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Polar 3-alkylidene-5-pivaloyloxymethyl-5′-hydroxymethyl-γ-lactones as protein kinase C ligands and antitumor agents

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ABSTRACT

A series of DAG-lactones with polar 3-alkylidene substituents have been investigated as PKC-α ligands and antitumor agents. Extensive analysis of structure–activity relationships for the 3-alkylidene chain revealed that polar groups such as ether, hydroxyl, aldehyde, ester, acyloxy, and amido were tolerated with similar binding affinities and reduced lipophilicities compared to the corresponding unsubstituted alkylidene chain. Among the derivatives, compounds **5**, **6** and **8** with an ether type of side chain showed high binding affinities in range of K_i = 3–5 nM and excellent antitumor profiles, particularly against the colo205 colon cancer and the K562 leukemia cell lines.

The protein kinase C (PKC) family of serine/threonine kinases plays a pivotal role in cellular signal transduction.[1,2] These enzymes are activated by diacylglycerol (DAG) which can be generated either by phospholipase C (PLC) mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) or indirectly by phospholipase D followed by phosphatidate phosphatase.[3] DAG binds to the C1 domains of both the calcium-dependent classical PKC isoforms α, β, and γ and the novel or calcium-independent PKC isoforms δ, ε, η and θ. The binding activates these enzymes and promotes their association with the membrane phospholipids, inducing the translocation of cytosolic PKC to cellular membranes.[4] In addition to the PKCs, other C1 domain-containing receptors, including members of the RasGRP, chimaerin, MUNC13, PKD and DAG kinase families, also function in DAG signaling and therefore represent potential sites of action for DAG analogs.[5]

Changes in the level or activity of PKC in tumors, its role in signaling pathways involved in cancer formation, and the effect of its overexpression or knockout in animal models all emphasize its important role in cancer.[6] Reflecting this importance, a variety of inhibitors of PKC have been tested in in vitro and in vivo cancer models and developed clinically as a new class of antitumor agents.[7] It is important to emphasize, however, that activators of PKC have also proven to have anticancer activity. Both bryostatin 1[8] and ingenol 3-angelate (PEP005)[9] are in multiple clinical trials. In addition, DPP (12-deoxyphorbol-13-phenylacetate) and prostratin have been shown to be potent inhibitors of tumor promotion in mice, contrasting with phorbol-12-myristate-13-acetate (PMA) which is the paradigm for a tumor promoter.[10,11] In addition, prostratin (12-deoxyphorbol-13-acetate), gnidimacrin,[12] and ingenol-3-angelate[9] have also demonstrated antitumor activities in selected cell lines.

Phorbol esters bind competitively to the DAG binding site on the C1 domains with affinities several orders of magnitude greater than those of DAGs and have provided powerful pharmacological tools for studying PKC function.[13,14] Phorbol esters function as potent DAG surrogates because their conformationally rigid scaffold, unlike the flexible glycerol backbone of DAG, is able to specifically direct the hydrophilic pharmacophores of the ligand. In an attempt to improve binding affinity of DAG through conformational restriction, we had previously investigated a series of conformationally constrained DAG analogues and finally obtained 'ultrapotent' DAG-lactone analogues built on a 5-[(acyloxy)methyl]-5-(hydroxymethyl)tetrahydro-2-furanone template.[15] Depending on the type of hydrophobic substitution, some of the compounds built with this template displayed low-nanomolar binding affinities and a variety of biological profiles (see Fig. 1).[16]

Branched DAG-lactones, for example HK-434, were shown to exhibit high binding affinities for PKC-α and demonstrated a

