NOVEL PHORBOL ANALOGS WHICH BIND TO PROTEIN KINASE C (PKC) WITHOUT ACTIVATION

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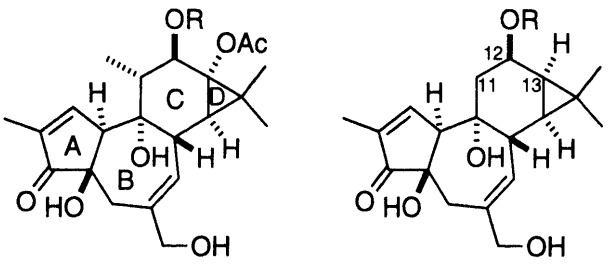
13-Deacetoxy-11-demethyl-phorbol derivatives with acyl groups of various lengths (from hexanoyl to tetradecanoyl) at the C-12 position were synthesized in an optically active form. Although considerable binding affinities to PKC were observed by analogs **3**-**7**, no activation of PKC was seen even at 10 μM.

KEY WORDS phorbol; PKC activation; PKC inhibitor; PMA analog

Protein kinase C (PKC), a serine/threonine phosphorylating enzyme, plays a vital role in cell metabolism and growth.[1] In addition, PKC is widely recognized as a target for phorbol 12-myristate 13-acetate (PMA; **1**) -type tumor promoters. Therefore, modulation of PKC by chemical agents is considered a standard method for examining the mechanisms of signal transduction and tumor promotion. PKC comprises a family of at least 11 closely related isozymes, which are thought to mediate separate biological effects in cells. Hence, PKC isozyme-specific modulators are essential for investigating the biological behavior of PKC. Computational modeling of the structure-activity relationship of PMA-type tumor promoters, such as ingenol derivatives, teleocidins, and aplysiatoxins, has been performed by many scientists.[2] For PMA, most of the proposed pharmacophores for PKC activation have been oxygen substituents at A,B-ring systems and a hydrophobic side chain at C-12. However, no experimental proof has been given. To clarify the pharmacophores of PMA and to develop selective PKC modulators, we have been studying the synthesis of structurally simplified phorbol analogs. Recently, we have successfully synthesized several phorbol analogs, including 13-deacetoxy-11-demethyl-PMA (**3**), and evaluated their binding affinity to PKC.[3] Although the affinity of **3** to PKC was somewhat lower than that of parent PMA by about two orders of magnitude, the result suggested that the methyl substituent at C-11 and the acetoxyl group at C-13 are not essential for phorbol analogs to bind with PKC. At this stage, we assumed that this relatively lower PKC-binding activity of **3** may result from the difference in polarity between the synthesized analog and PMA. Therefore, we synthesized several PMA analogs with hydrophobic side chains of various lengths at C-12 by a method similar to that used to synthesize **3** via **2** (Fig. 1).[3a]



1: PMA; R = $CO(CH_2)_{12}CH_3$

2: R = H
3: C-14 analog; R = $CO(CH_2)_{12}CH_3$
4: C-12 analog; R = $CO(CH_2)_{10}CH_3$
5: C-10 analog; R = $CO(CH_2)_8CH_3$
6: C-8 analog; R = $CO(CH_2)_6CH_3$
7: C-6 analog; R = $CO(CH_2)_4CH_3$

Fig. 1. Structure of PMA and Phorbol Analogs

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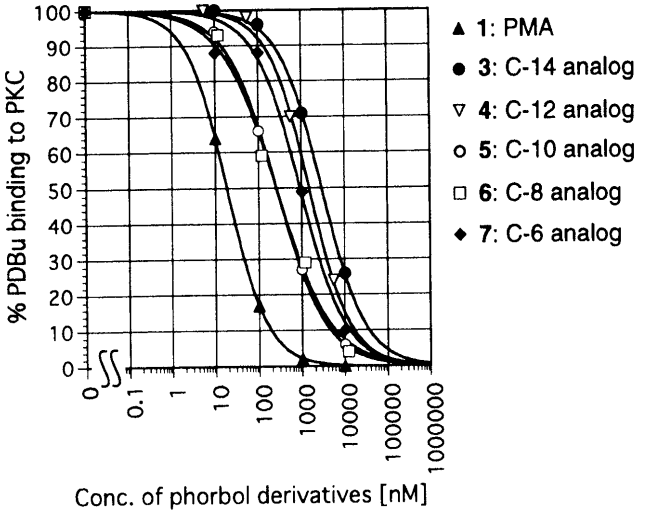
That is, (+)-3-carene was converted to **2** in a highly stereocontrolled manner, and phorbol analogs **3-7** were obtained from the common intermediate **2** in good yields. PKC-binding affinities of these analogs **3-7** were examined by their competitive inhibition of the binding of tritium-labeled phorbol-12,13-dibutyrate ([^{3}H]PDBu) to PKC (Fig. 2).[4,5]

