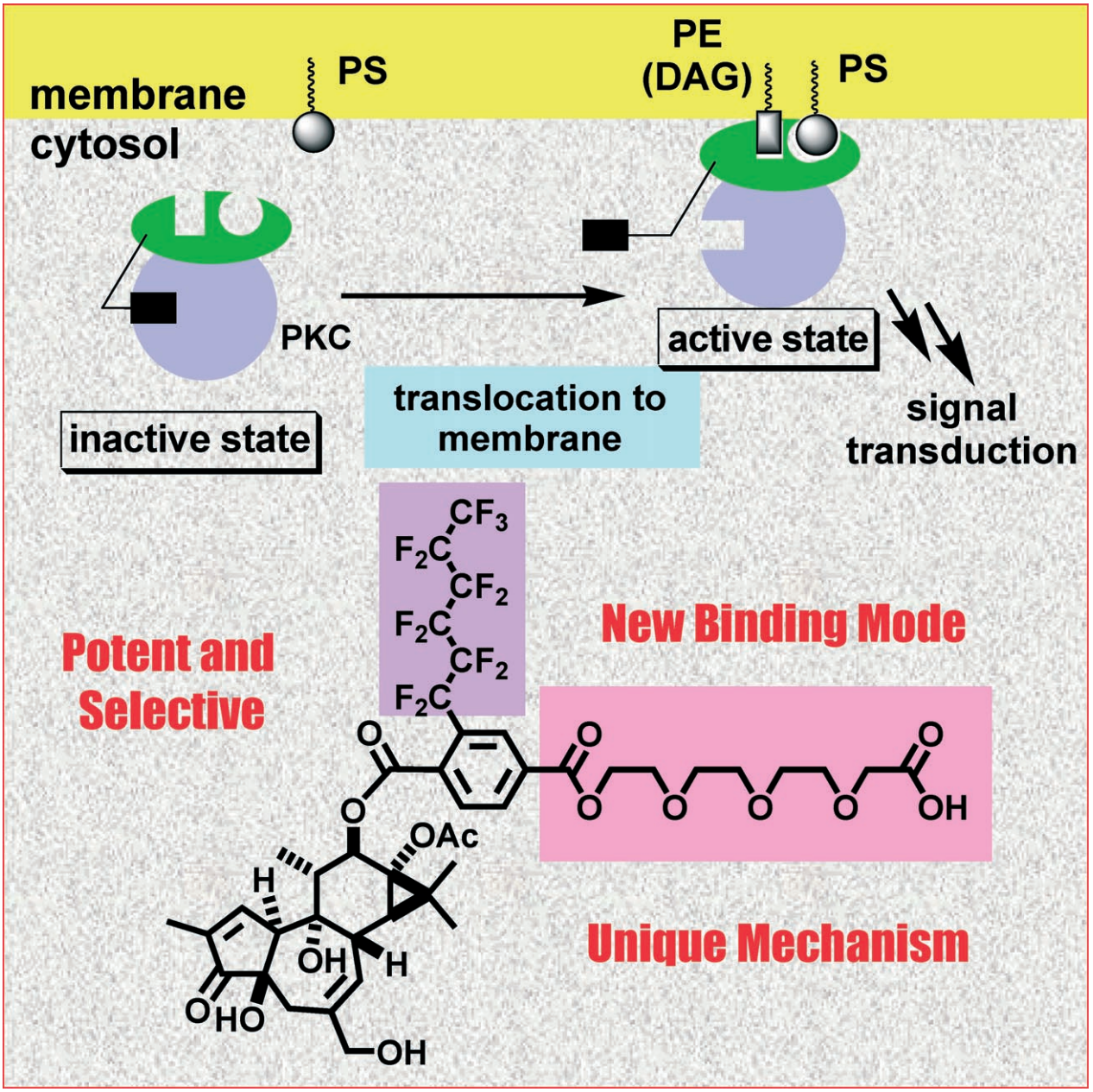

Abstract: The elucidation of specific functions of protein kinase C (PKC) subtypes in physiological processes is an important challenge for the future development of new drug targets. Subtype-selective PKC agonists and antagonists are useful biological tools for this purpose. Most of the currently used PKC modulators elicit their activities through binding to the ATP binding site of PKC, which shares many features with other kinases. PKC modulators that target the PKC regulatory domain are considered to be advantageous in terms of selectivity, because the structure of the regulatory domain is intrinsic to each PKC subtype. In this paper, we describe the identification of new potent and conventional PKC-selective inhibitors that target the regulatory domain. The inhibitors contain a phorbol skeleton, a naturally occurring potent and selective PKC regulatory domain binder, with a perfluorinated alkyl group and a polyether hydrophilic chain on a terephthaloyl aromatic ring at the C12 position. Both of these substituents are essential for the potent inhibitory activity. Specifically, the binding affinity between PKC and the phorbol ester analogues was improved by an electron-deficient aromatic ring at C12. This finding cannot be explained by the previously proposed binding model and suggests a new binding mode between phorbol esters and PKC.

Keywords: enzymes · fluoroalkyls · inhibitors · phorbol esters · protein kinase C

Introduction

Almost all responses of living cells, including cell growth, differentiation, and apoptosis, are induced by the reversible phosphorylation of proteins. The number of protein kinases encoded by the human genome is estimated to be 518 (1.7% of the human genome),[1] and kinases either crosstalk, cooperate, or compete with each other to determine the fate of the cell. Clarification of the specific role of each protein kinase, and more specifically each kinase subtype, is essential for detailed understanding of the signal transduction pathway. This achievement should further lead to the development of new drug targets.

Serine/threonine kinases constitute the protein kinase C (PKC) family, which has pivotal roles in intracellular signal transduction cascades.[2] The PKC family comprises three subclasses, depending on their structure and cofactor requirements for activation: conventional, novel, and atypical PKC. These subclasses have a common catalytic domain at the C-terminal, whereas the structure of the regulatory domains at the N-terminal is different. Conventional PKC, including PKCα, βI, βII, and γ isozymes, contains two tandem cystein-rich C1 domains (C1A and C1B) and a Ca^{2+}-binding C2 domain in the regulatory site, and physiologically requires diacylglycerol (DAG; binding to C1A and B) and Ca^{2+} for its activation. Novel PKC, which includes the δ, ε, η, and θ isozymes, contains only the C1 domain and lacks the C2 domain, thus its activation does not require Ca^{2+} ions. The atypical ζ and ι/λ isozymes contain neither functional C1 nor C2 domains, and their regulatory mechanism is not known.

