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CONFORMATIONALLY CONSTRAINED ANALOGUES OF DIACYLGLYCEROL (DAG). 15.[1] THE INDISPENSABLE ROLE OF THE *sn*-1 AND *sn*-2 CARBONYLS IN THE BINDING OF DAG-LACTONES TO PROTEIN KINASE C (PK-C)

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Abstract: The binding mode of DAG-lactones to PK-C was investigated using the C1b domain from the X-ray structure of the phorbol ester/C1b complex of PK-Cδ as a template. Modeling experiments revealed two binding alternatives in which one of the carbonyls of the DAG lactones remained uninvolved with the protein. Experimentally, however, the removal of either *sn*-1 or *sn*-2 carbonyls caused a dramatic drop in binding affinity towards PK-C. Although it was not possible to discriminate between the two binding alternatives of the DAG-lactones, the study demonstrates an important role for the additional carbonyl group. The function of this group could be equivalent to that of the C-9(OH)/C-13 (C=O) motif in phorbol esters, which also appears free of interactions in the phorbol ester/C1b complex. This role presumably reflects interaction with the phosholipid head groups required for high affinity binding under the conditions of the biological assays.

The lipophilic second messenger *sn*-1,2-diacylglycerol (DAG) plays a prominent role in cellular signal transduction.[2] Generated both through G-protein coupled and tyrosine kinase activated isoforms of phospholipase C, as well as indirectly by phospholipase D, DAG binds to C1 domains in members of the protein kinase C (PK-C) family activating their downstream pathways.[3,4] The importance of these pathways in cellular responses, including proliferation, differentiation, gene expression, and tumor promotion, has been highlighted by the remarkable effects of the phorbol esters that function as potent and metabolically more stable DAG surrogates.[5] The phorbol esters bind to the classical (α, β, and γ) as well as the novel (δ, ε, η, and θ) PK-C isozymes with binding affinities three to four orders of magnitude higher than those of DAG.[6] Recently, however, this gap has been reduced approximately two orders of magnitude by constraining the glycerol backbone into lactones that overcome some of the energy penalty associated with loss of entropy.[7–9] These potent DAG-lactones were designed using a pharmacophore-guided approach that was based on the spatial correspondence between critical oxygen atoms in the phorbol and the DAG-lactones.[10] The recent X-ray structure of phorbol-13-*O*-acetate bound to the C1b domain of PK-Cδ[11] now permits the mode of binding of these DAG-lactones to be evaluated by a computer-guided molecular docking approach. As anticipated, the X-ray structure of the phorbol/C1b complex confirmed the importance of hydrogen bonds involving the key phorbol ester pharmacophores C-20 (OH) and C-3 (C=O), the latter in combination with C-4 (OH).[11] However, the presumed critical C-9 (OH) pharmacophore was not found to be involved in hydrogen bonding to the protein. Instead, this OH group formed an intramolecular hydrogen-bond to the C-13 carbonyl ester .[11] Since the DAG-lactones compete with phorbol esters for the same binding site,[5] a common binding mode between

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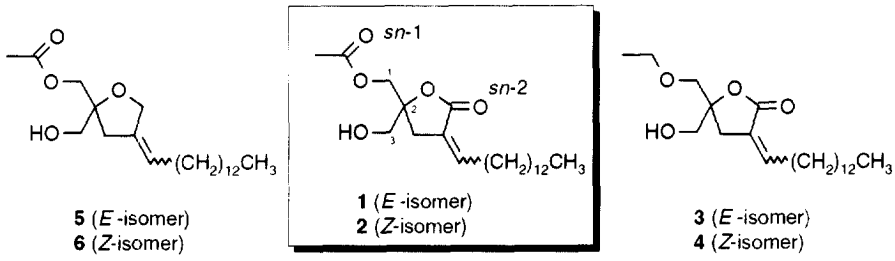
these two classes of ligands would require at least one C=O group of a DAG-lactone to be involved in binding to the C1b domain of the enzyme. We decided to test the individual importance of these nonequivalent carbonyl functions by synthesizing DAG-lactone analogues of **1** and **2** in which a single carbonyl function is deleted (compounds **3**–**6**).