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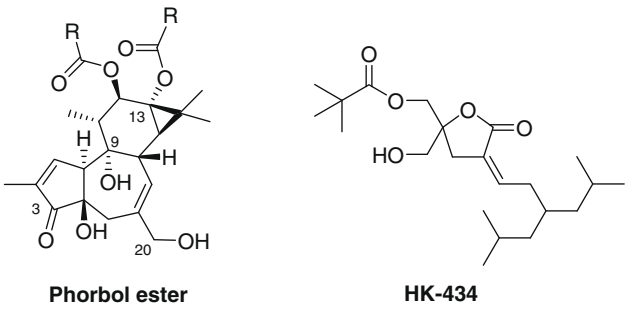
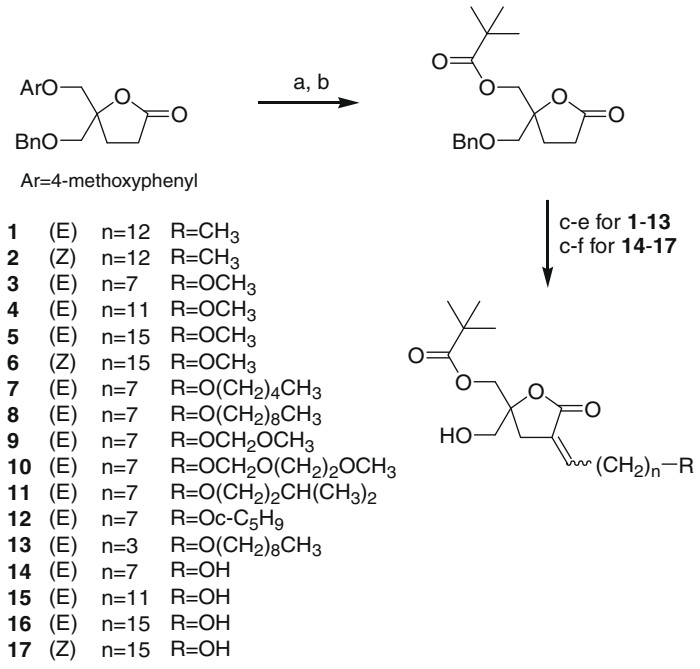


Figure 1.

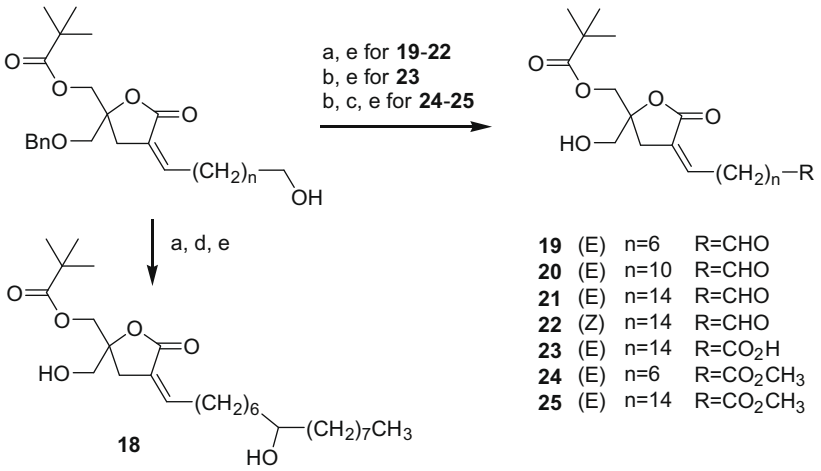
selective and potent antitumor profile in the National Cancer Institute (NCI) 60-cell line in vitro screen. The NCI's computerized, pattern-recognition program COMPARE showed that the mean-graph profile of DAG-lactones matched with that of prostratin, the nontumor-promoting antitumor phorbol ester, indicating that the antitumor mechanism of DAG-lactone results from PKC activation.[17]

The 3-alkylidene DAG-lactones such as compounds **1** and **2** are highly lipophilic. In an effort to develop more drug-like DAG-lactones, we have reduced the lipophilicity of the DAG-lactones by incorporating a variety of polar groups into the 3-alkylidene chain. In this Letter, we describe the structure–activity relationships for PKC binding and for inhibition of growth of a variety of tumor cell lines by these DAG-lactones possessing polar 3-alkylidene chains.