To elucidate the specific function of each PKC subtype, selective PKC agonists and antagonists are important biological tools.[3,4] Currently, however, there is only a limited number of selective PKC agonists or antagonists available for use. This is partly due to the fact that the PKC modulators elicit their activities through binding to the ATP binding site in the catalytic domain, which shares many features with other kinases. For example, a potent and selective PKC inhibitor, Gö6976,[5] binds to the ATP binding site of PKC. Gö6976 also potently inhibits checkpoint kinase and phosphorylase kinase.[4] On the other hand, the structure of the kinase regulatory domain is intrinsic to each kinase and/or subclass type. Therefore, inhibitors that target the PKC regulatory domain should be more selective.[6]

Phorbol esters (PEs; Figure 1) such as phorbol myristate acetate (PMA; **1**) and phorbol dibutylate (PDBu; **2**) exist abundantly in plant-derived croton oil and can be isolated in significant amounts through relatively easy procedures.[7] PEs are extremely potent conventional PKC-selective agonists that strongly bind to the C1 regulatory domain (the same site as DAG).[8] The binding affinity of PEs is at least three orders of magnitude greater than that of DAG. Crystallographic[9] and molecular modeling[10] studies of PKC–PE complexes revealed atom-level pictures of the interaction; PEs bind strongly to PKC by a hydrogen-bond network mainly through the 20-OH group and the 3-ketone oxygen

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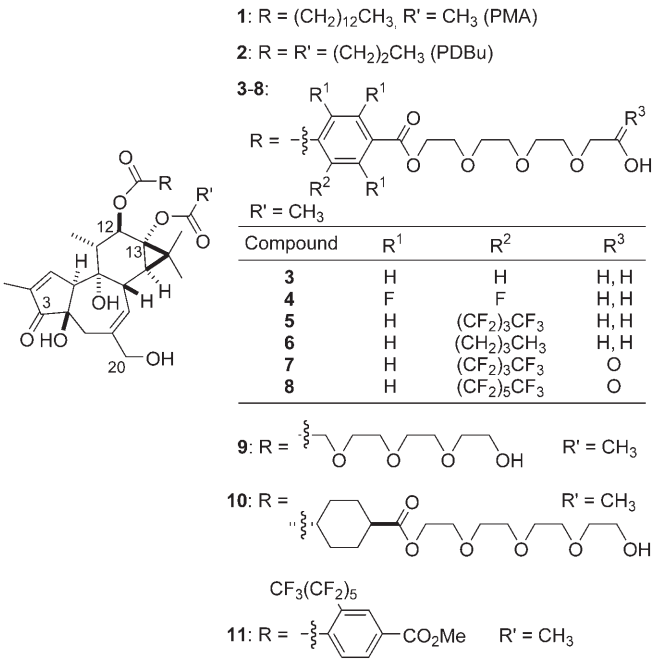


Figure 1. Phorbol esters and their analogues.

atom. Those binding models did not involve any interactions between the ester chain at the C12 position of PE and PKC.

PKC activation by PE (**1** or **2**) requires several independent events.[11] An increase in the intracellular Ca^{2+} concentration triggers the redistribution of PKC from the cytosol, where PKC is maintained in an inactive conformation, to the cell membrane, where PKC becomes allosterically activated by interactions with PE and phosphatidyl-L-serine (L-PS). Alternatively, interactions of PKC and PE occur first in the cytosol, followed by translocation of the PKC–PE complex to the membrane. In both cases, the activation of PKC requires translocation to the hydrophobic environment, and the hydrophobic ester chain at C12 of **1** and **2** has a key role for retaining the PKC–PE complex in the membrane. Although DAG is metabolized immediately through phosphorylation or hydrolysis, PEs persistently activate PKC due to the absence of a terminating pathway in animal cells. Thus, PEs produce malignant cell growth and are the most potent tumor promoters known to date. Despite their tumor-promoting activity, the extremely high binding affinity and selectivity of PEs for the PKC C1 domain have attracted the interest of many synthetic chemists, including our group, to utilize this molecule as a scaffold to develop new PKC modulators.[12]

The proposed binding feature of PEs to PKC and the scenario for PKC activation provided a basis for our previous development of a PE-derived PKC inhibitor **3** (Figure 1).[12a] Thus, because the proposed binding motifs (ketone at C3 and OH at C20) of PE are intact, the PKC inhibitor **3** had a binding affinity for PKC. Due to the hydrophilic polyether chain at C12, however, the PKC–**3** complex did not translocate to the hydrophobic environment (see below). As a result, **3** was a pure PKC antagonist. Despite the presence of the proposed binding motifs, the binding affinity of **3** was significantly lower ($K_i = 3.2$ μM) than that of naturally occurring PE **1** ($K_i = 0.01$ μM). The low binding affinity resulted in the weak inhibitory activity of **3**, which hampered further evaluation of kinase selectivity and application as a biological tool. In this paper we report that the binding affinity of PE analogues is significantly enhanced by the introduction of an electron-deficient aromatic group with a perfluoroalkyl substituent at the C12 ester. This finding led to the identitification of new C1 domain-binding PKC inhibitors that exhibit potent binding affinity, practical inhibitory activity, and subclass selectivity.

Abstract in Japanese:

プロテインキナーゼC（PKC）は、さまざまな細胞内情報伝達経路に関与する極めて重要なタンパク質リン酸化酵素である。特定のサブクラス選択的 PKC 阻害剤の開発は、PKC サブクラスの機能解明を促進するばかりでなく、医薬のリード創出にもつながりうる。我々は PKC のレギュレトリードメインに選択的かつ強力に結合する天然物であるホルボールエステルの骨格を母核として、12位のエステル部位を親水的なポリエーテル鎖で修飾することにより、PKC 阻害剤（**3**）を創製しうることをすでに報告している。しかしながら **3** の PKC への結合能は低く、それゆえに阻害活性も中程度にとどまっていた。今回、12位に電子欠損型芳香環を導入することによりホルボール誘導体の PKC に対する結合能が向上することを見いだした。この知見をもとに阻害剤の構造最適化をおこなったところ、12位芳香環上にパーフルオロアルキル基を導入し、さらにポリエーテル鎖の末端をカルボン酸とすることで、**3** に比較して25～50倍阻害活性の向上した **7**, **8** を開発した。これらの阻害剤は conventional PKC サブクラス選択的阻害剤であることを明らかとした。

Results and Discussion

In the course of our study to improve the binding affinity of phorbol-derived inhibitors, we encountered unexpected results that deviated from the proposed binding model of the PKC–PE complex;[9,10] the terephthalate aromatic ring of the ester at C12 has a key role in PKC binding. Thus, compounds **9** and **10**, which lack the aromatic ring, exhibited 10–100 times lower binding affinity to PKCα than the weak antagonist **3**. Conversely, these initial findings suggested that the binding affinity of phorbol analogues to PKC might be enhanced through modifying the C12 terephthalate aromatic ring.

