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The stereochemical requirement for protein kinase C activation by 3-methyldiglycerides matches that found in naturally occurring tumor promoters aplysiatoxins

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Protein kinase C is stereospecifically activated by *sn*-1,2-(S)-diglycerides. A second chiral center was introduced into the diglycerides by preparing the 3-methyl derivatives. The activation of protein kinase C was also stereospecific with respect to the new chiral center established at the C3 position of the methylated diglycerides. The stereospecificity of protein kinase C directed towards the C2 and C3 positions of the diglycerides is matched in the analogous C29 and C30 stereocenters of the tumor promoting debromoaplysiatoxins. This finding strengthens the view that the structurally diverse tumor promotors contain the embedded diglyceride-like pharmacophore.

3-Methylglyceride; Glyceride; Aplysiatoxin; Tumor promoter

1. INTRODUCTION

Protein kinase C (PKC) serves as an important control element in cellular function [1]. The enzyme is transiently and physiologically activated by diglycerides, which are generated by hydrolysis of endogenous polyphosphoinositides by a temporally regulated phospholipase C [1,2]. Liberated diglycerides bind to the regulatory domain of PKC and cause its activation, allowing the enzyme to phosphorylate its natural substrates at serine or threonine residues [3]. PKC is deactivated by the metabolism of the effector diglycerides by diglyceride kinase or diglyceride lipase [1,4].

The nature of the diglyceride binding-site has been probed with a substantial number of diglycerides. The interaction is stereospecific, with only the naturally occurring *sn*-1,2-(S)-enantiomer being active, both in vitro and in vivo [5-7]. Studies on the structure/activity relationship have demonstrated great specificity of interaction with respect to diglycerides. For example, 1,3-diglycerides are inactive, and the addition of a single methyl group at the *sn*-1 position of an otherwise active diglyceride almost completely abolishes activity [8,9]. PKC's great specificity with respect to diglycerides must be contrasted, however, to its apparent lack of specificity with regard to the structurally diverse tumor promoters [10]. The tumor promoters capable of potently activating PKC include phorbol esters, ingenols, teleocidins, and aplysiatoxins [1,11-13]. These molecules function in vivo by activating PKC permanently on a physiological time scale, since they are not readily metabolized and are tightly bound to the enzyme [14-16].

An important issue to resolve is how the same effector binding-site in PKC can specifically recognize both the diglycerides and the apparently structurally dissimilar tumor promoters. Recently, we have presented a stereochemical model which addresses this issue and explains how the structurally diverse tumor promoters and the diglycerides can all be accommodated by the same binding-site [10]. Debromoaplysiatoxin (DAT; **1**) and 3-deoxydebromoaplysiatoxin play a central role in this model [10]. The aplysiatoxins provide synthetically manipulable templates which are crucial in the development of the structural hypothesis. The model draws attention to the structural correlation between DAT and its cognate *sn*-1,2-diglyceride **2**, as shown in Fig. 1. Of considerable interest is the C30 stereochemistry of DAT. It would be predicted that this stereochemical preference should carry over to diglycerides if our model is cogent. In this communication, it is demonstrated that the binding of 3-methyldiglycerides to PKC and the subsequent activation of PKC are stereospecific with respect to both the C2 position and the C3 position of the diglycerides. Furthermore, the absolute stereochemical preference is exactly as predicted by our model. This study thus establishes the stereochemistry of PKC activator binding and, in addition, strongly supports out model.

