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Article

Design, Synthesis, and Characterization of Novel *sn*-1 Heterocyclic DAG-Lactones as PKC Activators

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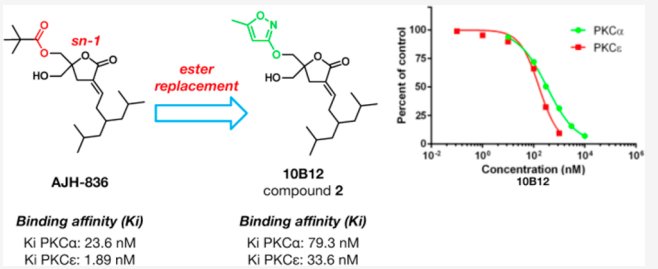
ABSTRACT: DAG-lactones represent useful templates for the design of potent and selective C1 domain ligands for PKC isozymes. The ester moiety at the *sn*-1 position, a common feature in this template, is relevant for C1 domain interactions, but it represents a labile group susceptible to endogenous esterases. An interesting challenge involves replacing the ester group of these ligands while still maintaining biological activity. Here, we present the synthesis and functional characterization of novel diacylglycerol-lactones containing heterocyclic ring substituents at the *sn*-1 position. Our results showed that the new compound **10B12**, a DAG-lactone with an isoxazole ring, binds PKCα and PKCε with nanomolar affinity. Remarkably, **10B12** displays preferential selectivity for PKCε translocation in cells and induces a PKCε-dependent reorganization of the actin cytoskeleton into peripheral ruffles in lung cancer cells. We conclude that introducing a stable isoxazole ring as an ester surrogate in DAG-lactones emerges as a novel structural approach to achieve PKC isozyme selectivity.



INTRODUCTION

Protein kinase C (PKC) isozymes comprise a family of signaling proteins that play essential roles in the control of cellular functions as diverse as cell cycle progression, differentiation, apoptosis, proliferation, motility, and gene expression.[1] The central role of PKC in cellular signal transduction has established it as a validated therapeutic target for various pathologies such as cancer, diabetes, Alzheimer's, and cardiovascular diseases.[2−5]

All members of the PKC family contain in their *N*-terminal regulatory domain one or two copies of C1 domains, a zinc-binding, cysteine-rich motif of 50−51 amino acid residues. In classical/conventional (cPKCs: α, β1, β2, and γ) and novel isoforms (nPKCs: δ, ε, η, and θ), the second messenger diacylglycerol (DAG) binds to the C1a and C1b domain present in their regulatory region, which promotes their translocation to the plasma membrane or other intracellular compartments and subsequent enzyme activation. The C1 domains in cPKCs and nPKCs are also high-affinity binding sites for the phorbol ester tumor promoters, natural compounds that bind to the same site as DAG. Unlike the C1 domains in cPKCs and nPKCs, a single C1 domain present in atypical isoforms (aPKCs: ζ and ι/λ) is unable to bind either phorbol esters or DAG.[6] Individual members of the PKC family have been shown to confer distinctive patterns of cellular responses, including opposite responses in the context of tumor growth.[1]Although PKCs are the best-studied class of signaling proteins with C1 domains,[7] other families of proteins containing DAG/phorbol ester-responsive C1 domains have been later identified. These include RasGRPs, which are guanyl exchange factors for Ras; chimaerins, which are GTPase activating proteins for Rac; DAG kinases (DGKs), enzymes responsible for DAG phosphorylation and the generation of phosphatidic acid; MRCK (myotonic dystrophy kinase-related cdc42-binding kinase), protein kinase D isoforms (PKDs), and Munc-13 isoforms.[8,9] Despite the similar overall structure of C1 domains in these proteins, subtle differences may confer differential ligand binding and membrane association properties to individual members of the family, thus encouraging the generation of agents capable of displaying binding selectivity among the different phorbol ester receptors and specificity for activation. This would also be useful to dissect PKC isozyme-specific responses in cellular models.