5 (*E*-isomer)
6 (*Z*-isomer)

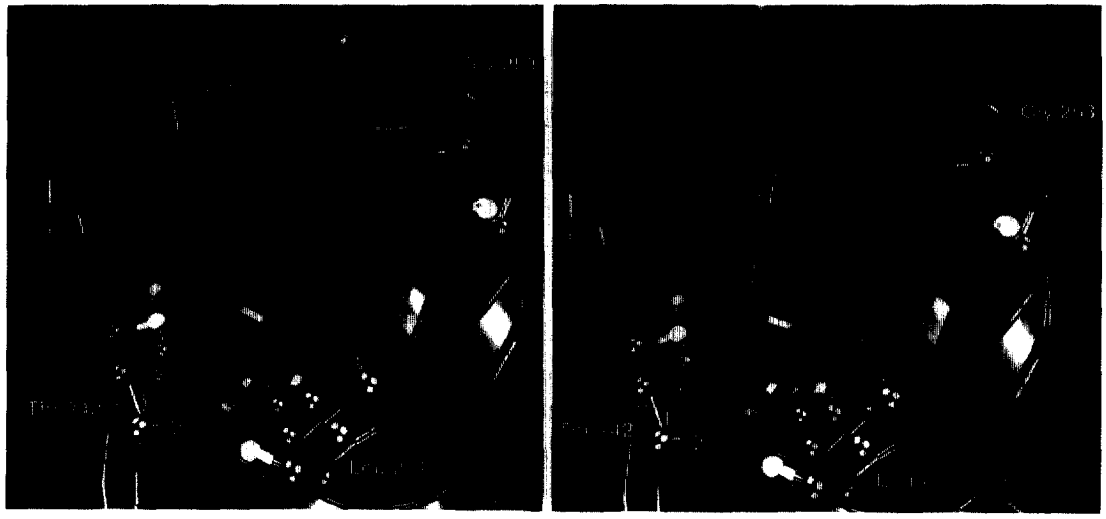
1 (*E*-isomer)
2 (*Z*-isomer)

3 (*E*-isomer)
4 (*Z*-isomer)

Molecular modeling

Hydrogen bonding. Using the crystal coordinates of the C1b domain complexed with phorbol-13-*O*-acetate, the more potent *Z*-isomer (**2**) was docked into an empty C1b domain using the program AutoDock 2.4.[12] This program combines a Monte Carlo simulated annealing algorithm to search the conformational space with a fast evaluation of the interaction energy. Partial flexibility can be applied to the ligand by specifying non-ring rotatable bonds. The search strategy consisted of performing a random walk of the ligand on the surface of the receptor, which was kept rigid. Using this procedure, the crystallographic position of phorbol-13-*O*-acetate[11] was reproduced with a RMS deviation of 0.62 Å. Since DAG-lactone **2** contains an embedded glycerol backbone, the two nonequivalent carbonyl functions were identified, as in DAG, as *sn*-1 and *sn*-2. In order to

Figure 1. Docking results showing hydrogen bonding interactions of **2** with the C1b domain (PK-Cδ) in binding modes *sn*-1 (left) and *sn*-2 (right). The molecular graphics were generated with the program VMD.[13]



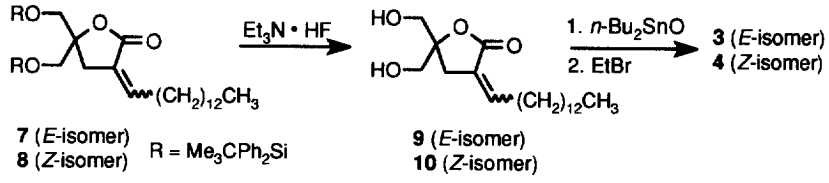
reduce the number of degrees of freedom during docking, the long alkyl chain was shortened to 3 carbons. The docking simulation was performed 100 times. Fifty trials led to a binding mode involving the *sn*-1 carbonyl (Figure 1, left); 43 led to an alternative binding mode involving the *sn*-2 carbonyl (Figure 1, right); and the remaining complexes were energetically less favorable and formed outside the binding pocket. Both *sn*-1 and *sn*-2 binding modes had a comparable AutoDock scoring energy and displayed a similar network of hydrogen bonds to Thr 242, Leu 251, and Gly 253, as observed with phorbol-13-*O*-acetate,[11] with participation of the primary OH and one of the carbonyl functions. In both binding modes one carbonyl function always remained free from hydrogen bonding to the protein.

