Mapping Phorbol Ester Binding Domains of Protein Kinase C (PKC): The Design, Synthesis and Biological Activity of Novel Phorbol Ester Dimers

Paul A.Wender,*[a] Michael F.T. Koehler,[a] Dennis L. Wright,[a] Kazuhiro Irie[b]

[a] Department of Chemistry, Stanford University, Stanford, California 94305–5080, USA

Fax +1(650)7250259; E-mail: Wender@leland.stanford.edu

[b] Department of Applied Life Sciences, School of Agriculture, Kyoto University, Kyoto 606–8502, Japan

Received 8 April 1999

Abstract: The design and synthesis of a new class of protein kinase C (PKC) activators, phorbol ester dimers, is described. These dimers were found to bind to and activate PKC with affinities depending dramatically on their tether length and composition. In two cases, the binding affinities of these novel compounds exceeded that of phorbol dibutyrate.

Key words: bioorganic chemistry, dimerizations, esterifications, antitumor agents, lipids

Over the past two decades, the protein kinase C isozymes (PKCs) have emerged as one of the most important families of enzymes involved in cellular regulation.[1] These serine/threonine kinases have been implicated in a diverse array of biological processes ranging from learning to tumor promotion and are being pursued as therapeutic targets in the treatment of cancer, diabetes and neuropathic pain. It appears that this diversity of function derives from the fact that at least 11 PKC isozymes are expressed and that individual isozymes can have different functions. Therefore, the identification of isozyme selective agents is of great fundamental and therapeutic importance.

The PKCs are grouped according to their activator and cofactor requirements:[2] the conventional isozymes (α, βI, βII, γ) are activated by diacyl glycerol (DAG) and calcium, the novel isozymes (δ, ε, θ) are also activated by DAG but are calcium independent, and the atypical (ζ, λ) PKCs require neither DAG nor calcium for activation. The conventional and novel PKCs bind DAG and other activators using their first conserved (C1) region, which contains two homologous cysteine rich domains (CRD1 and CRD2). These CRDs are also referred to individually as C1A and C1B.

In 1995, we demonstrated that a machine synthesized 51-mer corresponding to PKC-$\gamma_{101–151}$, (i.e. PKC-γ C1B), binds phorbol esters with affinities and phospholipid requirements comparable to the natural protein.[3] These studies have more recently been extended to include the synthesis and [^{3}H]-phorbol 12,13 dibutyrate ([^{3}H]-PDBu) binding assay of both CRDs of all conventional and novel PKCs.[4] Intriguingly, in all but one of the isozymes, only the second CRD binds phorbol esters with high affinity. In the exception, PKC-γ, the first and second CRDs bind phorbol esters with comparably high affinities.[5] This finding provides the conceptual basis for the design of a new class of potentially isozyme selective agents in which two binding subunits would be linked together with a tether of appropriate length and composition. The two subunits could thus bind to individual CRDs or to one CRD and a secondary site. To the extent that the CRDs or secondary binding sites differ among the isozymes, isozyme selective binding would be possible. Our interest in this approach to PKC regulation was further stimulated by studies on a PKC-γ knockout mouse which suggest a crucial role for this isozyme in mediating neuropathic pain.[6]

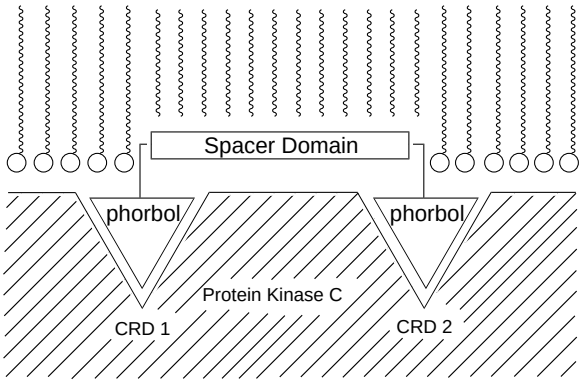
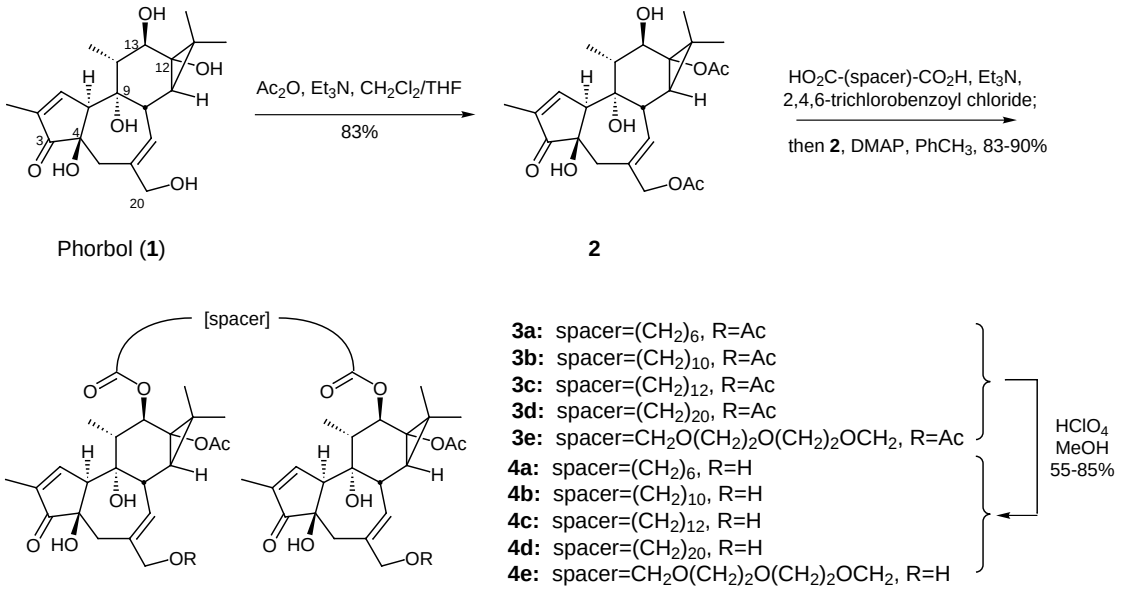