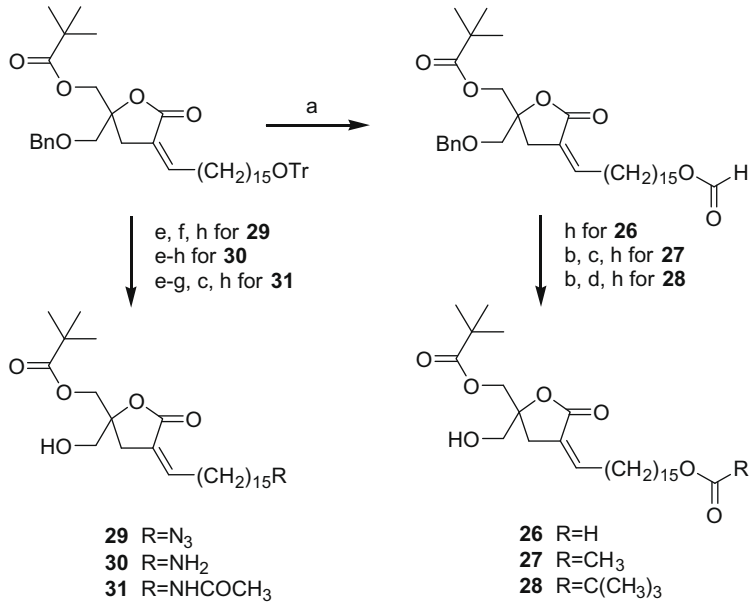
The syntheses of DAG-lactones with polar 3-alkylidene chains are described in Schemes 1–3. Basically, alkylation of the protected 5,5-disubstituted γ-lactone, previously reported, with various polar side chains followed by routine work-up provided the corresponding final compounds.



Scheme 1. Syntheses of hydroxyl and ether analogues. Reagents and conditions: (a) CAN, CH_3CN–H_2O, 0 °C; (b) $(CH_3)_3CCOCl$, Et_3N, DMAP, CH_2Cl_2; (c) LiHMDS, $CH_3(CH_2)_{12}CHO$ for **1**–**2**, $RO(CH_2)_nCHO$ for **3**–**13**, $TrO(CH_2)_nCHO$ for **14**–**17**, THF, −78 °C; (d) (i) MsCl, NEt_3, CH_2Cl_2, (ii) DBU; (e) BCl_3, CH_2Cl_2, −78 °C; (f) CF_3CO_2H, CH_2Cl_2, 0 °C.



Scheme 2. Syntheses of aldehyde and carboxylate analogues. Reagents and conditions: (a) PCC, 4 Å MS, CH_2Cl_2; (b) PDC, DMF; (c) TMS–diazomethane, MeOH; (d) $C_8H_{17}MgBr$, ether, −10 °C; (e) BCl_3, CH_2Cl_2, −78 °C.



Scheme 3. Syntheses of O-acylated and amino analogues. Reagents and conditions: (a) HCO_2H, CH_2Cl_2, rt; (b) K_2CO_3, MeOH, rt; (c) Ac_2O, NEt_3, DMAP, CH_2Cl_2, rt; (d) $(CH_3)_3CCOCl$, NEt_3, CH_2Cl_2, rt; (e) CF_3CO_2H, CH_2Cl_2, 0 °C; (f) PPh_3, DEAD, DPPA, THF, rt; (g) PPh_3, H_2O, THF, rt; (h) BCl_3, CH_2Cl_2, −78 °C.