2. MATERIALS AND METHODS

2.1. *PKC purification and enzyme assays*

PKC was partially purified from rat brain as previously described

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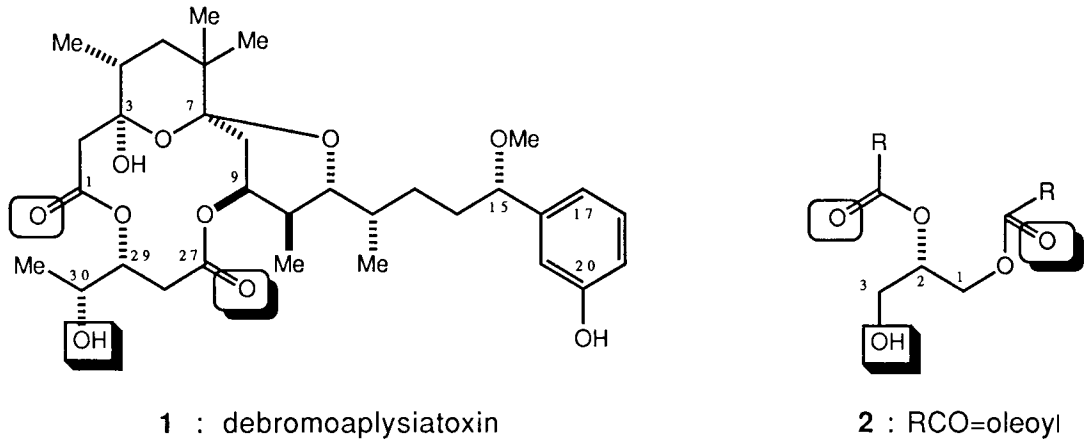


Fig. 1. Proposed PKC activation model. Identical symbols indicate equivalent atoms.

[6]. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into lysine-rich histone type VS, essentially as reported [6], with the following modifications: for incorporation into the assay, mixtures of PS and diglycerides in chloroform were dried down under nitrogen for 30 min and resuspended in 60 mM Tris-HCl pH 7.5, 3 mM EDTA by sonication for 2 min with a Kontes micro-ultrasonic cell disrupter. Incubations were carried out at 30°C for 5 min.