Figure 1 Design Concept for Isozyme Selective Compounds. Two separate ligands for CRD1 and CRD2 are covalently linked through a tether which serves to properly position the ligands relative to the CRDs (or secondary binding sites) as well as to the membrane.

The goals of the initial study reported herein were to synthesize a series of dimeric PKC activators and to determine if they bound and activated PKC in a fashion different from the corresponding monomeric phorbol esters as a prelude to and screen for more complex isozyme selective functional assays. Initially, it was decided to examine ligands which potently bind PKC as monomers to better enable us to detect weak binders as we refined our designs. With this in mind, we selected the phorbol esters, which are known to be nanomolar binders of the CRDs, and additionally have several functional groups which could serve to link two monomers together. It should be noted that this concept can also be generally applied to other PKC activators to produce homodimeric (e.g. DAG-linker-DAG) or heterodimeric (e.g. DAG-linker-phorbol) conjugates. [7]



Scheme Synthesis of phorbol ester dimers **4a–e** using a modular synthetic approach.

We previously reported a comprehensive analysis of the structural features of phorbol esters that are required for recognition at the PKC regulatory domain. According to this analysis, the heteroatom triad whose spatial orientations correspond best with PKC binding are the oxygens at C4 (or C3), C9, and C20 as well as a lipophilic domain at C12 which is proposed to associate with the membrane.[8] Based on this model, we sought to link two phorbol moieties by esterification at the C12 position of phorbol thereby introducing the crucial hydrophobic region at C12 while leaving the putative pharmacophoric atoms free to interact with the protein. This modular approach provides dimeric activators wherein all three functional domains, the two CRD binding ligands and the spacer connecting them, can be readily varied. Our initial synthetic efforts were directed at the incorporation of linkers of different length, while using phorbol esters on both sides as our CRD binding domains (Scheme).

Phorbol possesses five hydroxyl groups which can be selectively modified by careful choice of reaction conditions.[9] The allylic hydroxyl at C20 and the tertiary C13 hydroxyl group typically show the highest level of reactivity, allowing for their direct conversion of phorbol to the 13,20-bisacetate **2** by exposure to acetic anhydride and triethylamine. The hydroxyl positioned at C12 is sufficiently more reactive than the C4 and C9 hydroxyl groups that dimer formation would be expected to occur at the C12 position without further protection of the diacetate **2**.

Several methods for diester formation were examined including the use of diacids activated with DCC or 2-iodopyridinium salts, as well as direct condensation with the corresponding diacid chlorides. Although these methods did deliver the dimeric phorbol esters, the reactions were somewhat capricious and typically yielded the dimers in only moderate yields (40–50%). However, conversion of the diacid to the bis-mixed anhydride by the method of Yamaguchi[10] and direct exposure to the 13,20-diacetate routinely provided the dimers **3a–e** in excellent yields (83–90%). This dimerization protocol is general and several different types of linkers have been successfully employed including those containing rigid groups or heteroatom functionality in the tether.

Final deblocking of the C20 acetate group is then required to generate the desired phorbol dimers. The high inherent reactivity of this position allows application of an acid catalyzed transesterification ($HClO_4$/MeOH) to deliver the unprotected dimers in just three steps overall from phorbol. The resultant first generation dimers are pseudo-C2 symmetric dimers in which both binding units are identical. However, this synthetic scheme is easily extended to allow for the linking of different activators at the ends of the tether.

As a first test of whether these dimers would exhibit novel activities, we elected to examine their affinity in a conventional binding assay based on the inhibition of [^{3}H]-PDBu binding to a PKC isozyme mixture isolated from a rat brain homogenate.[11] In general, those dimers with largely lipophilic linkers including polymethylene chains, alkynes, or aromatic rings appeared highly active in preliminary screens yielding apparent inhibition constants in the low nanomolar region. Interestingly, compound **4e**, incorporating a glycol based tether, failed to displace PDBu at concentrations as high as 100 nM in these screens. This is consistent with the tether subunit serving as a lipophilic anchor, facilitating binding of the phorbol domains at either end.