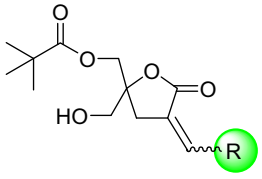
The interaction of the synthesized DAG-lactones with PKC was assessed in terms of the ability of the ligand to displace bound [20-^{3}H]phorbol 12,13-dibutyrate (PDBu) from the recombinant PKC-α isoform in the presence of phosphatidylserine as previously described.[17] The IC_{50} values were determined by fitting the data points to the theoretical competition curve, and the K_i values for inhibition of binding were calculated from the corresponding IC_{50} values (Table 1). The antitumor activity of selected compounds was evaluated against eight cancer lines, including leukemia (K562, MOLT 4F, CCRF-CEM and HL-60), breast (HS 578T), colon (Colo#205), melanoma (SK-MEL-5) and lung (NCI-H322) cell lines and is expressed as GI_{50}, the concentration that yields 50% inhibition of cell growth (Table 2).

The incorporation of a tetradecyl alkyl chain into the DAG-lactone template provided reference compounds **1** (*E*-form) and **2** (*Z*-form). These compounds possessed a lipophilicity, expressed in terms of a calculated *c* log *P*[18] of 6.71 and yielded binding affinities of K_i = 7.83 and 7.15 nM, respectively.

DAG-lactones (**3**–**13**) were designed with oxygenated side chains. Incorporation of an oxygen into compound **1** at three different positions provided the compounds **4, 7** and **13**. They were calculated to be less lipophilic by 1.74 log units; nevertheless, they possessed binding affinities similar to the parent. Compounds **5** and **8** showed similar behavior. Additional oxygens rendered the DAG-lactone much less lipophilic, as shown for compounds **9** and **10**; however, they reduced their binding affinities dramatically as well. The pattern of the alkyl chain next to the oxygen did not affect the binding affinities, as observed with compounds **7** (linear, K_i = 8.1 nM), **11** (branched, K_i = 7.74 nM) and **12** (cyclic, K_i = 10.2 nM). The result indicated that incorporation of an oxygen into the 3-alkylidene chain of the DAG-lactone, regardless of position, appears to be optimal for lowering lipophilicity without affecting the binding affinity.

We next investigated the behavior of DAG-lactones with polar groups, such as hydroxyl (**14**–**18**), aldehyde (**19**–**22**), carboxylate (**23**–**25**), acyloxy (**26**–**28**), azido (**29**) and amino (**30**–**31**), at the terminus of the side chain. The hydroxyl analogues (**14**–**18**) had similar binding affinities compared to the corresponding alkyl analogues (compound **1** vs **16** and **2** vs **17**). Interestingly, for the hydroxyl analogues, the *E*-isomer (**16**) showed modestly better binding affinity than the *Z*-isomer (**17**) in contrast to the general finding[15] with aliphatic side chains. Positioning of the hydroxyl