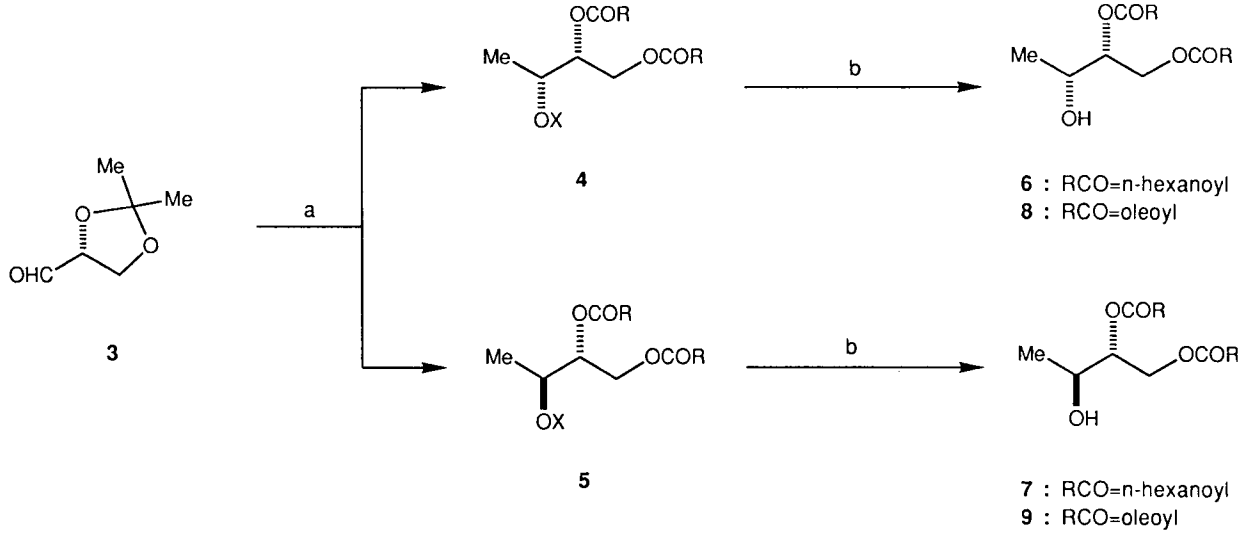
2.2. *Synthesis of analogs*

The diglyceride analogs **6**~**9** were synthesized as summarized in Scheme 1. Addition of methylmagnesium bromide on D-glyceraldehyde acetonide, i.e. step a.1, yielded a ca 3:1 mixture of the two expected diastereomers, which were separated by silica gel chromatography after introduction of the two acyl groups. In order to assign the stereochemistry of these diastereomers, the stereochemically-defined synthesis of the intermediate **4** (X = $C_6H_5CH_2$), starting from diethyl L-(+)-tartrate, was carried out as outlined in Scheme 2. Comparison of their spectroscopic data (^1H and ^{13}C NMR, MS, IR, and α_D) established unambiguously that the minor diastereomer **4** obtained in the first synthesis is the antipode of the threo diastereomer **12**.

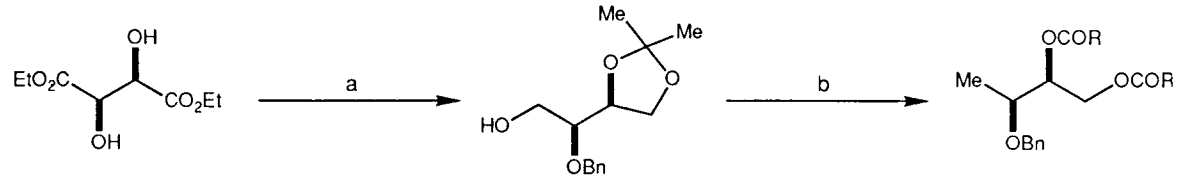
The four diglyceride analogs **4**~**7** thus synthesized gave satisfactory spectroscopic data, including the following: **6**: $[\alpha]_D$ +7.5° (c 1.5, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ 4.97 (1H, dt, J = 4.3, 6.7 Hz), 4.40 (1H, dd, J = 4.2, 11.9 Hz), 4.12 (1H, dd, J = 6.7, 11.9 Hz), 3.94 (1H, br), 2.36 (2H, t, J = 7.6 Hz), 1.29 (2H, t, J = 7.5 Hz), 1.62 (4H, m), 1.30 (8H, m), 1.21 (3H, d, J = 6.5 Hz), and 0.89 (6H, m). **7**: $[\alpha]_D$ −20.7° (c 2.1, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ 4.94 (1 H, dt, J = 4.7, 5.5 Hz), 4.32 (2H, d, J = 4.7 Hz), 3.90 (1H, m), 2.34 (2H, t, 6.5 Hz), 2.31 (2H, t, J = 7.7 Hz), 1.64 (4H, m), 1.31 (8H, m), 1.22 (3H, d, J = 4.4 Hz), and 0.90 (6H, m). **8**: $[\alpha]_D$ + 1.6° (c 0.43, CH_2Cl_2; ^1H NMR (500 MHz, $CDCl_3$) δ 5.24 (4H, m), 4.87 (1H, dt, J = 4.3, 6.6 Hz), 4.30 (1H, dd, J = 4.3, 11.9 Hz), 4.02 (1H, dd, J = 6.7, 11.9 Hz), 3.85 (1H, dt, J = 4.3, 6.4 Hz), 2.26 (1H, t, J = 7.5 Hz), 2.19 (1H, t, J = 7.6 Hz), and 1.11 (3H, d, J = 6.5 Hz). **9**: $[\alpha]_D$ −11.0° (c 1.2, CH_2Cl_2); ^1H NMR (500 MHz, $CDCl_3$) δ 5.34 (4H, m), 4.94 (1H, dt, J = 4.8, 9.9 Hz), 4.32 (2H, d, J = 4.6 Hz), 3.90 (1H, dq, J = 5.7 Hz), 2.34 (2H, t, J = 7.5 Hz), 2.31 (2H, t, J = 7.7 Hz), and 1.22 (3H, d, J = 6.4 Hz).