Based on the initial results, we systematically tuned the characteristics of the aromatic ring by introducing substituents onto it. To assess the electronic effect, we synthesized compound **4**, which contains a tetrafluoroterephthalate moiety (Figure 1). This compound should have significantly lower π-electron density than the original **3** without changing the steric factor and hydrophobicity.[13] Thus, the binding affinity of **4** ($K_i = 0.42$ μM) to PKCα was approximately 10 times higher than that of the original **3** (Table 1). These results indicated that the binding affinity of phorbol analogues to PKC is enhanced by introducing an electron-deficient aromatic ring at the C12 position.

Table 1. Binding affinities and inhibitory activities of phorbol esters to PKCα.[a]

PE	K_i [μM]	IC_{50} [μM]	PE	K_i [μM]	IC_{50} [μM]
3	3.2±0.3	5.1±0.4	**7**	0.45±0.17	0.11±0.06
4	0.42±0.77	1.5±1.0	**8**	0.06±0.01	0.19±0.07
5	0.13±0.24	0.24±0.01	**11**	0.03±0.02	–[b]
6	10±0.3	1.8±1.1			

[a] K_i and IC_{50} values (IC_{50} = 50% inhibitory concentration) were determined by at least three independent experiments, and the mean values and error limits are shown. [b] Functioned as a full agonist.

To improve the binding affinity further, a strongly electron withdrawing perfluorobutyl group was introduced (**5**; Figure 1). The binding affinity of **5** (K_i = 0.13 μM) was higher than that of **4** (Table 1). The effect of fluorine substitution on PKC binding affinity was significant; the control analogue **6**, with a butyl instead of a perfluorobutyl group, had a K_i value of 10 μM. The observed difference in binding affinity of **5** and **6** of two orders of magnitude is attributed to the electronic factor of the aromatic ring at the C12 position, because the steric difference between butyl and perfluorobutyl groups is small.[14]

Next, PKCα agonist activities of these phorbol derivatives were evaluated (see Supporting Information). The previous inhibitor **3** and fluorine-containing **4** and **5** exhibited no agonist activity even at high concentrations; however, the butyl-substituted **6** exhibited partial agonist activity, producing about 20% PKC activation at 10 μM concentration.[15] Therefore, the introduction of a perfluorinated alkyl group is beneficial for enhancing PKC binding affinity without inducing any PKC agonist activity. Due to this favorable feature of **5** as a PKC inhibitor, the inhibitory activity of **5** (IC_{50} = 0.24 μM) was significantly higher than that of the previous weak inhibitor **3** (IC_{50} = 5.1 μM) (Table 1). The potency of **5** is comparable to or even higher than widely used PKC inhibitors such as H7 (IC_{50} = 6 μM)[16] and chelerythrine chloride (IC_{50} = 0.7 μM).[17]

Having identified a novel substituent effect of the perfluorobutyl group on the potency of PKC inhibitors, we next investigated the effect of the perfluorinated alkyl chain length and the terminal hydrophilic group on PKC binding affinity and inhibitory activity by comparing the properties of **5**, **7**, and **8** (Figure 1). Compound **7**, a carboxylic acid analogue of **5**, exhibited almost comparable binding affinity (K_i = 0.45 μM) to **5** with slightly higher inhibitory activity (IC_{50} = 0.11 μM). Compound **8**, which contains a perfluorohexyl group on the aromatic ring and a carboxylic acid at the ester terminal, exhibited the highest binding affinity (K_i = 0.06 μM) with potent inhibitory activity (IC_{50} = 0.19 μM). Indeed, **7** and **8** are the most potent PKCα inhibitors containing the phorbol skeleton reported to date. The polyether chain was also essential for the inhibitory activity; a control compound **11** with a methyl group instead of the polyether chain exhibited very strong PKC agonist activity that was comparable to that of **1**.

Moreover, **8** was shown to be a conventional PKC-selective inhibitor (Table 2). Evaluation of inhibitory activities of **8** against PKA, PKCδ, and PKCζ demonstrated that **8** does not inhibit PKA and PKCζ at all, and only very weakly inhibits PKCδ at high concentration (80% activity in the presence of 10 μM of **8**). The selectivity of **8** was attributed to the selectivity of natural PEs, which bind only to conventional PKC with high affinity. Thus, **8** is a potent and conventional PKC-selective inhibitor that might be useful as a biological tool.

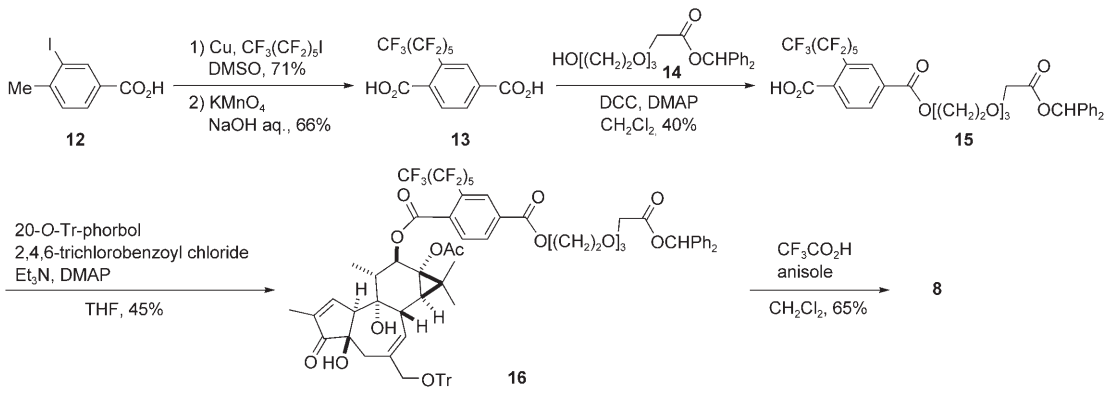
Table 2. Kinase and subtype selectivity of inhibitor **8**.

	PKCα	PKA	PKCδ	PKCζ
IC_{50} [μM]	0.19±0.07[a]	N.I.[b]	⩾10	N.I.[b]

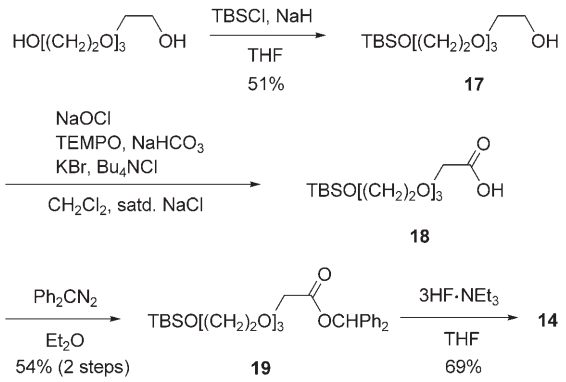
[a] Mean value determined from four independent experiments. [b] No inhibition.