The simplest polymethylene derivatives **4a–d** exhibited the most potent binding, and they were thus selected for further evaluation. Standard competition binding assays employing [^{3}H]-phorbol-12,13-dibutyrate as the reporter

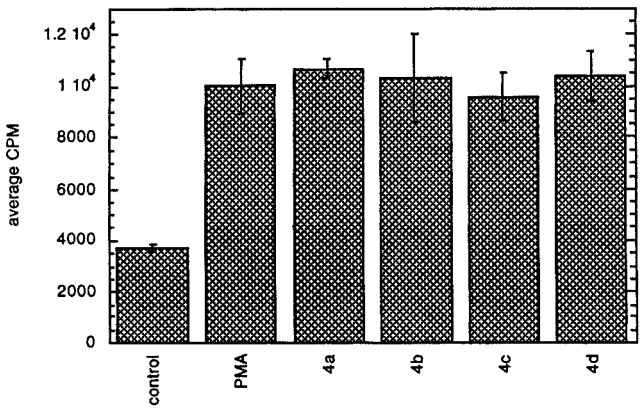


Figure 2 Incorporation of ^{32}P into PKC substrate $MBP_{(4–14)}$ in the presence of 0.1 mM Ca^{2+}, PS and 100 nM dimers **4a–d**

molecule[12] yielded dose-response curves for the 6 and 20 methylene derivatives with K_i values in the low nanomolar region. However, dose-response curves generated for the 10 and 12 methylene linkers produced data which, despite good internal consistency, did not fit satisfactorily to the theoretical curves for competitive binding, especially at very low concentrations of inhibitor. In addition, it was found that the observed IC_{50} values for these two dimers was dependent on the concentration of PKC employed in the assay.

Both of these observations are consistent with the occurrence of ligand depletion, which arises when high affinity ligands, which must be tested at extremely low concentrations, are used at concentrations so low that a significant portion of that ligand is sequestered by its receptor in the assay. As a result, the assumption that the amount of free ligand in the assay solution is equal to the amount added breaks down.[13] Ligand depletion can be suppressed by minimizing the concentration of the receptor in the assay system, and so the binding assays were performed with the lowest concentration of PKC possible while maintaining good signal to noise ratios. Binding assays conducted under these optimized conditions delivered K_i values in the picomolar region for these designed activators. Competition binding assays resulted in values of 4.5 nM, 0.1 nM, 0.4 nM and 2.7 nM for dimers **4a**, **4b**, **4c** and **4d** respectively (Table). Under identical assay conditions, the K_d for PDBu was determined to be 1.31 ± 0.24 nM.

These studies provide several important findings. First, they demonstrate that phorbol esters dimerized through esterification at their C12 positions are able to competitively bind to protein kinase C. Indeed, these are among the tightest binders of PKC measured.[14] Second, a significant relationship was observed between the length of the tether employed and the binding constant that pointed to an optimal length of 10–12 methylene groups. The difference of just 4 methylenes from dimer **4a** to dimer **4b** increased the binding affinity 45 fold, suggesting that a second binding site is being accessed.

An important issue related to this design is whether the binding of these high affinity ligands actually results in the activation or inhibition of PKC. To address this point, a standard assay for enzymatic activity involving the incorporation of a radiolabeled phosphate onto a serine/threonine residue of a suitable substrate was conducted.[15] Under standard conditions and concentrations identical to those used with phorbol-12-myristate-13-acetate, the dimers were found to promote phosphorylation of a myelin basic protein peptide fragment, $MBP_{(4–14)}$. This indicates that their binding of PKC was accompanied by a normal catalytic response (Figure 2) and the dimers can be considered as activators of the enzyme.

This study represents the first synthesis and evaluation of a new class of exceptionally potent dimeric activators of PKC based on regulatory domain binding motifs. Preliminary biological studies show that these compounds bind to PKC with affinities varying in a manner dependent on their tether length, and, in two cases, exceeding the affinity of phorbol dibutyrate. They additionally activate the enzyme in a manner comparable to phorbol esters. The modular synthesis can deliver the compounds quickly while retaining a high degree of efficiency, as is required for combinatorial synthesis. The relationship between tether length and PKC affinity as well as the molecules' extreme potency suggest that these compounds are engaging multiple binding sites on the enzyme. Synthesis of this type of dimeric compound therefore provides a method for mapping distances between binding sites. It is not yet clear whether the enhanced binding is attributable to association of the phorbol ester subunits of the dimer with the C1A and C1B subdomains or with one of these and an as yet unrecognized region of PKC. However, to the extent that these sites differ in distance or affinity in the different isozymes, this study provides the basis for a rational approach to the design of PKC isozyme selective binders. Efforts to differentiate these possibilities and to determine the isozyme binding affinities of these new ligands are in progress.

Table 1 Inhibition Constants for the Binding of Phorbol Ester Dimers to Rat Brain PKC

Dimer	n	K_i (nM) ± SD
4a	6	4.5 ± 0.1
4b	10	0.1 ± 0.06
4c	12	0.4 ± 0.07
4d	20	2.7 ± 0.02

Oxygen- and moisture-sensitive reactions were carried out in oven dried flasks sealed with rubber septa under a positive pressure of dry N_2 or Ar. Correspondingly sensitive liquids were transferred via syringe or cannula through rubber septa. Organic solutions were concentrated on a Büchi rotary evaporator connected to a ChemGlass diaphragm pump, followed by exposure to high vacuum (<1 torr) for at least 15 minutes.

All commercially available reagents were used without further purification unless otherwise noted. Et_2O and THF were distilled from

Na–benzophenone ketyl prior to use. MeCN, CH_2Cl_2, diisopropylamine, Et_3N and pyridine were distilled from CaH_2. Toluene was distilled over sodium metal. Flash chromatography grade hexanes were distilled from technical grade hexanes.