Hydrophobic interactions. The two binding modes revealed by the docking experiments showed an identical pattern of hydrogen bonds without regard to the disposition of the aliphatic alkyl chain. Even though the role of the aliphatic chains in DAG has been correlated mostly with partition or transport between biological phases, the two docking alternatives appear to propel the aliphatic chain in opposite directions (Figure 1). Since the phorbol-13-*O*-acetate/C1b domain complex does not contain a lipid matrix, it is impossible, at present, to discriminate between these two binding alternatives based on hydrophobic interactions with the lipid membrane. Similarly, the normally higher affinity observed with the *Z*-isomer (**2**) cannot be explained with this model.

Chemistry

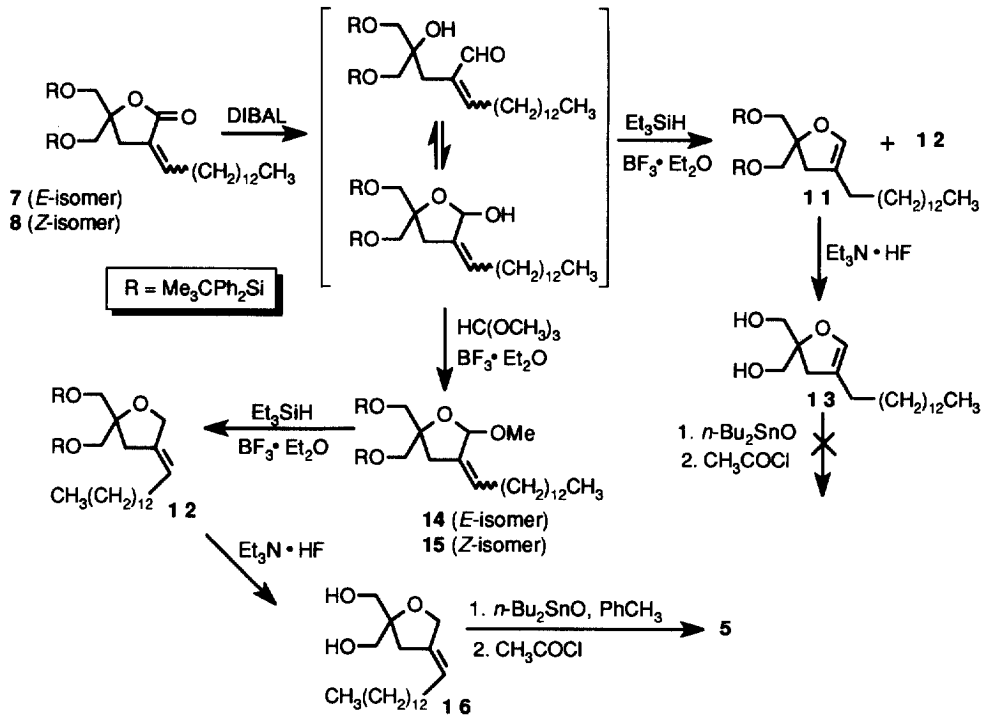
In order to expedite the study, all the target compounds (**3**–**6**) were synthesized as racemates, which were intended to be compared with the corresponding racemates of **1** and **2**. Scheme 1 outlines the synthesis of the *sn*-1-deleted targets **3**[14] and **4**,[15] starting with known compounds **7** and **8**, respectively.[7] The isolated yield in the monoalkylation step was 52% for both isomers.

Scheme 1



The synthesis of the *sn*-2-deleted targets was somewhat more involved (Scheme 2). Starting with the *E*-isomer **7**, diisobutylaluminum hydride (DIBAL) reduction gave a mixture of open aldehyde and lactol in a ratio that changed as a function of time. A ^{1}H NMR of the isolated product in $CDCl_3$ revealed a predominance of the open aldehyde that gradually shifted with time into an equilibrium, still in favor of the open form. In deuterated benzene, addition of a Lewis acid was accompanied by complete conversion to the lactol. This equilibrium could be easily followed by observing the intensity of the CHO signal at δ 9.40. Reduction of this mixture with triethylsilane in the presence of a Lewis acid produced a low yield of the desired product **12** (13%) and glycal **11** (7%), the latter resulting from an initial S_N2' hydride attack on the olefin. Alternatively, acid-catalyzed ring