The synthesis of **8** is straightforward (Scheme 1).[18] Ullmann coupling of aryl iodide **12** with perfluorohexyl iodide,[19] followed by oxidation with $KMnO_4$, produced terephthalic acid **13**. After selective monoester formation with **14** (which was prepared as outlined in Scheme 2), coupling of the resulting **15** with the phorbol moiety was conducted under Yamaguchi esterification conditions to give **16**. Deprotection of **16** under acidic conditions afforded **8**.

Finally, to gain preliminary insight into the mechanism of PKC inhibition by **8**, a partition assay with a cell/buffer biphasic system was performed.[12a] This assay evaluates the



Scheme 1. Synthesis of **8**. DCC = dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, DMSO = dimethyl sulfoxide, Tr = triphenylmethyl.



Scheme 2. Synthesis of **14**. TBS = *tert*-butyldimethylsilyl, TEMPO = 2,2,6,6-tetramethylpiperidin-1-oxyl radical.

translocation ability of compounds from the aqueous buffer phase to the hydrophobic cell membrane. Previously, **3** was determined to exist mainly (92%) in the buffer phase owing to the hydrophilic polyether chain.[12a] The hydrophilic property of **3** led us to propose that **3** exhibits PKC inhibitory activity by preventing the PKC–**3** complex from translocating to the hydrophobic environment, where PKC is transformed to an active conformation. However, another potent PKC agonist, which contains a hydrophobic C12 ester (**1**-mimic), was distributed mainly (75%) in the cell membrane. Contrary to our expectation, the potent inhibitor **8** was also predominantly distributed in the membrane (89–99%).[20] The result of the partition assay indicated that **8** should exhibit its PKC inhibitory activity in a hydrophobic environment. Therefore, the new inhibitor **8** may function through a different mechanism to the previous hydrophilic, weak antagonist **3**.

The results described in this paper raise two main issues concerning the mode of PKC–PE interaction and PKC inhibition. First, the fact that the binding affinities of PE analogues to PKC strongly depend on the characteristics of the C12 ester moiety suggests a direct interaction between the ester moiety and PKC. This binding mode is distinct from the one that has been previously proposed,[9,10] but may be consistent with our previous observations in photocross-linking studies.[21] Therein, PKC was labeled by a PE-derived photoaffinity probe bearing a photosensitive group on the C13 ester moiety. Thus, we conclude that the C13 (and C12) esters are positioned proximal to PKC in the PKC–PE complex. The second mechanistic issue raised by our studies concerns inhibitor **8**, which inactivates PKC despite its ability to translocate PKC to the hydrophobic environment. We attribute this surprising inhibitory activity to the presence of the polyether on the C12 ester moiety (compare the inhibitory activities of **8** and **11**). The strong metal-binding ability of polyethers[22] may be relevant in this case. It is known that there are Zn^{2+} ions in both the C1A and C1B domains of PKC, which are essential for its function.[23] One of the Zn^{2+} ions is located near the PE binding site.[9,10] It seems possible, therefore, that the polyether chain of **8** may coordinate to Zn^{2+}, thus modulating the function of PKC.[24]

Conclusions

We have developed potent, conventional PKC-selective inhibitors **7** and **8**. These compounds contain a phorbol skeleton with perfluorinated alkyl and hydrophilic polyether chains on an electron-deficient aromatic ring at the C12 ester. Both of the substituents are essential for the potent inhibitory activity. The inhibitory activity of these compounds was 25- to 50-fold stronger than the previous lead compound **3**, which bears a polyether chain but not the perfluoroalkyl chain. To our knowledge, **7** and **8** are the first phorbol derivatives that exhibit practical and selective PKC inhibitory activity.[25] Partition studies to address the origin of the enhanced inhibitory activity of **8** relative to **3** revealed a contrasting difference in the cytosol/membrane distribution of these compounds. Studies to clarify the binding mode and the inhibitory mechanism as well as the application of **8** as a biological tool are ongoing.

Experimental Section

General

NMR spectra were recorded on a JEOL JNM-LA500 spectrometer operating at 500 MHz for ^{1}H and 125.65 MHz for ^{13}C. Chemical shifts were reported downfield from $Si(CH_3)_4$ (=0 ppm) for ^{1}H NMR spectra. For ^{13}C, chemical shifts were reported relative to the solvent as an internal reference. ESI mass spectra were recorded on a Water-ZQ4000 spectrometer. IR spectra were recorded on a JASCO FT/IR 410 Fourier-transform infrared spectrophotometer. Column chromatography was performed with silica gel Merck 60 (230–400 mesh ASTM).

Syntheses

4-Methyl-3-perfluorohexylbenzoic acid: The Ullmann coupling between **12** and perfluorohexyl iodide was conducted according to the reported procedure.[19] A mixture of **12** (3.0 g, 11.4 mmol), perfluorohexyl iodide (2.74 mL, 12.6 mmol), and copper powder (3.64 g, 57.2 mmol) in DMSO (22.9 mL) was heated at 100 °C for 6 h. After dilution with $CHCl_3$, HCl (1 M) was added, and the precipitates were filtered off through celite. Brine was added to the filtrate, and the products were extracted with $CHCl_3$. The combined organic layer was dried with Na_2SO_4. Filtration, evaporation, and purification by SiO_2 column chromatography (CH_2Cl_2/MeOH = 20:1) gave the product as a white solid (3.67 g, 71%). IR (KBr): $\tilde{\nu}$ = 1687 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz): δ = 8.16 (br s, 1H), 8.13 (br d, J = 8.1 Hz, 1H), 7.51 (d, J = 8.1 Hz, 1H), 2.54 ppm (m, 3H); ^{13}C NMR (CD_3OD, 125 MHz): δ = 168.0, 144.6, 144.6, 134.4, 134.3, 130.8 (m), 128.1 (m), 109.8–121.1 (multipeak derived from perfluoroalkyl chain), 20.7 ppm (m); MS (ESI): m/z: 453 $[M-H]^-$.

13: The benzoic acid synthesized above (1.0 g, 2.2 mmol) was dissolved in NaOH (1.5 M, 22 mL), and $KMnO_4$ (5.22 g, 33.0 mmol) was added. The mixture was heated at 100 °C for 2 h, followed by the further addition of $KMnO_4$ (1.7 g, 11 mmol) and stirring for 30 min. MeOH was added at 0 °C, and the precipitates were filtered off through celite, after which the excess MeOH was evaporated. HCl (1 M) was added to acidify the mixture, and a white precipitate emerged. The precipitate was filtered and washed with H_2O. A white solid was obtained after the resulting powder was dried (699 mg, 66%). IR (KBr): $\tilde{\nu}$ = 2999, 1709 cm^{-1}; ^{1}H NMR (CD_3OD, 500 MHz) δ = 8.33 (br d, J = 8.0 Hz, 1H), 8.25 (br s, 1H), 7.77 ppm (d, J = 8.0 Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ = 170.2, 167.2, 139.7 (m), 134.5, 133.9, 130.6, 130.5, 126.8, 110–120 (multipeak derived from perfluoroalkyl chain); MS (ESI): m/z: 483 $[M-H]^-$.