Among the multiple C1 domain ligands generated as agents capable of activating PKC isozymes, diacylglycerol lactones (DAG-lactones) represent a unique class of DAG mimetics in which the flexibility of the structure has been constrained to reduce the entropic loss due to binding. Indeed, the natural second messenger DAG displays a rather weak affinity for cPKCs and nPKCs, largely reflecting the flexibility of the

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glycerol backbone, with the consequence that the immobilization of the glycerol upon binding is entropically unfavorable. The design of DAG-lactones, pioneered by the Marquez and Blumberg laboratories, constitutes the greatest synthetic efforts to date, leading to the development of potent C1 domain ligands in which the glycerol backbone of DAG has been cyclized, leading to a rigid structure that eliminates the entropic penalty. Through appropriate manipulation of the hydrophobic side chains, it has been possible to obtain DAG-lactone analogues displaying higher affinity than the natural DAGs and, in many cases, achieving nanomolar binding affinities similar to those of phorbol esters or related compounds derived from natural sources.[10−12]

Modeling and docking experiments have revealed two different modes of binding for diacylglycerol itself and the DAG-lactones, defined by the interaction between the C1 domain and either the *sn*-1 carbonyl or the *sn*-2 carbonyl in the DAG-lactone structure (Figure 1).[13] These two comparable

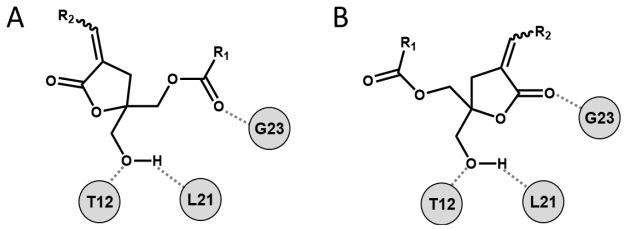

Figure 1. Schematic illustration of the C1 domain hydrogen bonding interactions in the alternative *sn*-1 (A) and *sn*-2 (B) binding modes for DAG-lactones.

binding modes form identical networks of hydrogen bonds with receptor amino acids Thr 12, Leu21, and Gly23, as is observed in the crystal structure of PKCδ C1b with phorbol 13-*O*-acetate.[14] For convenience in comparing C1 domains from different isozymes, we use residue numbering internal to the C1 domain, with residue 1 corresponding to the first zinc-binding histidine and residue 50 corresponding to the last zinc-binding cysteine.

The carbonyl that is not directly involved in C1 domain binding contributes to the binding energy through interactions with the C1 domain surface and the lipid bilayer in the C1 domain−ligand−membrane ternary complex. Thus, both carbonyl groups have been shown to be essential for a strong interaction with PKC. The distinct patterns of acyl (R1) and alkyl (R2) substitution are key elements that determine selectivity, providing significant differences in biological outcome patterns. When this approach was applied, combinatorial libraries of DAG-lactones were produced, leading to compounds with marked selectivity for the RasGRP family relative to their affinity for PKCα and other PKC isozymes.[11,12,15] A study by Ann et al.[16] designed to generate isozyme-specific PKC ligands by the incorporation of linoleic acid derivatives as well as saturated and unsaturated alkyl chains into the side chains of DAG-lactones led to the identification of DAG-lactones with prominent isozyme selectivity. One of these compounds, the DAG-lactone (*E*)-(2-(hydroxymethyl)-4-(3-isobutyl-5-methylhexylidene)-5-oxotetrahydrofuran-2-yl)methyl pivalate (referred to as **AJH-836**), exhibits pronounced selectivity *in vitro* for novel PKCε and PKCδ relative to classical PKCα and PKCβ.[17] Remarkably, **AJH-836** displayed significant selectivity for the translocation of PKCε to the plasma membrane (a readout of activation) relative to PKCα. This selectivity could also be observed at a functional level in cellular models. For example, **AJH-836** is capable of inducing major changes in the reorganization of the actin cytoskeleton, specifically inducing the formation of peripheral membrane ruffles, a response that is mediated primarily by PKCε.[17] On the other hand, **AJH-836** has limited ability to induce transcriptional activation of genes in lung cancer cells, a response that is mainly mediated by PKCα,[18] ultimately reflecting its selectivity for the nPKCs relative to cPKCs.