Phorbol 13,20-Diacetate 2 (89%)

To a stirred solution of phorbol•EtOH (50.0 mg, 0.122 mmol) in CH_2Cl_2 (1 mL) and THF (1 mL) under N_2 was added Et_3N (230 µL, 1.65 mmol) followed by Ac_2O (0.15 ml, 1.59 mmol) and the solution was allowed to stir for 8 h. The reaction was concentrated under reduced pressure to give a dark orange oil which was immediately chromatographed (1:1 hexane: EtOAc) to give the 13, 20 diacetate **2** as an amorphous white solid (89%).

^{1}H NMR ($CDCl_3$): δ = 7.58 (s, 1H), 5.7 (d, 1H, *J* = 4.6 Hz), 4.5 (dd, 2H, *J* = 4.5, 12 Hz), 3.9 (dd, 1H, *J* = 3.6, 9.5), 3.21 (app t, 1H, *J* = 5 Hz), 3.14 (br s, 1H), 2.85 (s, 1H), 2,7 (s, 1H), 2.4 (dd, 2H, *J* = 18, 6 Hz), 2.1 (s, 3H), 2.0 (m, 1H), 1.6 (br s, 1H), 1.2 (s, 3H), 1.2 (m, 4H).

^{13}C ($CDCl_3$): δ = 208, 174.3, 171.1, 160.5, 136.2, 133.1, 132.5, 78.3, 77.3, 73.2, 69.4, 68.2, 56.5, 45.1, 39.2, 38.9, 35.1, 25.5, 23.4, 21, 20.7, 16.5, 14.9, 10.1.

Dimer Formation; General Procedure

To a solution of Et_3N (4 equivalents) in toluene (1mL/12 mg diacetate) was added the diacid (0.6 equivalents) and the resulting solution was allowed to stir until it became homogenous. At that time, 2,4,6-trichlorobenzoyl chloride (1.4 equivalents) was added dropwise via syringe and the solution was allowed to stir for 1 h at r.t. over which time it took on a light yellow color. A solution of the diacetate (1 equivalent) in toluene (1 mL/12 mg diacetate) was prepared and to this was added DMAP (1:1 (w/w) with the diacetate) and a few microliters of CH_2Cl_2 to dissolve the diacetate. This solution was then added dropwise via syringe to the preformed anhydride and the solution became highly turbid. The reaction was monitored by TLC and was typically complete within 1 h. Upon completion, the solution was applied directly to a column of silica gel and the column was eluted with hexane: EtOAc (1:1) and the product containing fractions concentrated to give the dimer (85–95%) as either a colorless oil or a foam.

C8 Dimer-20 Acetate 3a

From suberic acid and phorbol 13,20-diacetate, (87%)

^{1}H NMR (500 MHz $CDCl_3$): δ = 7.62 (s, 1H), 5.72 (d, 1H, *J* =4.1 Hz), 5.56 (br s, 1H), 5.42 (d, 1H, *J* =10.5 Hz), 4.48 (ab quartet, 2H, *J* =12.5 Hz), 3.25 (m, 2H), 2.52 (ab quartet, *J* =19 Hz), 2.3 (8 lines, 2H), 2.11 (s, 3H), 2.08 (s, 3H), 1.81 (s, 3H), 1.6 (m, 2H), 1.38 (m, 1H),1.24 (s, 3H), 1.23 (s, 3H), 1.38 (m, 2H), 1.09 (d, *J* =5.5 Hz,1H), 0.9 (m, 4H). ^{13}C (125 MHz $CDCl_3$): δ = 209.4, 174.4, 174.1, 171.5, 161.4, 136.3, 133.6, 133.3, 78.7, 77.3, 74.2, 70.1, 66.2, 56.7, 43.6, 40, 39.6, 36.8, 35.1, 29.3, 26.3, 25.6, 24.6, 21.8, 21.7, 17.4, 15.1, 10.8.

IR(neat): ν = 3403(m), 2929(m), 1723(s), 1628(w), 1376(s), 1260(s).

HRMS (FAB) Calcd for $C_{56}H_{74}O_{18}Na$, 1057.4772; Found, 1057.4787, TLC R_f=0.17 (hexane/EtOAc: 1/1)

C12- Dimer-20 Acetate 3b

From 1,12-dodecanedioic acid and phorbol 13,20-diacetate, (85%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.58 (s, 1H), 5.69 (d, 1H, *J* =4 Hz), 5.51 (br s, 1H), 5.38 (d, 1H, *J* =10.5 Hz), 4.43 (ab quartet, 2H, *J* =12.5 Hz), 3.23 (m, 2H), 2.5 (ab quartet, 2H, *J* =19.2 Hz), 2.29 (8 lines, 2H), 2.14 (m, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 1.76 (m, 3H), 1.6 (m, 7H), 1.31 (m, 1H), 1.28 (m, 3H), 1.26 (s, 3H), 1.19 (s, 3H), 1.04 (d, *J* =5.2 Hz,1H), 0.9 (m, 2H).

^{13}C (125 MHz, $CDCl_3$): δ = 208.7, 173.7, 173.6, 170.7, 160.8, 135.5, 132.8, 132.6, 78.1, 76.5, 73.5, 69.4, 65.5, 56, 42.9, 39.3, 38.9, 34.5, 29.4, 29.2, 28.9, 25.6, 25.1, 23.8, 21.1, 20.9, 16.7, 14.4, 10.1.