17: Tetraethylene glycol (2.67 mL, 0.0154 mol) was added to a suspension of NaH (60% oil dispersion, 0.65 g, 0.016 mol) in THF (30.8 mL), and the resulting solution was stirred for 1 h at room temperature. After cool-

ing the mixture to 0 °C, TBSCl (2.32 g, 0.0154 mol) was added. The reaction was continued at room temperature for 30 min, and H_2O was added. The products were extracted with EtOAc, and the combined organic layer was washed with brine, dried with Na_2SO_4, and filtered. The filtrate was evaporated and the residue was purified by SiO_2 column chromatography (CH_2Cl_2/MeOH = 25:1) to afford **17** as a colorless oil (2.44 g, 51 %). IR (neat): $\tilde{\nu}$ = 3435, 2928, 2858 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 3.72 (t, J = 5.5 Hz, 2H), 3.67 (t, J = 4.6 Hz, 2H), 3.63–3.60 (m, 8H), 3.56 (t, J = 4.6 Hz, 2H), 3.51 (t, J = 5.5 Hz, 2H), 2.64 (s, 1H), 0.85 (s, 9H), 0.02 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 77.2, 72.6, 72.5, 70.7, 70.6, 70.3, 62.7, 61.7, 25.9, 18.3, 5.3 ppm ; MS (ESI): m/z: 331 $[M+Na]^+$.

18: TEMPO (4.8 mg, 0.0324 mmol), a solution of KBr (34.8 mg, 0.29 mmol) and Bu_4NCl (44.8 mg, 0.16 mmol) in saturated $NaHCO_3$ (6.5 mL), and a solution of NaOCl (5 %, 15.7 mL) in saturated $NaHCO_3$ (3.78 mL) and saturated NaCl (7.39 mL) were added to a solution of **17** (1.0 g, 3.24 mmol) in CH_2Cl_2 (10.9 mL) at 0 °C.[26] After 2.5 h, the reaction mixture was diluted with $CHCl_3$, and H_2O and $KHSO_4$ (3.2 equiv) were added. The products were extracted with $CHCl_3$, and the combined organic layer was washed with saturated NaCl, dried with Na_2SO_4, and filtered. The filtrate was evaporated to give the product **18** (1.1 g). This crude mixture was used without purification in the next step.

19: Ph_2CN_2[27] (3.15 g, 16.2 mmol) in Et_2O (9.8 mL) was added to **18** (without purification, 3.24 mmol) at room temperature, and the mixture was stirred for 15 h. AcOH was added, and the volatile compounds were evaporated. Purification by SiO_2 column chromatography (EtOAc/hexanes = 1:4) gave **19** as a colorless oil (848 mg, 54 % in 2 steps). IR (neat): $\tilde{\nu}$ = 2927, 2857, 1759 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.28–7.20 (m, 10H), 6.91 (s, 1H), 4.20 (s, 2H), 3.70–3.67 (m, 4H), 3.63–3.62 (m, 2H), 3.57 (m, 4H), 3.48 (t, J = 5.5 Hz, 2H), 0.83 (s, 9H), 0.00 ppm (s, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 169.6, 139.8, 128.5, 128.0, 127.1, 77.1, 72.6, 71.0, 70.7, 70.7, 70.6, 68.8, 62.7, 25.9, 18.3, 5.3 ppm; MS (ESI): m/z: 511 $[M+Na]^+$.

14: $3HF{\cdot}NEt_3$ (2.83 mmol, 17.4 mmol) was added to a solution of **19** (848 mg, 1.74 mmol) in THF (17.4 mL) at room temperature. After 7 h, the reaction mixture was diluted with EtOAc, and H_2O was added. The products were extracted with EtOAc, and the combined organic layer was washed with brine, dried with Na_2SO_4, and filtered. The filtrate was evaporated, and the residue was purified by SiO_2 column chromatography (CH_2Cl_2/MeOH = 10:1) to give **14** as a colorless oil (445 mg, 69 %). IR (neat): $\tilde{\nu}$ = 3388, 2874, 1751 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.30–7.22 (m, 10H), 6.93 (s, 1H), 4.22 (s, 2H), 3.70–3.60 (m, 10H), 3.55 (t, J = 4.6 Hz, 2H), 2.49 ppm (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 169.5, 139.7, 128.5, 128.0, 127.1, 77.1, 72.4, 70.9, 70.6, 70.5, 70.3, 68.7, 61.7 ppm; MS (ESI): m/z: 397 $[M+Na]^+$.

15: Compound **14** (10 mg, 0.027 mmol) in CH_2Cl_2 (0.53 mL) was added to **13** (26 mg, 0.0534 mmol), DCC (8.3 mg, 0.0401 mmol), and DMAP (3.3 mg, 0.027 mmol), and the mixture was stirred for 3 h. CH_2Cl_2 and H_2O were added, and the products were extracted with $CHCl_3$. The combined organic layer was washed with saturated NH_4Cl and brine, dried with Na_2SO_4, and filtered. The filtrate was evaporated, and the residue purified by preparative TLC (CH_2Cl_2/MeOH = 9:1) to give **15** as a colorless oil (9 mg, 40 %). IR (neat): $\tilde{\nu}$ = 3424, 2923, 1726, 1613 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ = 8.22 (br d, J = 7.9 Hz, 1H), 8.17 (br s, 1H), 7.58 (d, J = 7.9 Hz, 1H), 7.36–7.24 (m, 10H), 6.90 (s, 1H), 4.46 (t, J = 4.7 Hz, 2H), 4.27 (s, 2H), 3.80 (t, J = 4.7 Hz, 2H), 3.70–3.58 ppm (m, 8H); ^{13}C NMR (CD_3OD, 125 MHz): δ = 171.4, 171.3, 166.2, 155.7 (m), 151.6 (m), 141.5, 136.3, 134.2, 130.9(m), 130.3 (m), 129.6, 129.0, 128.1, 78.8, 71.9, 71.6, 71.6, 71.5, 70.1, 69.5, 65.8 ppm (peaks corresponding to the perfluorohexyl chain could not be seen because of heavy C–F couplings); MS (ESI): m/z: 839 $[M-H]^-$.