Table 1
Binding affinities of DAG-lactones



Compd #	Geo	R	K_i	(nM)	*c* Log *P*
1	*E*	$(CH_2)_{12}CH_3$	7.83	(±0.55)	6.71
2	*Z*	$(CH_2)_{12}CH_3$	7.15	(±0.51)	6.71
3	*E*	$(CH_2)_7OCH_3$	191	(±8.5)	3.01
4	*E*	$(CH_2)_{11}OCH_3$	8.2	(±0.3)	4.98
5	*E*	$(CH_2)_{15}OCH_3$	4.4	(±0.1)	6.93
6	*Z*	$(CH_2)_{15}OCH_3$	5.0	(±0.4)	6.93
7	*E*	$(CH_2)_7O(CH_2)_4CH_3$	8.1	(±0.8)	4.97
8	*E*	$(CH_2)_7O(CH_2)_8CH_3$	3.2	(±0.4)	6.94
9	*E*	$(CH_2)_7OCH_2OCH_3$	198.4	(±4.8)	2.75
10	*E*	$(CH_2)_7OCH_2O(CH_2)_2OCH_3$	323	(±17)	2.78
11	*E*	$(CH_2)_7O(CH_2)_2CH(CH_3)_2$	7.74	(±0.35)	4.84
12	*E*	$(CH_2)_7O$-*c*-C_5H_9	10.2	(±1.3)	4.33
13	*E*	$(CH_2)_3O(CH_2)_8CH_3$	7.44	(±0.11)	4.97
14	*E*	$(CH_2)_7OH$	378	(±15)	2.71
15	*E*	$(CH_2)_{11}OH$	14.5	(±0.4)	4.68
16	*E*	$(CH_2)_{15}OH$	6.4	(±1.4)	6.64
17	*Z*	$(CH_2)_{15}OH$	8.24	(±0.55)	6.64
18	*E*	$(CH_2)_6CH(OH)(CH_2)_7CH_3$	118	(±6.8)	6.57
19	*E*	$(CH_2)_6CHO$	39.3	(±0.72)	2.28
20	*E*	$(CH_2)_{10}CHO$	30.3	(±2.5)	4.25
21	*E*	$(CH_2)_{14}CHO$	6.38	(±0.74)	6.21
22	*Z*	$(CH_2)_{14}CHO$	11.4	(±0.79)	6.21
23	*E*	$(CH_2)_{14}CO_2H$	29.7	(±0.49)	6.46
24	*E*	$(CH_2)_6CO_2CH_3$	533	(±19)	2.82
25	*E*	$(CH_2)_{14}CO_2CH_3$	6.83	(±0.18)	6.75
26	*E*	$(CH_2)_{15}OCOH$	8.0	(±1.1)	6.69
27	*E*	$(CH_2)_{15}OCOCH_3$	9.3	(±1.1)	7.24
28	*E*	$(CH_2)_{15}OCOC(CH_3)_3$	10.5	(±0.58)	8.60
29	*E*	$(CH_2)_{15}N_3$	15.6	(±1.5)	7.67
30	*E*	$(CH_2)_{15}NH_2$	36.4	(±3.1)	6.23
31	*E*	$(CH_2)_{15}NHCOCH_3$	8.25	(±0.44)	6.17

Table 2
Antitumor activities of DAG-lactones (GI_{50})

Compd #	PKCα	Leukemia				Breast	Colon	Melanoma	Lung
		K562	MOLT 4F	CCRF-CEM	HL-60	HS 578T	Colo #205	SK-MEL-5	NCI-H322
	K_i (nM)	GI_{50} (μg/ml)							
ADR		0.375	0.277	0.181	0.413	0.596	0.523	0.481	0.615
HK-434	2.9	0.118	1.098	<0.1	3.469	3.304	0.138	2.153	4.236
1	7.83	<0.1	0.227	0.140	2.398	2.266	0.253	0.779	>10
4	8.2	0.516	2.384	0.701	5.217	7.337	0.541	2.808	>10
5	4.4	0.189	0.498	4.261	4.653	5.449	0.118	1.753	>10
6	5.0	0.138	1.639	<0.1	3.967	6.770	0.178	2.134	>10
7	8.1	1.149	1.029	0.877	2.960	3.002	0.491	2.994	9.520
8	3.2	0.156	2.139	0.304	4.525	4.637	0.264	1.431	>10
9	198	3.694	1.842	>10	>10	>10	2.008	8.326	>10
15	14.5	1.090	1.343	0.650	>10	>10	2.487	5.813	>10
17	8.24	0.872	1.374	6.630	6.389	>10	2.337	5.336	>10
18	118	7.303	7.910	5.715	>10	>10	8.908	>10	>10
21	6.38	5.106	>10	0.933	>10	>10	>10	>10	>10
22	11.4	2.579	2.022	>10	>10	>10	5.144	>10	>10
25	6.83	0.856	>10	0.691	>10	>10	3.861	7.796	>10
27	9.3	0.959	1.546	7.55	>10	>10	2.099	5.618	>10
28	10.5	0.352	0.605	>10	7.783	5.238	0.597	3.456	>10
30	36.4	1.518	3.167	2.820	>10	>10	1.535	5.104	8.428
31	8.25	3.384	6.352	1.076	>10	>10	4.735	>10	>10