IR(neat): ν = 3405(m), 2931(m), 1724(s), 1626(w), 1376(s), 1260(s).

HRMS (FAB) Calcd for $C_{60}H_{82}O_{18}Na$, 1113.5398; found, 1113.5456, TLC R_f = 0.26 (hexane/EtOAc: 1/1)

C14 Dimer-20 Acetate 3c

From 1,14 tetradecanedioic acid diacid and phorbol 13,20-diacetate, (90%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.63 (m, 1H), 5.74 (d, 1H, *J* =4 Hz), 5.56 (br s, 1H), 5.43 (d, 1H, *J* =10.5 Hz), 4.48 (ab quartet, 2H, *J* =12.5 Hz), 3.27 (m, 2H), 2.5 (ab quartet, 2H, *J* =19 Hz), 2.4 (8 lines, 2H), 2.14 (m, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 1.81 (m, 3H), 1.6 (m, 6H), 1.31 (m, 1H), 1.28 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H), 1.09 (d, *J* =5 Hz,1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 208.7, 173.7, 173.6, 170.7, 160.8, 135.5, 132.8, 132.6, 78.1, 76.5, 73.5, 69.4, 65.5, 56, 42.8, 39.3, 38.9, 36.6, 36, 34.5, 29.4, 29.2, 28.9, 25.6, 25.1, 23.8, 21.1, 20.9, 16.7, 14.4, 10.1.

IR(neat): ν = 3403(m), 2926(m), 2855(w),1728(s), 1628(w), 1377(m), 1260(s).

HRMS (FAB) Calcd for $C_{62}H_{86}O_{18}Na$, 1141.5712; found, 1141.5671, TLC R_f = 0.26 (hexane/EtOAc: 1/1)

C22 Dimer-20 acetate 3d

From 1,22-docosanedioic acid and phorbol 13,20-diacetate, (83%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.63 (m, 1H), 5.74 (d, 1H, *J* = 4 Hz), 5.57 (br s, 1H), 5.43 (d, 1H, *J* = 10 Hz), 4.48 (ab quartet, 2H, *J* = 12.5 Hz), 3.28 (m, 2H), 2.51 (ab quartet, 2H, *J* = 19 Hz), 2.34 (8 lines, 2H), 2.14 (m, 1H), 2.11 (s, 3H), 2.08 (s, 3H), 1.81 (m, 3H), 1.6 (m, 14H), 1.31 (m, 1H), 1.28 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H), 1.09 (d, *J* = 5 Hz, 1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 208.7, 173.7, 173.6, 170.7, 160.8, 135.5, 132.8, 132.7, 78.1, 76.4, 73.5, 69.4, 65.5, 56.1, 42.9, 39.3, 38.9, 36, 34.6, 29.7, 29.67, 29.65, 29.4, 29.3, 29, 25.6, 25.1, 23.8, 21.1, 20.9, 16.7, 14.4, 10.1.

IR(neat): ν = 3408(m), 2925(m), 2854(m), 1731(s), 1377(s), 1260(s).

HRMS (FAB) Calcd for $C_{70}H_{102}O_{18}Na$, 1253.6963; found, 1253.7000, TLC R_f = 0.35 (hexane/EtOAc: 1/1)

PEG-Dimer-C20 acetate 3e

From the PEG diacid chloride and phorbol 13,20-diacetate, (54%)

To a solution of phorbol 13,20 diacetate (3.0 mg. 6.7µM) in 600 µL of CH_2Cl_2 was added a solution of the bis-acid chloride (0.8 mg in 80 µL of CH_2Cl_2) followed by a small quantity of DMAP. The solution was allowed to stir under N_2 at r.t. for 5h and then quenched by the addition of sat. $NaHCO_3$. The layers were separated and the aqueous layer extracted (3 x 5 mL) with CH_2Cl_2 and the organic layer concentrated. Chromatography of the resultant oil (50% EtOAc:hexane to 70% EtOAc:hexane) to give the dimer (2.0 mg, 54%).

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.58 (m, 1H), 5.685 (d, 1H, *J* = 5 Hz), 5.5 (br s, 1H), 5.48 (d, 1H, *J* = 10.5 Hz), 4.4–4.0 (m, 4H), 3.79 (s, 2H), 3.55 (m, 4H), 3.27 (m, 2H), 2.5 (ab quartet, 2H, *J* = 20 Hz), 2.2 (m, 1H), 2.14 (s, 3H), 2.07 (s, 3H), 1.79 (m, 3H), 1.6–1.5 (m, 4H), 1.31–1.21 (m, 4H), 1.1 (d, *J* = 5 Hz,1H), 0.9 (m, 1H).

^{13}C (125 MHz, $CDCl_3$): δ = 209.4, 174.4, 171.5, 171.1, 161.1, 136.5, 133.7, 133.1, 78.7, 74.1, 71.6, 71.6, 70, 69.2, 66, 64.4, 56.7, 54.1, 43.5, 39.9,36.9, 30.4, 26.6, 24.5, 21.8, 21.7, 17.4, 15.1, 10.8.

IR(neat): ν = 3403(m), 2926(m), 1722(s), 1629(m), 1458(w), 1378(s), 1260(s).