16: A solution of 2,4,6-trichlorobenzoyl chloride (1.7 μL, 0.011 mmol) and Et_3N (3 μL, 0.0214 mmol) in THF (60 μL) was added to **15** (9 mg, 0.0107 mmol) at room temperature. After 1 h, 20-*O*-Tr-phorbol (3.5 mg, 0.0054 mmol)[12b] in THF (50 μL) and DMAP (20 mg, 0.16 mmol) were added. After 3 days, saturated NH_4Cl was added, and the products were extracted with EtOAc. The combined organic layers were washed with saturated NH_4Cl and brine, dried with Na_2SO_4, and filtered. The filtrate was evaporated, and the residue was purified by preparative TLC (SiO_2, hexanes/EtOAc = 1:1) to give **16** (3.4 mg, 45 %). IR (neat): $\tilde{\nu}$ = 3419, 2922, 1726 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz): δ = 8.29 (br s, 1H), 8.25 (br d, J = 8.0 Hz, 1H), 7.59 (m, 1H), 7.50 (d, J = 8.0 Hz, 1H), 7.40 (d, J = 7.5 Hz, 6H), 7.31–7.19 (m, 19H), 6.94 (s, 1H), 5.68 (d, J = 10.3 Hz, 1H), 5.64 (m, 1H), 4.49 (t, J = 4.9 Hz, 2H), 4.22 (s, 2H), 3.80 (t, J = 4.9 Hz, 2H), 3.70–3.62 (m, 8H), 3.50 (s, 2H), 3.25 (m, 1H), 3.15 (m, 1H), 2.48 (d, J = 18.3 Hz, 1H), 2.39 (d, J = 19.5 Hz, 1H), 2.23 (m, 1H), 2.08 (s, 3H), 1.76 (m, 3H), 1.24 (s, 3H), 1.22 (s, 3H), 1.10 (d, J = 5.2 Hz, 1H), 1.01 (d, J = 6.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 208.7, 173.8, 169.6, 166.9, 164.9, 164.4, 161.0, 160.5, 150.2, 144.0, 139.8, 133.1, 132.8, 132.0, 129.3, 128.7, 128.5, 128.1, 127.8, 127.1, 127.0, 126.6, 120.7, 86.9, 78.1, 77.6, 77.5, 77.2, 73.8, 71.0, 70.7, 70.7, 70.6, 69.0, 64.9, 68.8, 56.1, 42.8, 39.3, 39.1, 36.4, 29.7, 23.6, 20.8, 20.3, 16.7, 14.5, 10.1 ppm (peaks corresponding to the perfluorohexyl chain could not be seen because of heavy C–F couplings); MS (ESI): m/z: 1493 $[M+H]^+$; HRMS (FAB): calcd for $C_{76}H_{71}F_{13}O_{15}Cs$: 1603.3634 $[M+Cs]^+$; found: 1603.3621.

8: Trifluoroacetic acid in CH_2Cl_2 (10 %, 0.1 mL) was added to **16** (3.4 mg, 0.0024 mmol) and anisol (5.3 mL, 0.048 mmol) at room temperature, and the mixture was stirred for 12 h. The reaction mixture was directly subjected to reverse-phase preparative TLC (MeOH/H_2O = 4:1) to give **8** (1.7 mg, 65 %). IR (neat) $\tilde{\nu}$ = 3408, 2925, 1725, 1616 cm^{-1}; ^1H NMR (CD_3OD, 500 MHz): δ = 8.40 (br d, J = 7.5 Hz, 1H), 8.30 (br s, 1H), 7.80 (d, J = 7.5 Hz, 1H), 7.58 (m, 1H), 5.71 (d, J = 10.3 Hz, 1H), 5.64 (m, 1H), 4.53 (m, 2H), 4.01 (s, 2H), 3.94 (m, 2H), 3.87 (t, J = 4.9 Hz, 2H), 3.71–3.65 (m, 8H), 3.30 (m, 1H), 3.17 (m, 1H), 2.50 (m, 2H), 2.33 (m, 1H), 2.08 (s, 3H), 1.74 (m, 3H), 1.25 (s, 3H), 1.22 (d, J = 5.2 Hz, 1H), 1.17 (s, 3H), 1.04 (d, J = 6.9 Hz, 3H); ^{13}C NMR (CD_3OD, 125 MHz): δ = 210.2, 182.1, 177.5, 174.1, 170.6, 165.6, 162.8, 160.3, 143.0, 134.7, 130.5, 129.1, 124.3, 123.5, 120.6, 85.4, 85.2, 79.9, 74.6, 71.6, 71.5, 71.5, 70.1, 68.0, 66.9, 66.2, 57.4, 44.3, 38.9, 38.5, 34.2, 33.6, 30.8, 23.9, 20.8, 17.2, 15.0, 10.2 ppm (peaks corresponding to the perfluorohexyl chain could not be seen because of heavy C–F couplings); MS (ESI): m/z: 1062 $[M-H]^-$; HRMS (FAB): calcd for $C_{44}H_{47}F_{13}O_{15}Cs$: 1195.1756 $[M+Cs]^+$; found: 1195.1781.

Biological Assay

Binding assay:[28] Plastic tubes of each binding-assay mixture (100 μL) contained Tris/HCl (50 mM, pH 7.5), $CaCl_2$ (1 mM), L-PS (100 μg mL^{-1}), [^{3}H]PDBu (20 nM, 20.7 Ci $mmol^{-1}$; NEN), DMSO (0.5 %), bovine serum albumin (BSA; 1 mg mL^{-1}), PKCα (0.8 μg mL^{-1}; PanVera), and PE analogue (10 nM–10 μM). L-PS was sonicated in Tris/HCl (50 mM, pH 7.5) at 0 °C prior to use. After incubation at 30 °C for 30 min, the mixture was filtered through a glassfiber filter, which was pretreated with freshly prepared polyethyleneimine (0.3 %) for 1 h. The residue was washed three times with of ice-cold DMSO (0.5 %, 3 mL). The radioactivity of each residue was counted in a scintillation vial with of scintillator (Aquasol-2, NEN; 10 mL) by using a liquid scintillation counter. The count in the presence of PMA (10 μM) accounted for nonspecific binding and was subtracted from each of the counts to account for the specific bindings.