group was critical. The hydroxyl in the middle of the side chain caused a dramatic reduction in binding affinity (compound **16** vs **18**). The aldehyde analogues (**19**–**22**) showed an SAR pattern similar to that of the hydroxyl analogues. In particular, compound **21** exhibited better binding affinity despite of lower log *P* value compared to compound **1**. The carboxylic acid analogue (**23**) showed moderately reduced binding affinity compared to the parent compound; however, the ester analogue (**25**) had a similar affinity. The acyloxy analogues (**26**–**28**) showed activities similar to those of the other polar compounds. However, a higher log *P* rather caused a lower binding affinity. Whereas the azide (**29**) and amino (**30**) analogues showed moderate decreases in binding affinities, the amide analogue (**31**) displayed binding affinity even better than that of the corresponding ester (**27**).

We conclude that incorporation of polar groups at the terminus of the linear alkyl chain had little effect on binding affinity in most cases. However, charged polar groups, such as carboxylate and amino, caused a moderate reduction in binding affinity. The position of the polar groups is also critical. Whereas the polar groups are tolerated at the terminus of the chain, there was a dramatic reduction in binding affinity when they were present in the middle of the chain. These results suggest that whereas the middle part of side chain needs to be inserted into the hydrophobic interior of the membrane, the terminus may be able to fold back to the region of the hydrophilic lipid head groups.

The comparison between *c* log *P* and binding affinity indicated that the overall log *P* seems to be critical for binding affinity. The binding affinity of DAG-lactones was correlated with the log *P* of the compounds and a value of 6–7 appeared to be optimal for partitioning between enzyme and membrane. Importantly, the *E*-isomer in this series was more potent than the *Z*-isomer, a result different from our previous findings in the alkylidene series. It is of substantial practical significance because the *E*-isomer is more synthetically accessible and can be obtained exclusively during the alkylation step in the DAG-lactone synthesis.

The antitumor activities of the PKC ligands were evaluated in eight different cell lines and compared with adriamycin and HK-434 as reference compounds. Compound **1** showed significant antitumor activity in a broad range of cell lines with the exception of the lung cancer cell line. In general, the DAG-lactones (compounds **5**, **6**, and **8**) with high affinities (K_i = 3.2–5) showed significant antitumor activities as expected whereas ligands (**9**, **18**, **30**) with low affinities (K_i = 36–198 nM) displayed moderate to low activities. The antitumor activity of the DAG-lactones was selective for the colon cancer and several of the leukemia cell lines compared to adriamycin. In particular, compounds **5**, **6** and **8** demonstrated excellent tumor inhibition against the colon cancer line which was several times more potent than adriamycin.

Interestingly, although compounds **21** and **25** had similar PKC binding affinities and lipophilicities compared to compound **5**, they showed weak cell growth inhibition. The role of intracellular localization in determining functional activity is critical, and the ability of PKCs to phosphorylate their substrates depends not only on their intrinsic level of catalytic activity but also on their proximity to their potential substrates. Typically, PKCs translocate to different cellular compartments in response to ligand binding to the C1 domains. Stability under the conditions of the cellular assays may also be a factor.

In summary, in order to find less lipophilic DAG-lactones with high affinity binding, a series of polar 3-alkylidene DAG-lactones containing ether, hydroxyl, aldehyde, acid, ester, acyloxy, azido, amino or amide functionalities have been synthesized and evaluated as PKC-α ligands and as antitumor agents. Most polar groups except the charged groups were tolerated and found to be comparable to the corresponding linear alkyl ones in terms of binding affinity. However, positioning of the polar groups and the overall log *P* of the compounds were critical for binding to the enzyme. The etheric DAG-lactones **5**, **6** and **8** showed not only high binding affinity but also significant antitumor activities, particularly against the leukemia and colon cancer cell lines.

Acknowledgments

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