Scheme 2



closure of the mixture of open aldehyde and lactol with triethylorthoformate produced the methyl glycoside **14** (*E*-isomer). All attempts to purify **14** led back to the formation of the lactol and open aldehyde forms. Compound **14** was therefore used as a crude material that underwent reduction with triethylsilane in the presence of BF_3•OEt_2 to give **12** (23% yield from **7**). Deblocking of the silyl ether groups and monoacylation with acetyl chloride, via the dibutylstannylene, afforded the target *E*-isomer **5**[16] in 55 % yield (2 steps). A similar approach starting with the *Z*-isomer **8** gave the methyl glycoside **15**. However, during the BF_3•OEt_2/triethylsilane reduction, the olefin isomerized to the same *E*-isomer **12**, obtained previously. Finally, attempted monoacylation of glycal **13**, either indirectly through the intermediate dibutylstannylene, or directly in pyridine, failed to give the corresponding monoacylated isomer. This compound was attractive because, if active, it would have obviated the need to separate geometrical isomers.

Biological activity and discussion

This investigation was prompted by the results obtained from molecular docking experiments using computer modeling to discern the binding mode of DAG lactones **1** and **2** at the C1b domain of PK-C. The study revealed two binding alternatives in which one of the carbonyl esters remained uninvolved with the protein. Attempts to assess the individual importance of each carbonyl function led to the synthesis of DAG-

lactone analogues **3–5**, in which a single carbonyl function is missing. The K_i values for compounds **3–5** revealed a ca.100-fold drop in binding affinity relative to the parent racemates **1** and **2** (Table 1). The affinity of these ligands for PK-C was assessed in terms of their ability to displace [20-^{3}H]phorbol 12,13-dibutyrate (PDBU) from a recombinant single isozyme (PK-Cα) in the presence of phosphatidylserine, as already described.[7–9] The clear conclusion from this work is that both *sn*-1 and *sn*-2 carbonyls in the DAG-lactones are essential for a strong interaction with PK-C. While the results do not resolve the issue of which binding mode depicted in Figure 1 is involved in binding to PK-C, it suggests that the intramolecularly hydrogen-bonded C-9 (OH)/C-13 (C=O) motif in phorbol-13-*O*-acetate —the missing pharmacophore!— may be interacting at the same binding site as the ester carbonyl in the DAG-lactones which does not appear to be directly hydrogen bonded to the protein.[17] This binding domain probably occurs with the phospholipid headgroups at the membrane interface, and since the crystal structure of the phorbol-13-*O*-acetate/C1b complex does not contain lipid, the complete picture of how the third pharmacophore interacts in the PK-C/membrane complex remains unresolved. An important implication of our findings is that the third pharmacophore may provide a means for incorporating selectivity targeted to the lipid microenvironment rather that the PK-C binding domain itself. Additional approaches for discrimination between *sn*-1 and *sn*-2 binding modes have already been initiated in our laboratory[1] and future experiments may help resolve this issue.

Table 1. Apparent K_i values for ligands **1-5** as inhibitors of PDBU binding to PK-Cα

Compound	K_i (nM)
1 (*E*-isomer)	78 ± 4.7
2 (*Z*-isomer)	35 ± 1.3
3 (*E*-isomer)	4,979 ± 97
4 (*Z*-isomer)	7,460 ± 2,000
5 (*E*-isomer)	7,582 ± 45