Activation of PKCα: PKC activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into EGF-R fragment peptide (651–658; Biomol) using the reported method[29] with minor modifications. The amorphous powder of L-PS (Sigma) in triton X-100 solution (0.3 %) in Tris/HCl buffer (50 mM, pH 7.5) was vortexed for 1 min and sonicated on ice, followed by the addition of $CaCl_2$, dithiothreitol (DTT), and EGF-R. Various concentrations of PE analogue in DMSO were added to this mixture, after which it was vigorously vortexed. PKCα solution (25 μL) in Tris buffer was then added. The enzyme reaction was started by the addition of a solution (25 μL) containing ATP, [γ-^{32}P]ATP, and $MgCl_2$. The final reaction mixture (75 μL) contained $CaCl_2$ (1 mM), $MgCl_2$ (15 mM), DTT (2.5 mM), ATP (50 μM), [γ-^{32}P]ATP (0.2 μCi), EGF-R (75 μM), L-PS (12–20 mol %) dispersed in triton X-100 (0.025 % *w*/*v*) in Tris/HCl buffer (50 mM, pH 7.5), and drug (100 nM–10 μM). The reaction was conducted for 15 min at 30 °C and stopped by the addition of ice-cold trichloroacetic acid (25 %, 25 μL). The resulting precipitate was collected on binding paper (P-81, Whatman Ltd., England) and washed twice with phosphoric acid (75 mM, 250 mL). The residue was placed in a scintillation vial in a solution of Aquasol-2, and ^{32}P radioactivities were quantitated by a scintillation counter. The count for the control assay without PMA was deter-

mined and was subtracted from each of the counts to account for the nonspecific kinase activities.

Inhibitory assay of PKCα: PKC activity in the presence of PMA (10 nM) and drug (100 nM–10 μM or none) was determined as above.

Inhibitory assay of PKCδ: PKCδ (Calbiochem) activity in the presence of PMA (1 μM) and drug (100 nM–10 μM or none) was determined in the same way as for PKCα, except that the final reaction mixture contained ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 100 μM) instead of $CaCl_2$. The result shown (Table 2) is representative of at least three sets of experiments.

Inhibitory assay of PKCζ: PKCζ (Calbiochem) activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into peptide ε (149–164; Biomol) using the reported method.[30] The reaction mixture (100 μL) contained Tris/HCl (25 mM, pH 7.5), peptide ε (30 μM), [γ-^{32}P]ATP (50 μM, 200 cpm pmol^{-1}), $MgCl_2$ (5 mM), EGTA (0.5 mM), DTT (1 mM), L-PS (2 μg), and PKCζ (0.04 μg). The amorphous powder of L-PS in Tris/HCl (25 mM, pH 7.5) was vortexed for 1 min and sonicated on ice, followed by the addition of $MgCl_2$, EGTA, DTT, and peptide ε. Various concentrations of PE analogue in DMSO were added to this mixture, after which it was vigorously vortexed. PKCζ solution (25 μL) in Tris buffer was then added. The enzymatic reaction was started by the addition of a solution (25 μL) containing ATP and [γ-^{32}P]ATP. The reaction mixture was incubated at 30 °C for 10 min. The reaction was stopped with the addition of NaF (30 mM, 25 μL) and EDTA (100 mM, pH 7.5). The reaction mixture was then applied to binding paper (P-81, Whatman Ltd., England) and washed twice with phosphoric acid (75 mM, 250 mL). The residue was placed in a scintillation vial in a solution of Aquasol-2, and ^{32}P radioactivities were quantified by a scintillation counter. As PKCζ activity in vitro shows limited dependence on the presence of PMA, the total incorporation of ^{32}P into peptide ε was taken as PKCζ activity. The result shown (Table 2) is representative of at least three sets of experiments.

Inhibitory assay of PKA: PKA catalytic subunit (Sigma) activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into Kemptide (65189-71-1, Sigma) using the reported method[31] with minor modifications. The reaction mixture (75.5 μL) contained MOPS buffer (50 mM, pH 6.9), $Mg(OAc)_2$ (2 mM), DTT (1 mM), NaCl (50 mM), BSA (0.166 mg mL^{-1}), Kemptide (100 μM), [γ-^{32}P]ATP (132 μM, 200 cpm pmol^{-1}), and PKA catalytic subunit (16.6 nM). Various concentrations of drug solution in DMSO (0.5 μL) were added to the mixture of MOPS buffer (25 μL, pH 6.9), NaCl, $Mg(OAc)_2$, and DTT, after which it was vigorously vortexed. PKA catalytic subunit solution (25 μL) in MOPS buffer containing NaCl, $Mg(OAc)_2$, DTT, and BSA, was added to this mixture, which was then incubated for 5 min at 30 °C. The reaction was started by the addition of [γ-^{32}P]ATP solution (400 μM) and was continued for 15 min at 30 °C. After incubation, the reaction mixture (30 μL) was absorbed onto binding paper (P-81, Whatman Ltd., England), and the samples were washed four times with phosphoric acid (75 mM, total of 500 mL) followed by ethanol prior to drying. The residue was placed in a scintillation vial in a solution of Aquasol-2, and ^{32}P radioactivities were quantified by a scintillation counter. The result shown (Table 2) is representative of at least three sets of experiments.

Partition assay: A solution of PE analogue (15 μM,10 μL) in water was added to a suspension of Hela cells in PBS buffer (140 μL, 2×10^6 cells mL^{-1}), and the resulting mixture was incubated for 30 min at 30 °C. After centrifuging the mixture at 1000 rpm for 5 min at 4 °C, 20 μL of the supernatant was subjected to reverse-phase HPLC to quantify the amount of drugs in the buffer phase (detection at 254 nm; mobile phase, methanol/water (7:3 for **8**, $t_R = 19.2$ min) at a flow rate of 0.5 mL min^{-1}).

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[1] G. Manning, D. B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, *Science* **2002**, *298*, 1912.

[2] a) Y. Nishizuka, *FASEB J.* **1995**, *9*, 484; b) A. Toker, *Front. Biosci.* **1998**, *3*, D1134; c) *Protein Kinase C: Current Concepts and Future Perspectives* (Eds.: D. S. Lester, R. M. Epand), Ellis Horwood, Chichester, **1992**; d) A. C. Newton, *Curr. Opin. Cell Biol.* **1997**, *9*, 161.

[3] For a review of protein kinase inhibitors, see: A. J. Bridges, *Chem. Rev.* **2001**, *101*, 2541.

[4] For selectivity evaluation of protein kinase inhibitors, see: a) S. P. Davies, H. Reddy, M. Caivano, P. Cohen, *Biochem. J.* **2000**, *351*, 95; b) L. Saravia, P. Fresco, E. Pinto, J. Goncalves, *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 475.

[5] G. Martiny-Baron, M. G. Kazanietz, H. Mischak, P. M. Blumberg, G. Kochs, H. Hug, D. Marme, C. Schachtele, *J. Biol. Chem.* **1993**, *268*, 9194. Gö6976 is a potent, subtype-selective inhibitor of PKCα ($IC_{50} = 2.5$ nM), but does not exhibit inhibitory activity against PKCδ, ε, and ζ.