A fundamental feature common to DAG-lactones is the presence of an ester moiety at the *sn*-1 position that, as discussed above, is important in mediating membrane and receptor interactions; however, this makes the DAG-lactones susceptible to hydrolysis by endogenous esterases, reducing their stability in cells.[19] Previous attempts to replace this labile functional group led to a significant loss in biological activity. Interestingly, the isosteric replacement of the side-chain ester group at the *sn*-1 position with an amide or an *N*-hydroxyl amide group, developed by Marquez and colleagues, revealed a dramatic drop in binding affinity of more than 3 orders of magnitude, suggesting that the new functional groups were not able to adequately recapitulate the polar interactions that the *sn*-1 carbonyl makes in the C1 domain−membrane−DAG-lactone ternary complex.[19,20] In the present study, we designed and synthesized heterocycle-containing DAG-lactones in order to expand the chemical space covered by these ligands, leading to the development of novel activators of DAG-responsive C1 domain-containing proteins. Using **AJH-836** as a lead DAG-lactone structure, we synthesized six new compounds in which we varied the nature of the heterocyclic ring at the *sn*-1 position, specifically replacing the ester moiety with isoxazole (**1**−**2**), triazole (**3**−**4**), and tetrazole (**5**−**6**) groups (Figure 2).

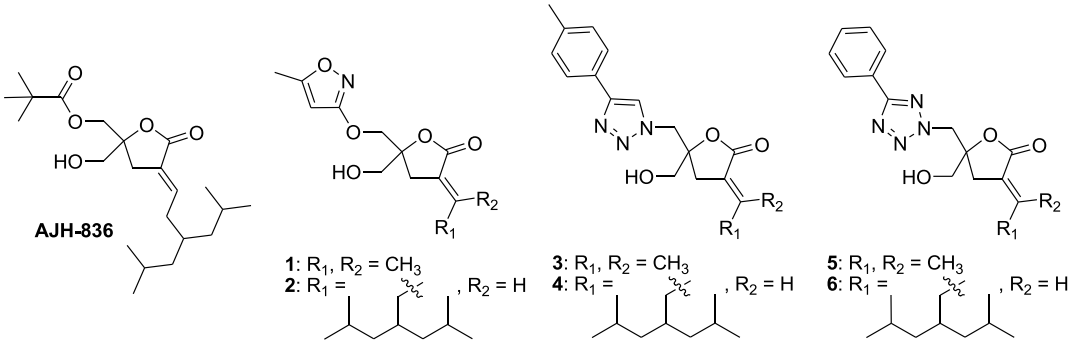

Figure 2. Structure of new heterocyclic DAG-lactones **1**−**6** and **AJH-836**.

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Evaluation of their binding and functional properties in cellular models revealed that a stable isoxazole ring can function as an ester surrogate to allow binding to the C1 domain.

RESULTS AND DISCUSSION

Design of the Compounds. Heterocycle rings play a central role in modern drug design. According to statistics, more than 85% of all biologically active chemical entities contain a heterocycle. The presence of heterocycles provides a useful tool for the modification of solubility, lipophilicity, polarity, and hydrogen bonding capacity of biologically active agents, which results in the optimization of the ADME/Tox properties of drugs or drug candidates.[21] With the aim of increasing metabolic stability, we selected three popular heterocyclic ring moieties present in numerous biologically active compounds for the design of the new DAG-lactones derivatives. In this sense, isoxazole, triazole, and tetrazole heterocycles have provided improved pharmacological properties that have resulted in the development of wide synthetic strategies and methods for the synthesis of these valuable building blocks.[22]

Isoxazole is an azole with an oxygen atom attached to nitrogen often found in natural products and in various pharmaceutical and therapeutic products such as insecticides, antibacterials, antibiotics, antifungals, among others. It is interesting to obtain the isoxazolol derivative since this group represents a bioisostere of the ester group present in our lead structure at the *sn*-1 position.[23,24]

The 1,2,3-triazole unit is a fundamental building block found in different bioactive compounds. One of the most interesting features of triazoles is their ability to mimic the characteristics of different functional groups. This aromatic heterocycle is stable to metabolic and chemical degradation and is capable of acting as a hydrogen bond donor and acceptor, which can be convenient in the binding of biomolecular targets. This explains its wide use as a bioisostere for the synthesis of new active molecules, mainly amide bonds, although there are also some examples where it is used as an ester group isostere to reduce the susceptibility to enzymatic degradation *in vivo*.[25]

Tetrazole is a five-member, planar heterocycle with four nitrogen atoms arranged in a regular fashion rarely present in nature. It has attracted significant attention, especially in medicinal chemistry, because it increases drug bioavailability and is resistant to biological degradation, extending their action.[26,27]

Molecular Modeling. After planning the synthesis, a series of heterocycle-containing DAG-lactones was docked into the crystal structure of the PKCδ C1b domain bound to phorbol ester.[14] This domain has a 62% sequence identity with the PKCα and PKCε C1b domains, and the ligand-binding residues are strictly conserved. These docking experiments allowed us to ensure that the designed heterocyclic compounds fit into the binding site, to compare their binding mode to other known and previously studied DAG-lactones, and to analyze the interactions formed by the new heterocyclic rings and the C1 domain.