HRMS (FAB) Calcd for $C_{56}H_{74}O_{21}$, 1082.4824; found, 1082.4723, TLC R_f = 0.25 (hexane/EtOAc: 1/1)

C20 Deprotection; General Procedure

To a solution of the dimer in MeOH (3.2 mL / 10 mg of dimer) was added a solution of perchloric acid in MeOH (prepared by adding 28 µL of concentrated perchloric acid to 10 mL of MeOH) in one portion (3.2 mL/ 10 mg dimer) and the flask was wrapped in aluminum foil and was allowed to stir under a positive pressure of N_2 for 48 h. Upon completion, a small quantity of solid $NaHCO_3$ was added and allowed to stir for 1 h. The solution was then filtered through a 1 inch pad of silica gel and the filter cake was washed repeatedly (5–6 times) with EtOAc. The solution was concentrated to give a white residue which was purified by silica gel chromatography (EtOAc as eluent) to give the dimer (65–75%) as a colorless oil or as a foam.

C8 Dimer 4a
(85%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.61 (s, 1H), 5.7 (d, 1H, *J* = 5 Hz), 5.57 (br s, 1H), 5.41 (d, 1H, *J* = 10 Hz), 4.04 (ab quartet, 2H, *J* = 12.5 Hz), 3.26 (m, 2H), 2.54 (ab quartet, 2H, *J* = 19 Hz), 2.35 (8 lines, 2H), 2.11 (s, 3H), 1.81 (d, *J* = 1.5 Hz, 3H), 1.6 (m, 2H), 1.38 (m, 1H),1.27 (s, 3H), 1.23 (s, 3H), 1.22 (s, 3H), 1.09 (d, *J* = 5 Hz,1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 208.9, 173.7, 173.4, 160.7, 140.5, 132.8, 129, 78.1, 73.7, 67.9, 65.6, 56, 42.9, 38.9, 39.5, 36.2, 34.5, 29.7, 28.6, 25.6, 24.8, 23.8, 21.1, 16.8, 14.4, 10.1.

IR(neat): ν = 3396(m), 2925(m), 1716(s), 1628(w), 1377(s), 1261(s).

HRMS (FAB) Calcd for $C_{52}H_{70}O_{16}Na$, 973.4561; found, 973.4601, TLC R_f = 0.26 (100% EtOAc).

C12 Dimer 4b
(59%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.56 (s, 1H), 5.651 (d, 1H, *J* =4.8 Hz), 5.6 (br s, 1H), 5.52 (d, 1H, *J* =10.5 Hz), 4.0 (ab quartet, 2H, *J* =13.1 Hz), 3.22 (m, 2H), 2.49 (ab quartet, 2H, *J* =18.5 Hz), 2.43 (br s, 1H), 2.31 (m, 2H), 2.13 (m, 1H), 2.07 (s, 3H), 1.74 (m, 3H), 1.53 (m, 6H), 1.31 (m, 1H), 1.27 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H), 1.06 (d, *J* =5 Hz,1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 209, 173.8, 173.7, 160.7, 140.5, 132.8, 129.1, 78.2, 76.4, 73.7, 68, 65.5, 56, 42.8, 38.9, 38.5, 36.2, 34.6, 29.3, 29.1, 28.9, 25.6, 25.1, 23.8, 21.1, 16.8, 14.4, 10.1.

IR(neat): ν = 3396(m), 2925(m), 1716(s), 1628(w), 1377(s), 1261(s).

HRMS (FAB) Calcd for C56H78O16Na1029.5187; found, 1029.5227, TLC Rf=0.41 (100% EtOAc).

C14 Dimer 4c
(78%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.6 (s, 1H), 5.71 (d, 1H, *J* = 5 Hz), 5.6 (br s, 1H), 5.42 (d, 1H, *J* = 10.5 Hz), 4.05 (ab quartet, 2H, *J* = 13 Hz), 3.26 (m, 2H), 2.5 (ab quartet, 2H, *J* = 19 Hz), 2.64 (br s, 1H), 2.35 (m, 2H), 2.17 (m, 1H), 2.11 (s, 3H), 1.78 (d, 3H, *J* = 1.5 Hz), 1.6 (m, 6H), 1.31 (m, 1H), 1.28 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H), 1.1 (d, *J* = 5 Hz 1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 209, 173.8, 173.7, 160.8, 140.6, 132.8, 129.1, 78.2, 76.5, 73.6, 68, 65.6, 56, 42.8, 38.9, 38.4, 36.1, 34.6, 29.6, 29.5, 29.2, 28.9, 25.6, 25.1, 23.8, 21.1, 16.7, 14.4, 10.1

IR(neat): ν = 3405(m), 2925(m), 1717(s), 1457(w), 1376(s), 1261(s).

HRMS (FAB) Calcd for $C_{58}H_{82}O_{16}Na$, 1057.5500; found, 1057.5406, TLC R_f = 0.42 (100% EtOAc).

C22 Dimer 4d
(79%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.6 (s, 1H), 5.71 (d, 1H, *J* =4 Hz), 5.59 (br s, 1H), 5.43 (d, 1H, *J* =10 Hz), 4.03 (ab quartet, 2H, *J* =13 Hz), 3.27 (m, 2H), 2.55 (ab quartet, 2H, *J* =18.5 Hz), 2.34 (6 lines, 2H), 2.17 (m, 1H), 2.11 (s, 3H), 1.81 (m, 3H), 1.6 (m, 14H), 1.31 (m, 1H), 1.28 (m, 3H), 1.26 (s, 3H), 1.23 (s, 3H),.91 (d, *J* =5 Hz,1H), 0.9 (m, 4H).