2.3. Materials

Bovine phosphatidylserine (PS) was obtained from Avanti Polar



Scheme 1. Synthesis of 3-methylglycerides. Reagents for the dihexanoyl series: a. 1. MeMgBr/THF, 2. $C_6H_5CH_2Br$/KH/imidazole/ THF-DMF, 3. AcOH/MeOH, 4. *n*-hexanoyl chloride/py, followed by silica gel chromatography separation. b. 1. H_2/Pd(OH)$_2$ on C/MeOH. Reagents for the dioleoyl series: a. 1. same as above. 2. *p*-$MeOC_6H_4CH_2Br$/NaH/THF-DMF, 3. same as above, 4. oleoyl chloride/py, followed by silica gel chromatography separation. b. 1. DDQ/CH_2Cl_2.



10 : diethyl L-(+)-tartrate 11 12 : RCO=n-hexanoyl

Scheme 2. Stereochemically-defined synthesis of 3-methylated *sn*-1,2-diglycerides. Reagents: a. 1. $C_6H_5CH(OMe)2$/*p*-TsOH · py/ C_6H_6, 2. $NaBH_4$/EtOH, 3. DIBAL/CH_2Cl_2, 4. $MeC(OMe)_2Me$/*p*-TsOH · py/THF. b. 1. *p*-TsCl/py, 2. LAH/THF, 3. AcOH/MeOH, 4. *n*-hexanoyl chloride/py.

Lipids (Birmingham, AL). 1,2-Dioleoyl-*sn*-glycerol and histone type VS were from Sigma. Protease inhibitors were products of Boehringer. [γ-^{32}P]ATP (~3000 Ci/mmol) was from Amersham.

3. RESULTS

Rat brain PKC, partially purified as described in section 2, was found to have the same properties as the fully purified Ca^{2+}-phospholipid dependent protein kinase C [6,17]. With the rat brain preparation, *sn*-1,2-diolein bound to and activated PKC with a $K_d = 0.09$ μM and a $V_{max} = 9.36$ nmol/min/mg (Fig. 2A). The two diastereomeric 3-methyldiglyceride analogs **8** and **9** were prepared and studied as PKC activators (Fig. 2B). As can be seen here, only analog **8** was active. This compound bound to PKC approximately two-fold less well than (S)-diolein **2** ($K_d = 0.20$; $V_{max} = 7.70$ nmol/min/mg). The second methylated diastereomer **9** was basically inactive and elicited approximately 2% of the activity of the active 3-methyl analog **8**. This amount of activity is not considered to be significant, since only a 2% contamination of **8** would result in the observed activation, and possible contamination at this level would not have been detected by the analytical methods employed. The presence of a small contamination from **8** is probably the reason why saturation conditions could not be reached with the high concentrations (up to 20 μM) of **9** used (Fig. 2B, inset). The PKC activation observed when the different 3-methyldiglyceride analogs (**6**–**9**) were used at a concentration of 5 μM is shown in Fig. 3. The nature of the fatty acid groups of the diglyceride did not affect the observed stereospecificity, as can be seen with the dihexanoyl analogs (**6** and **7**).