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14. *rac*-(*E*)-5-(ethoxymethyl)-5-(hydroxymethyl)-3-tetradecylideneoxolan-2-one (**3**): solid mp 56–58 °C; IR ($CHCl_3$) 3448, 1735, 1679 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.86 (distorted t, 3 H, $C\underline{H}_3(CH_2)_{10}CH_2CH_2CH$=), 1.15 (t, *J* = 7.0 Hz, 3 H, $C\underline{H}_3CH_2O$), 1.20–1.30 (m, 20 H, $CH_3(C\underline{H}_2)_{10}CH_2CH_2CH$=), 1.50 (m, 2 H, $CH_3(CH_2)_{10}C\underline{H}_2CH_2CH$=), 1.77 (br s, 1 H, OH), 2.15 (distorted q, 2 H, $CH_3(CH_2)_{10}CH_2C\underline{H}_2CH$=), 2.72 (AB m, 2 H, H-$4_{a,b}$), 3.45–3.60 (m, 4 H, $CH_3C\underline{H}_2O$ and $C\underline{H}_2OH$), 3.70 (AB q, *J* = 12.0 Hz, 2 H, $EtOC\underline{H}_2$), 6.72 (m, 1 H, $C_{13}H_{27}C\underline{H}$=); ^{13}C NMR ($CDCl_3$) δ 14.10, 14.99, 22.67, 28.08, 29.30, 29.33, 29.39, 29.52, 29.63, 29.96, 30.21, 31.90, 65.81, 67.46, 72.61, 83.33, 126.29, 141.66, 170.26; FABMS (*m/z*, relative intensity) 369 (MH^+, 35), 305 ($MH^+ - H_2O$ + EtOH, 26). Anal calcd for $C_{22}H_{40}O_4$: C, 71.70, H, 10.94. Found: C, 71.39; H, 10.77.
15. *rac*-(*Z*)-5-(ethoxymethyl)-5-(hydroxymethyl)-3-tetradecylideneoxolan-2-one (**4**): solid mp 54–56 °C; IR ($CHCl_3$) 3676, 3502 and 1745 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (distorted t, 3 H, $C\underline{H}_3(CH_2)_{10}CH_2CH_2CH$=), 1.26 (t, *J* = 6.9 Hz, 3 H, $C\underline{H}_3CH_2O$), 1.30-1.45 (m, 20 H, $CH_3(C\underline{H}_2)_{10}CH_2CH_2CH$=), 1.50 (m, 2 H, $CH_3(CH_2)_{10}C\underline{H}_2CH_2CH$=), 1.80 (br s, 1 H, OH), 2.78 (distorted q, 2 H, $CH_3(CH_2)_{10}CH_2C\underline{H}_2CH$=), 2.88 (AB d, *J* = 2.0 Hz, 2 H, H-$4_{a,b}$), 3.55–3.70 (m, 4 H, $CH_3C\underline{H}_2O$ and $C\underline{H}_2OH$), 3.78 (AB q, *J* = 12.0 Hz, 2 H, $EtOC\underline{H}_2$), 6.29 (m, 1 H, $C_{13}H_{27}C\underline{H}$=); ^{13}C NMR ($CDCl_3$) δ 14.06, 14.96, 22.62, 27.63, 28.99, 29.03, 29.20, 29.28, 29.39, 29.51, 29.58, 29.79, 29.85 29.87, 31.84, 33.24, 65.60, 67.36, 72.45, 82.98, 124.07, 145.06, 168.84; FABMS (*m/z*, relative intensity) 369 (MH^+, 100), 305 ($MH^+ - H_2O$ + EtOH, 89). Anal calcd for $C_{22}H_{40}O_4$: C, 71.70, H, 10.94. Found: C, 71.82; H, 10.92.
16. *rac*-(*E*)-(1-(hydroxymethyl)-4-tetradecylidene-2-oxolanyl)methyl acetate (**5**): oil; IR (neat) 3458 and 1747 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.96 (distorted t, 3, $C\underline{H}_3(CH_2)_{11}CH_2CH$=), 1.25–1.50 (m, 22 H, $CH_3(C\underline{H}_2)_{11}CH_2CH$=), 1.60 (br s, 1 H, OH), 2.09 (m, 2 H, $CH_3(CH_2)_{11}C\underline{H}_2CH$=), 2.18 (s, 3 H, CH_3CO_2), 2.52 (AB q, *J* = 16.1 Hz, 2 H, H-$5_{a,b}$), 3.68 (AB q, *J* = 11.7 Hz, 2 H, $C\underline{H}_2OH$), 4.20 (br s, 2 H, $CH_3COOC\underline{H}_2$), 4.47 (br s, 2 H, H-$3_{a,b}$), 5.39 (m, 1 H, $C_{13}H_{27}C\underline{H}$=); ^{13}C NMR ($CDCl_3$) δ 14.05, 20.79, 22.62, 29.23, 29.25, 29.28, 29.38, 29.46, 29.58, 29.60, 29.72, 31.84, 32.94, 64.09, 65.11, 71.28, 84.06, 121.14, 136.39, 170.94; FABMS (*m/z*, relative intensity) 369 (MH^+, 100). Anal. calcd for $C_{22}H_{40}O_4$: C, 71.70, H, 10.94. Found: C, 71.61; H, 10.84).
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