^{13}C (125 MHz, $CDCl_3$): δ = 209.7, 174.5, 174.4, 161.5, 141.2, 133.6, 129.8, 78.9, 77.2, 74.4, 68.7, 66.3, 56.8, 43.5, 39.7, 39.4, 39.2, 36.9, 35.3, 30.36, 30.31, 30.3, 29.9, 29.7, 26.3, 25.9, 24.5, 21.8, 20.9, 17.5, 15.1, 10.8.

IR(neat): ν = 3404(m), 2925(m), 1716(s), 1628(w), 1377(s), 1262(s).

HRMS (FAB) Calcd for $C_{66}H_{98}O_{16}Na$, 1169.6752; found, 1169.6703 TLC R_f = 0.63 (100% EtOAc).

PEG-Dimer 4e
(55%)

^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.58 (m, 1H), 5.685 (d, 1H, *J* = 4.5 Hz), 5.57 (br s, 1H), 5.48 (d, 1H, *J* =10.5 Hz), 4.4–4.0 (m, 4H), 3.79 (s, 2H), 3.55 (m, 4H), 3.27 (m, 2H), 2.5 (ab quartet, 2H, *J* = 20 Hz), 2.2 (m, 1H), 2.14 (s, 3H),1.79 (m, 3H), 1.6–1.5 (m, 4H), 1.31–1.21 (m, 4H), 1.1 (d, *J* = 5 Hz, 1H), 0.9 (m, 1H).

^{13}C (125 MHz, $CDCl_3$): δ = 209.4, 174.6, 171.4, 161, 141.4, 131.6, 129.5, 78.8, 77.1, 75.7, 75.6, 74.3, 69.1, 68.6, 56.8, 56.7, 43.6, 39.7,39.2, 37.1, 23.6, 21.8, 17.4, 15.1, 10.8.

IR(neat): ν = 3412(m), 2932(m), 1718(s), 1629(m), 1438(w), 1377(s), 1261(s).

HRMS (FAB) Calcd for $C_{52}H_{70}O_{19}$, 1021.4409; found, 1021.4409, TLC R_f = 0.28 (100% EtOAc).

PKC Binding Assay Protocol
A. Preparation
Filters (30–35) were prepared by soaking for 30 min in a solution obtained by dissolving 3 mL of 10% polyethyleneimine in 100 mL of distilled water and swirling periodically.

Wash buffer was prepared by diluting 20 mL of 1 M Tris pH=7.4 to a volume of 500 mL and then cooling the resultant solution on ice for at least 30 min.

Phosphatidyl serine was prepared by concentrating 400 µL of 10 (mg/ml) PS (Avanti Polar Lipids) in $CHCl_3$ with a stream of N_2, then suspending it in 4 mL of Tris buffer followed by sonication at 0 °C (4 x 30 sec; 30 sec on then 30 sec off; power = 6; 40% duty cycle).

PKC (rat brain, purified by DEAE-cellulose chromatography) is prepared by dilution of the stock solution by adding 50 µL of stock to 10 mL of PKC buffer. This buffer was prepared from 1 mL of 1 M Tris 7.4, 2 mL of 1 M KCl, 30 µL of 0.1 M $CaCl_2$, 40 mg of BSA, and dilution to 20 mL total volume.

The compound to be assayed was diluted to the desired concentrations in absolute EtOH.

B. PKC Binding Assay
Each data point is obtained in triplicate and, in addition, a zero point, where no competitor was added, and a non-specific point, where all specific binding was eliminated through addition of excess phorbol myristate acetate, are also acquired.

60 µL of PS vesicles, 200 µL of diluted PKC, and 20 µL total volume of the compound to be assayed are added to an Eppendorf tube (1.5 mL**)**. The tubes used to measure non-specific binding receive 5 µL of a 1 mM PMA solution (in 5% DMSO/H_2O). To the solution was added 20 µL of tritiated PDBu (30 nM, final concentration = 2

nM, DuPont NEN) and each tube was momentarily vortexed to obtain good mixing. The samples are then incubated for 30 min at 37° C and the reaction was stopped by cooling on ice for at least 5 min. The contents of each tube was then filtered by applying the mixture uniformly to the filter disk that is placed on a Hirsch funnel. The tube was rinsed with 500 µL of ice-cold 20 mM Tris•HCl, pH 7.4 buffer and the wash was applied to the filter disk. The disk was then washed by slowly dripping 5 mL of the ice-cold buffer solution onto the filter disk. The disk was then placed in a scintillation vial and 3 mL of CytoScint fluid was added. The filteres were soaked for at least 4 h and were then quantitated in a scintillation counter. The data were then processed by averaging the three points, plotted and the theoretical curve fitted to the data using the standard non-linear regression algorithm in the KaleidaGraph v 3.0 package running on a Macintosh system. The following equation is used to fit the curve:

$$IC(50) = \quad B = B_{NS}+(B_{max}-B_{NS}) \ / \ 1+10^{(\log(dimer))-\log(IC50)}$$

B = avg CPM at concentration (dimer)

B_{NS} = CPM at saturating concentration of dimer (variable)