Docking predicted that the isoxazole-containing compounds, which retain an ether oxygen in the linkage between the heterocycle and the lactone ring, are capable of binding in both *sn*-1 and *sn*-2 orientations, like the parent DAG-lactone structures (Figure 3A,B). The triazole and tetrazole DAG-lactones, with a single methylene spacer between the two rings, bind exclusively *sn*-1 (Figure 3C,D). These compounds cannot bind *sn*-2 because the shorter linker does not give enough space

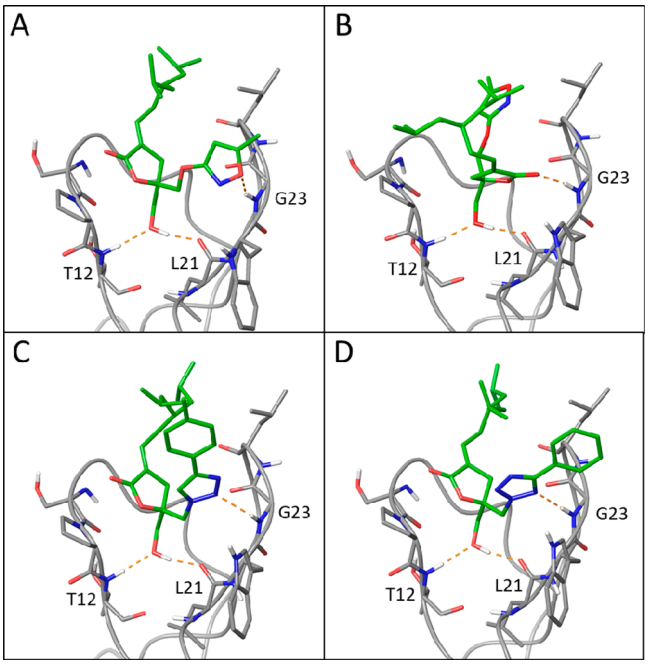

Figure 3. Docked structures of compound **2** in *sn*-1 (A) and *sn*-2 orientations (B) and compounds **4** (C) and **6** (D) in *sn*-1 orientation. Hydrogen bonds are indicated with dashed orange lines. The receptor crystal structure is the PKCδ C1b domain[14] (PDB ID: 1PTR).

to the heterocyclic ring, and instead of extending out of the back of the binding slot, it bumps against a side loop without making any productive interactions.

In the *sn*-1 orientation, the hydrogen bonding interaction between the C1 domain backbone at Gly23 and the DAG-lactone is stronger in the tri- and tetrazole compounds because, with the shorter linker, the ring can adopt an orientation such that the hydrogen bond is in the plane of the ring. With the additional ether oxygen in the linker, in the isoxazole compounds, the NH donor is not aligned with the ring plane, and the hydrogen bonding is a weaker, out-of-plane interaction with an isoxazole oxygen atom.[28]

Previous studies have shown that DAG-lactones prefer to bind in the *sn*-2 orientation, particularly when there is a large, branched alkyl chain at R_2, adjacent to the *sn*-2 carbonyl (Figure 1) as in compounds **2**, **4**, and **6**. In this binding mode, the entropic advantage of constraining the DAG backbone into a lactone ring is realized, with a concomitant increase in binding affinity relative to an equivalent open-chain DAG.[29] This suggested that the isoxazole compounds would exhibit stronger PKC C1 domain binding overall than the other compounds, even though their *sn*-1 binding is predicted to be weaker.