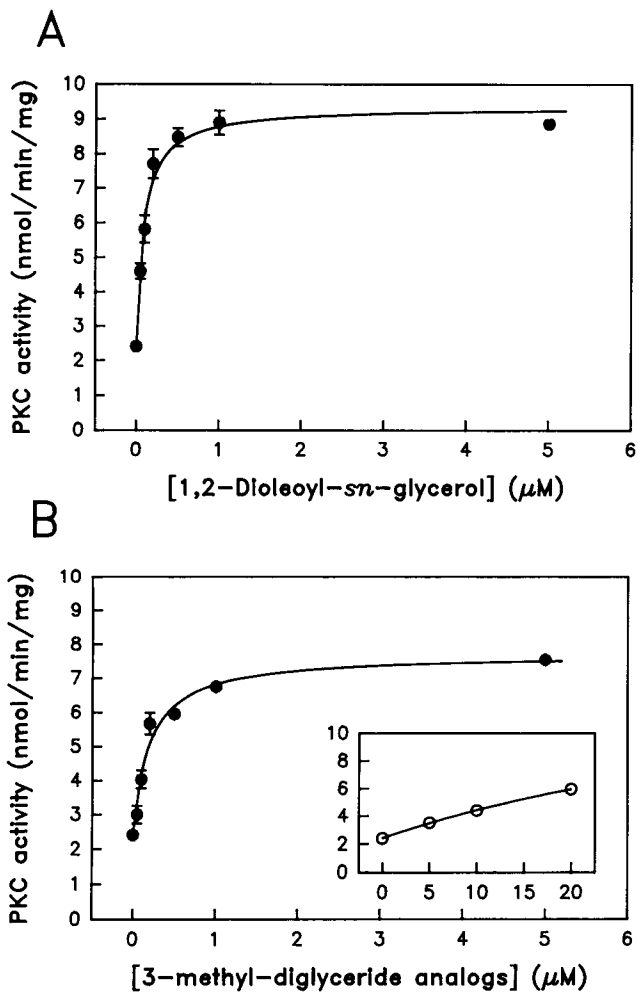


Fig. 2. Activation of PKC by diolein and 3-methyldiglyceride analogs. (A) Activation of PKC by diolein. (B) Activation of PKC by the 3-methyldiglyceride analogs **8** (•) and **9** (○, inset). Results are average values of a typical experiment done in triplicate ± the standard deviation from the mean.

4. DISCUSSION

The experiments reported here probed the nature of the diglyceride binding-site of PKC. The stereochemical requirement at the *sn*-2 position had already been demonstrated [6]. Although diglycerides do not possess a second chiral center, proposed tumor promoting diglyceride surrogates do. For example, compared with diglycerides, the proposed pharmacophore of DAT contains one additional chiral center at the C30 position (Fig. 1). It was thus important to probe the stereochemical requirement in the synthetic 3-methyldiglyceride series, to determine if it matches that found in the aplysiatoxin series. If this turned out to be the case, it would further strengthen the structural link forged between diglycerides and aplysiatoxins [10]. In this connection, it is interesting to note that methylated diglycerides **6** and **7** were studied previously, but their behavior

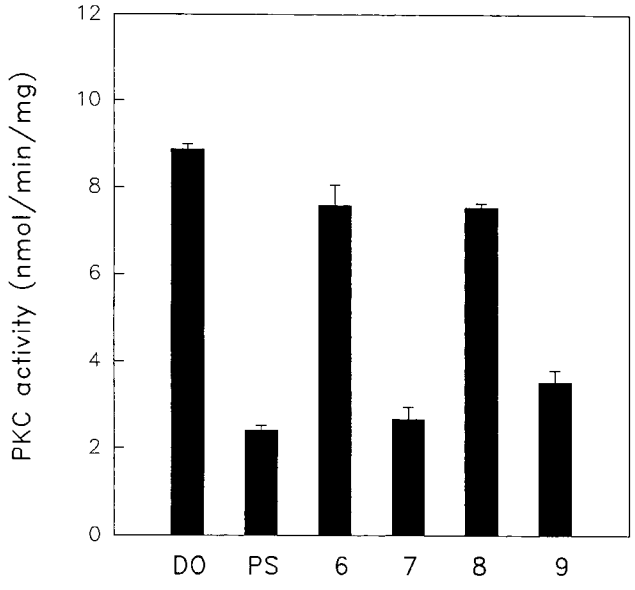


Fig. 3. Effect of diacylglycerol analogs as PKC activators. PKC was assayed as described in section 2 in the presence of PS and 2 mol% (5 μM) diolein (DO) or 3-methyldiglyceride analogs. Results are the average of triplicate experiments ± the standard deviation from the mean.

towards PKC appeared to be inconsistent with our hypothesis [10]. For this reason, we have independently synthesized methylated diglycerides **6** and **7**, established their stereochemistry unambiguously, and proved their biological behaviors to be consistent with our hypothesis. Furthermore, the corresponding diolein analogs **8** and **9** exhibit biological behavior parallel to that of **6** and **7**, demonstrating that the stereochemical requirement at the second asymmetric center of diglycerides is identical with that of aplysiatoxins. This finding lends strong support to the structural hypothesis that we have proposed which links structural elements of the diglycerides to the pharmacophore of the tumor promoters [10].

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