B_{max} = CPM when [dimer]=0

[dimer] = dimer concentration

IC50 = concentration of dimer at which 50% inhibition is observed

The K_i value was obtained form the IC_{50} as follows:

$$K_i = IC_{50} \ / \ 1+\{[PDBu]/K_d(PDBu)\}$$

Kinase Assay Protocol

The following stock solutions were required for the kinase assay:

1) Lipid was prepared by placing 12 µL phosphatidyl serine in $CHCl_3$ (Avanti Polar Lipids, 10 mg/ml), and 24 µL phosphatidyl choline in $CHCl_3$ (Avanti Polar Lipids, 20 mg/ml) in a glass test tube. A stream of dry N_2 was directed over the solutions until the organic solvents were removed. The remaining lipids were then suspended in 1 ml of 20mM Tris, pH 7.4 by vigorously pipetting the solution. This suspension was then sonicated at 0 °C (4 x 30 sec; 30 sec on then 30 sec off; power = 6; 40% duty cycle).

2) Substrate solution was prepared by mixing MBP^{4-14} (Sigma), 750 µL; 10 mM $CaCl_2$, 150 µL; and 100 µL of 20 mM Tris, pH 7.4.

3) The ATP solution is prepared with 75 µL of 1 M $MgCl_2$, 25 µL 3.75 µM ATP, 1.5 ml 20mM Tris, pH 7.4, and 1.5 µL γ^{32}P ATP.

The assay was performed in a Nunc V96 polypropylene 96 well plate designed to prevent protein adhesion to its surface. Into each well is placed 15 µL of the lipid vesicles, 5 µL of substrate solution, 20 µL of test compound, PKC solution (10 µL, 1:3 dilution form rat brain prep), and ATP solution (25 µL). The reactions were incubated at r.t. for 30 min and then stopped by the addition of 100 µL of 100 mM H_3PO_4. 150 µL of each stopped reaction is removed and blotted on 1 cm^2 Whatman P81 cation exchange paper. After air drying, the filters were washed three times with deionized water and once with absolute EtOH. The papers were then again allowed to air dry before being placed in a scintillation vial without scintillation fluid and counted.

Acknowledgment

We thank Professor Daria Mochly-Rosen for providing laboratory facilities for and invaluable assistance in performing our binding and kinase assays. Support of this work through a grant (CA31841) provided by the National Institutes of Health and a grant-in-aid for scientific research from the Ministry of Education, Science, Culture and Sports, Japan (K.I.) is also gratefully acknowledged. HRMS analyses were performed at the UC Riverside Mass Spectrometry Facility. Fellowship support from the Eli Lilly Corporation (M.F.T.K.) is gratefully recognized.

References

(1) Nishizuka, Y. *Science* **1986**, *233*, 305.
(2) Dekker, L. V.; Parker, P. J. *Trends Biochem. Sci.* **1994**, *19*, 73.
(3) Wender, P. A.; Irie, K.; Miller, B.L. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 239.
(4) Irie, K.; Oie, K.; Nakahara, A.; Yanai, Y.; Ohigashi, H.; Wender, P. A.; Fukuda, H.; Konishi, H.; Kikkawa, U. *J. Am. Chem. Soc.* **1998**, *120*, 9159.
(5) Quest, A. F.; Bell, R.M. *J. Biol. Chem.* **1991**, *266*, 18330.
Irie, K.; Yanai, Y.; Oie, K.; Ishizawa, J.; Nakagawa, Y.; Ohigashi, H.; Wender, P. A.; Kikkawa, U. *Bioorg. & Med. Chem.* **1997**, *5*, 1725.
(6) Malmberg, A. B.; Chen, C.; Tonegawa, S.; Basbaum, A.I. *Science* **1997**, *278*, 279.
(7) During the course of our investigations, the following example of these homodimeric species appeared in the literature:
Sodeoka, M.; Arai, M. A.; Adachi, K.; Uotsu, K.; Shibasaki, M. *J. Am. Chem. Soc.* **1998**, *120*, 457.
(8) Wender, P. A.; Koehler, K.F.; Sharkey, N. A.; Dell'Aquila, M.L.; Blumber, P. M. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 4214.
Wender, P. A.; Cribbs, C. M. *Adv. Med. Chem.* **1992**, *1*, 1.
(9) Hecker, E.; Schmidt, R. *Fortschr. Chem. Org. Naturst.* **1974**, *31*, 377.
(10) Yamaguchi, M. *J. Synth. Org. Chem. Jpn.* **1980**, ***38***, 22.
(11) Bollag, G. E.; Roth, R. A.; Beaudoin, J.; Mochly-Rosen, D.; Koshland, D.E. *Proc. Natl. Acad. Sci. USA* **1986**, *83*, 5822.
(12) Wender, P. A.; DeBranbander, J.; Harran, P. G.; Jimenez, J.-M.; Koehler. M.F.T.; Lippa, B.; Park, C.-M.; Siedenbiedel, C.; Pettit, G.R. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6624.
(13) Goldstein, A.; Barrett, R. W. *Mol. Pharm.* **1987**, *31*, 603.
(14) Sharkey, N. A.; Blumberg, P.M. *Carcinogenesis* **1986**, *7*, 677.
Sharkey, N. A.; Blumberg, P.M. *Cancer Res.* **1985**, *45*, 19.
(15) Epand, R. M. *Analytical Biochemistry* **1994**, *218*, 241.

Article Identifier:
1437-210X,E;1999,0,SI,1401,1406,ftx,en;xC02299SS.pdf