Chemistry. We started the synthesis of new analogues with orthogonally protected lactone **7** bearing a benzyl and a silyl ether group.[30] In order to explore the chemical compatibility between reaction conditions required to build the heterocycles and for hydroxyl group deprotection, we decided to synthesize simple derivatives bearing a propan-2-ylidene group at the *sn*-2 position. This alkyl substituent was introduced through a well-established procedure using an alkylation−elimination sequence to give lactone **8** according to published methods (Scheme 1).[13,31]

Considering that deprotection conditions used for silyl ethers cleavage were, in principle, compatible with the presence of the isoxazole ring, we decided first to selectively remove the benzyl

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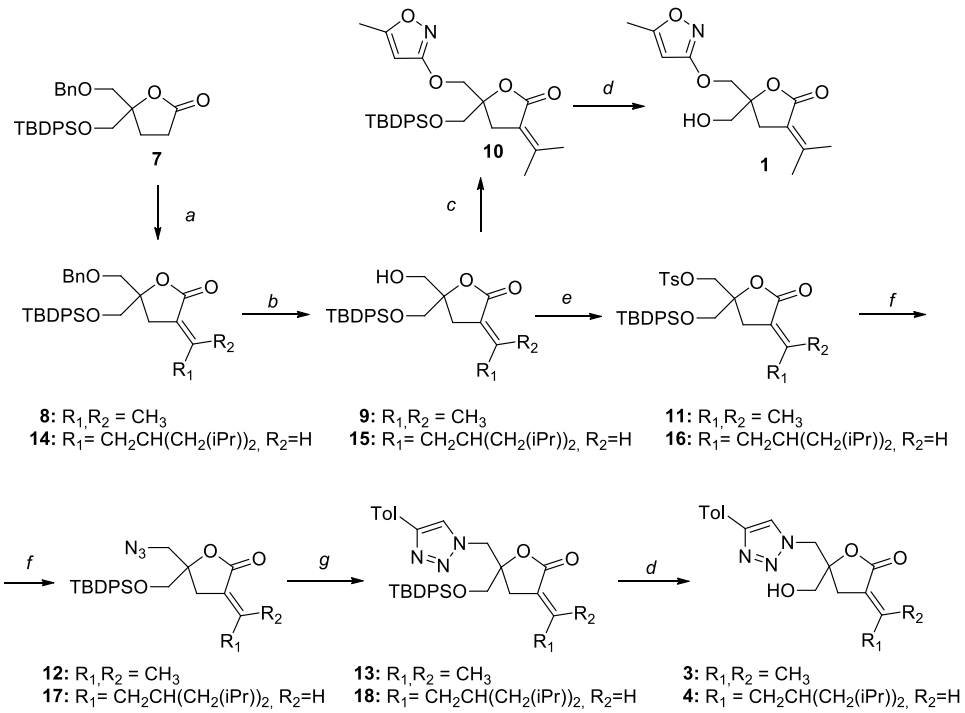
Scheme 1. Synthesis of Isoxazole (1) and Triazole (3−4) Derivatives Employing Orthogonally Protected Lactones[a]



[a]Reagents and conditions: (a) [1] R_1R_2CO, LiHMDS, THF, −78 °C, 2−4 h; [2] (i) CH_3SO_2Cl, Et_3N, 0 °C, 5 h; (ii) DBU, CH_2Cl_2, room temp, 24 h, 62% for **8**, 35% for **14** (*E*/*Z* regioisomers 69%); (b) BCl_3, CH_2Cl_2, −78 °C, 2 h, 97% for **9**, 89% for **15**; (c) 5-methy-3-isoxazolol, PPh_3, DIAD, THF, 24 h, 52%; (d) TBAF, THF, 0 °C, 1 h, 80% for **1**, 80% for **3**, 60% for **4**; (e) *p*-TsCl, pyr, CH_2Cl_2, 24 h, (40 °C) 88% for **11**, (90 °C) 90% for **16** (f) NaN_3, DMF, 100 °C, 48 h, 96% for **12**, 72% for **17**; (g) 4-ethynyltoluene, sodium ascorbate 1 M, $CuSO_4{\cdot}5H_2O$ 0.3 M, *t*-BuOH/H_2O, 48 h, 75% for **13**, 77% for **18**.

ether group, with BCl_3 at low temperatures, to obtain the known lactone **9**, already described in our previous work, in a good yield.[31]

The heterocyclic ring 5-methy-3-isoxazolol, a commercial product known as himexazole that is employed as a pesticide, was previously synthesized according to methods described in the literature.[32] A convergent strategy based on the Mitsunobu reaction was used to couple this heterocyclic ring to the free hydroxyl group of lactone **9**, using DIAD and PPh_3, to produce compound **10** in a moderate yield (52%). Selective desilylation of the *tert*-butyldiphenylsilyl ether (TBDPS) group under standard conditions (TBAF) was carried out in an excellent yield, obtaining the first heterocyclic-containing DAG-lactone **1** (Scheme 1).