[6] For C1-domain-binding artificial DAG analogues (agonists), see: a) V. E. Marquez, K. Nacro, S. Benzaria, J. Lee, R. Sharma, K. Teng, G. W. A. Milne, B. Bienfait, S. Wang, N. E. Lewin, P. M. Blumberg, *Pharmacol. Ther.* **1999**, *82*, 251; b) V. E. Marquez, P. M. Blumberg, *Acc. Chem. Res.* **2003**, *36*, 434; c) J. Lee, J. Kang, K. Han, Y. Kim, S. Y. Kim, H. Youn, I. Mook-Jung, H. Kim, J. H. L. Han, H. J. Ha, Y. H. Kim, V. E. Marquez, N. E. Lewin, L. V. Pearce, D. J. Lundberg, P. M. Blumberg, *J. Med. Chem.* **2006**, *49*, 2028; d) Y. Baba, Y. Ogoshi, G. Hirai, T. Yanagisawa, K. Nagamatsu, S. Mayumi, Y. Hashimoto, M. Sodeoka, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2963; e) Y. Baba, S. Mayumi, G. Hirai, H. Kawasaki, Y. Ogoshi, T. Yanagisawa, Y. Hashimoto, M. Sodeoka, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 2969.

[7] N. C. Mishra, R. D. Estensen, M. M. Abdelmonem, *J. Chromatography* **1986**, *369*, 435.

[8] a) U. Kikkawa, Y. Takai, Y. Tanaka, R. Miyake, Y. Nishizuka, *J. Biol. Chem.* **1983**, *258*, 11442. For reviews, see: b) M. G. Kazanietz, M. J. Caloca, P. Eroles, T. Fujii, M. L. Garcia-Bermejo, M. Reilly, H. Wang, *Biochem. Pharmacol.* **2000**, *60*, 1417; c) Y. Kishi, R. R. Rando, *Acc. Chem. Res.* **1998**, *31*, 163; d) P. A. Wender, K. F. Koehler, N. A. Sharkey, M. L. Dell′Aquila, P. M. Blumberg, *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4214.

[9] G. Zhang, M. G. Kazanietz, P. M. Blumberg, J. H. Hurley, *Cell* **1995**, *81*, 917.

[10] a) S. Wang, M. G. Kazanietz, P. M. Blumberg, V. E. Marquez, G. W. A. Milane, *J. Med. Chem.* **1996**, *39*, 2541; b) J. Hritz, J. Ulicny, A. Laaksonen, D. Jancura, P. Miskovsky, *J. Med. Chem.* **2004**, *47*, 6547.

[11] A. C. Newton, *Chem. Rev.* **2001**, *101*, 2353.

[12] For inhibitors based on the phorbol ester skeleton, see: a) R. Wada, Y. Suto, M. Kanai, M. Shibasaki, *J. Am. Chem. Soc.* **2002**, *124*, 10658; b) M. Sodeoka, M. A. Arai, K. Adachi, K. Uotsu, M. Shibasaki, *J. Am. Chem. Soc.* **1998**, *120*, 457; c) T. M. Bertolini, J. Giorgione, D. F. Harvey, A. C. Newton, *J. Org. Chem.* **2003**, *68*, 5028; d) P. A. Wender, T. A. Kirshberg, P. D. Williams, H. M. M. Bastiaans, K. Irie, *Org. Lett.* **1999**, *1*, 1009; e) M. Tanaka, K. Irie, Y. Nakagawa, Y. Nakamura, H. Ohigashi, P. A. Wender, *Bioorg. Med. Chem. Lett.* **2001**, *11*, 719.

[13] The calculated log*P* values of **3** and **4** are 2.49 and 2.18, respectively, which support our assumption.

[14] There may also be affinitive interactions between the perfluoroalkyl group and PKC. For a review of the effects of fluorine substituents on biological activity, see: a) B. E. Smart, *J. Fluorine Chem.* **2001**, *109*, 3. For recent reports of interesting effects of fluoroalkyl substituents on biological activity and molecular structure, see: b) S. Ortial, G. Durand, B. Poeggeler, A. Polidori, M. A. Pappolla, J. Böker, R. Hardeland, B. Pucci, *J. Med. Chem.* **2006**, *49*, 2812; c) K. Monde, N. Miura, M. Hashimoto, T. Taniguchi, T. Inabe, *J. Am.*

Chem. Soc. **2006**, *128*, 6000; d) C. Jäckel, M. Salwiczek, B. Koksch, *Angew. Chem.* **2006**, *118*, 4305; *Angew. Chem. Int. Ed.* **2006**, *45*, 4198.
[15] The apparently higher inhibitory activity deduced from the IC_{50} value of **6** than expected from its modest binding affinity is attributable to the partial agonistic character of **6**. Even in the presence of a high concentration of **6**, the PKC activity did not reach zero; see Supporting Information.
[16] H. Hidaka, M. Inagaki, S. Kawamoto, Y. Sasaki, *Biochemistry* **1984**, *23*, 5036.
[17] J. M. Herbert, J. M. Augereau, J. Gleye and J. P. Maffrand, *Biochem. Biophys. Res. Commun.* **1990**, *172*, 993.
[18] For detailed synthetic procedures and spectroscopic data of **8**, see Supporting Information.
[19] V. C. R. McLoughlin, J. Thrower, *Tetrahedron* **1969**, *25*, 5921.
[20] Fluoroalkyl groups can significantly increase the membrane-binding ability. For example, see: G. Gerebtzoff, X. Li-Blatter, H. Fischer, A. Frentzel, A. Seelig, *ChemBioChem* **2004**, *5*, 676.
[21] a) M. Sodeoka, K. Uotsu, M. Shibasaki, *Tetrahedron Lett.* **1995**, *36*, 8795; b) K. Uotsu, M. Sodeoka, M. Shibasaki, *Bio. Med. Chem.* **1998**, *6*, 1117.
[22] T. Okada, *Analyst* **1993**, *118*, 959.
[23] A. F. G. Quest, J. Bloomenthal, E. S. G. Bardes, R. M. Bell, *J. Biol. Chem.* **1992**, *267*, 10193.
[24] The same mechanism might be involved in the previous inhibitor **3**.
[25] Preliminary studies indicated that **8** has no significant cancer cell (K562) growth inhibitory activity.
[26] A. E. J. Nooy, A. C. Besemer, H. Bekkum, *Synthesis* **1996**, 1153.
[27] T. Kametani, H. Sekine, T. Honda, *Chem. Pharm. Bull.* **1982**, *30*, 4545.
[28] Y. Tanaka, R. Miyake, U. Kikkawa, Y. Nishizuka, *J. Biochem.* **1986**, *99*, 257.
[29] Y. A. Hannun, C. R. Loomies, R. M. Bell, *J. Biol. Chem.* **1985**, *260*, 10039.
[30] H. Nakanishi, J. H. Exton, *J. Biol. Chem.* **1992**, *267*, 16347.
[31] J. D. Scott, E. H. Fischer, J. G. Demaille, E. D. Krebs, *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 4379.

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