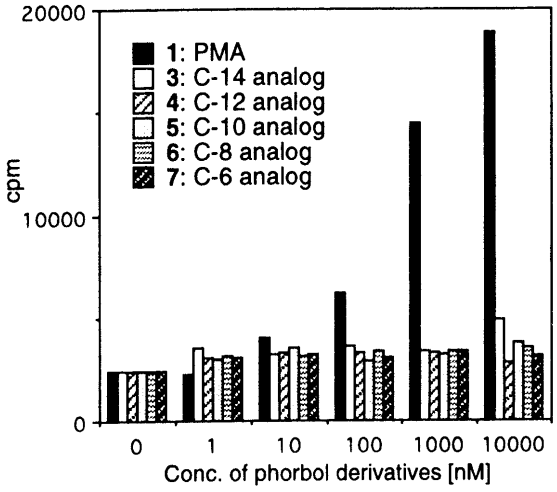


Binding assay conditions:
Various concentrations of phorbol analogs **3-7** were incubated with [^{3}H]PDBu (10 nM) and PKC (0.8 μg/mL) in 300 μL of buffer containing Tris•HCl (50 mM, pH7.5), $CaCl_2$ (4 mM), phosphatidylserine (100 μg/mL), bovine serum albumin (4 mg/mL), and DMSO (0.5%) on ice for 2h, and the PKC-bound [^{3}H]PDBu was counted using a scintillation counter after separating the complex by filtration through a polyethylene-imine-treated glassfiber filter paper. Data are shown as % of the [^{3}H]PDBu binding relative to that without cold competitor.

Fig. 2. Inhibition of [^{3}H]PDBu Binding to PKC by Phorbol Analogs

It is interesting to note that analogs **5** and **6,** which have suitably long side chains, exhibit stronger binding affinity to PKC than analogs **3**, **4**, and **7** (IC_{50}: **1** (PMA); 18 nM, **3**; 2.9 μM, **4**; 1.5 μM, **5**; 260 nM, **6**; 260 nM, **7**; 910 nM).

Having confirmed the validity of the pharmacophore model, we then sought to evaluate PKC activation by phorbol analogs **3-7** based on the incorporation of ^{32}P from [γ-^{32}P]ATP into 11 amino acid residues of the target peptide MBP.[6] Surprisingly, phorbol analogs **3-7** showed no activation under these conditions, even at 10 μM (Fig. 3).



PKC activation assay conditions:
Various concentrations of phorbol derivatives were incubated for 15 min at 25 °C with PKC (3.3 nM), [γ-^{32}P]ATP (50 μM), $CaCl_2$ (100 μM), Peptide MBP_{4-14} (QKRPSQRSKYL, 75 mM), DTT (2.5 mM), $MgCl_2$ (15 mM), and 3% Triton X-100 mixed micelle containing 12 mol% of phosphatidylserine. The reaction was terminated by adding trichloroacetic acid, and the phosphorylated peptide was separated using Whatman P8 binding paper. The radioactivity was measured by a scintillation counter.

Fig. 3. PKC Activation Assay of Phorbol Analogs

Although these synthesized phorbol analogs do not have an oxygen substituent at C-13, TLC analysis showed that they are more polar than PMA itself (*Rf* in TLC; silica-gel, AcOEt as an eluent: PMA; 0.79, **3**; 0.52, **4**; 0.51, **5**; 0.48, **6**; 0.46, **7**; 0.42). This phenomenon may be attributable to the fact that these

analogs lack intramolecular hydrogen bonding between the C-9 hydroxyl group and the C-13 acetoxyl group. Very recently, Zhang *et al.* reported the crystal structure of a complex of a 12-deacyl PMA with the cysteine-rich phorbol-binding domain of PKCδ, in which hydrogen bonding was observed between the C-9 hydroxyl group and the C-13 acetoxy carbonyl.[7] Although it is not yet clear why phorbol analogs **3-7** do not activate PKC, we presume that either the naked hydroxyl group at C-9 in the phorbol analogs affects the induced fit on PKC or the 13-acetoxyl group of PMA plays some role in the activation of PKC.[8] In any case, phorbol analogs **3-7** may act as inhibitors of PKC. Evaluation of the inhibitory activity of phorbol analogs **3-7** and their selectivity for other PKC isozymes is now under investigation.

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