In the past decade, the use of triazoles in medicinal chemistry has received marked attention, leading to the development of regioselective methodologies based on Huisgen 1,3-dipolar cycloaddition. We envisioned the synthesis of triazole analogues in the archetypical "click" reaction: the Huisgen [3+2] cycloaddition between alkynes and azides catalyzed by copper-(I) salts (Cu-AAC) that conduce to the regioselective formation of 1,4-regioisomer.[33] With that idea in mind, the hydroxyl group in compound **9** was transformed to the corresponding tosylate with tosyl chloride in pyridine, which in turn was treated with sodium azide in DMF at elevated temperatures, yielding azide **12** in 85% yield, for both steps. Preparation of triazole **13** was carried out using the click reaction as a key step, between azide **12** and a commercial alkyne, 4-ethynyl toluene, in the presence of $CuSO_4$ and sodium ascorbate, a well-known catalytic system.[34,35] The reaction proceeded regioselectively at room temperature to give **13**, as the 1,4-regioisomer exclusively, in 75% yield, although a long reaction time (48 h) was required to achieve full conversion (Scheme 1). Deprotection of the TBDPS group was accomplished under standard conditions (TBAF) to obtain triazole **3** in 80% yields.

The introduction of variations in the patterns of the substituents attached to the *sn*-1 and *sn*-2 positions on the DAG-lactone has resulted, over the years, in the development of very powerful agonists in the low nM range. One of the most potent analogues prepared to date, obtained by Márquez's group, was the DAG-lactone **AJH-836**. Encouraged by this, we decided to synthesize heterocyclic DAG-lactones maintaining the same alkyl substituent present in **AJH-836** at the *sn*-2 position. In this sense, aldehyde 5-methyl-3-(2-methylpropyl)-hexan-1-one was prepared according to published methods[13,19] and immediately employed in an aldol condensation with lactone **7**, followed by olefination through the alkylation−elimination sequence already mentioned, yielding 69% of the mixture of *E*/*Z* isomers (Scheme 1). Separation of *E*/*Z* geometric isomers was achieved at this stage by column chromatography. The vinyl proton corresponding to the *E*-isomer (**14**) appears in its ^{1}H NMR spectrum as a characteristic multiplet that is further downfield compared to that of the corresponding *Z*-isomer. Following the same synthetic sequence described for the preparation of triazole **3**, we obtained DAG-lactone **4** with similar yields for almost every step, except for TBDPS remotion with TBAF that renders a lower yield.

Attempts to obtain the branched isoxazole **2**, starting with compound **15** and following the strategy based on Mitsunobu coupling employed to synthesize DAG-lactone **1**, were unsuccessful. Consequently, we decided to change the starting material and prepared the known lactone **19**[36] protected with

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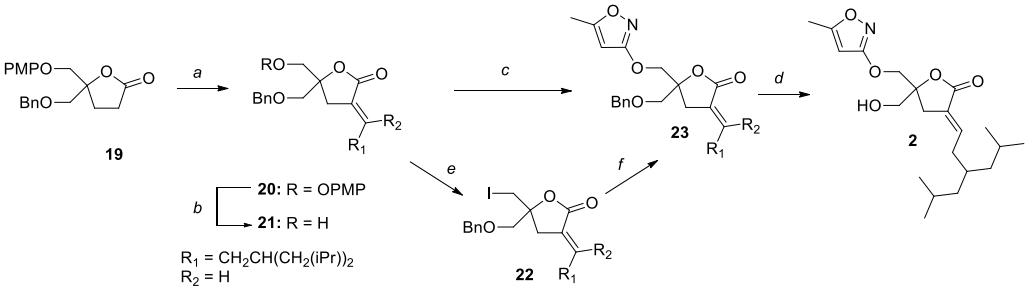
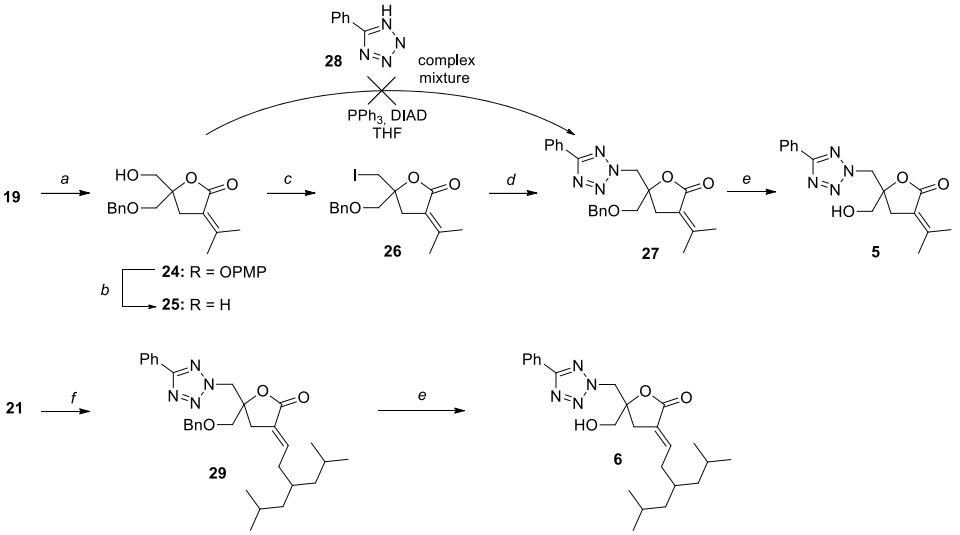
Scheme 2. Different Strategies to the Synthesis of Isoxazole 2[a]



[a]Reagents and conditions: (a) [1] 3-isobutyl-5-methylhexanal, LiHMDS, THF, −78 °C, 2−4 h; [2] (i) CH_3SO_2Cl, Et_3N, 0 °C, 5 h; (ii) DBU, CH_2Cl_2, room temp, 24 h, 35% for **20** (69% for *E*/*Z* mixture); (b) CAN, H_2O/CH_3CN, 75%; (c) 5-methy-3-isoxazolol, PPh_3, DIAD, THF, 24 h, 37% ; (d) BCl_3, CH_2Cl_2, −78 °C, 0.5 h, 67%; (e) I_2, PPh_3, imidazole, toluene, 90 °C, 1 h, 89%; (f) K_2CO_3, DMF, sealed tube, 140 °C, 4 h, 62%.

Scheme 3. Synthesis of Tetrazole Analogues DAG-Lactone 5 and DAG-Lactone 6[a]



[a]Reagents and conditions: (a) [1] acetone, LiHMDS, THF, −78 °C, 2−4 h; [2](i) CH_3SO_2Cl, Et_3N, 0 °C, 5 h; (ii) DBU, CH_2Cl_2, room temp, 24 h, 90% for **24**; (b) CAN, H_2O/CH_3CN, 92%; (c) I_2, PPh_3, imidazole, toluene, 90 °C, 1 h, 95%; (d) 5-phenyl-1*H*-tetrazole, K_2CO_3, DMF, sealed tube, 120 °C, 20 h, 74%; (e) BCl_3, CH_2Cl_2, −78 °C, 0.5 h, 90% for **5**, 78% for **6**; (f) 5-phenyl-1*H*-tetrazole, PPh_3, DIAD, THF, 24 h, 53%.

benzyl and *p*-methoxyphenyl (PMP) groups. The *sn*-2 alkyl substituent was introduced through condensation with the branched aldehyde mentioned before,[37] and after elimination of the *p*-methoxyphenyl (PMP)-protecting group, Mitsunobu coupling with isoxazolol was performed to yield compound **23** in 37% yield. Finally, deprotection of the benzyl group with BCl_3 afforded DAG-lactone **2** in a good yield (Scheme 2).

In view of the low yields obtained, it was decided to test an alternative synthetic route. The conversion of the hydroxyl group in compound **21** to an iodide group was carried out with iodine in the presence of PPh_3 and imidazole in excellent yield.[38] To favor the substitution reaction, compound **22** was treated with isoxazolol and a base (K_2CO_3) at an elevated temperature, in a sealed tube, obtaining the same intermediate **23** in higher yield (55% in two steps) than the Mitsunobu approach.[39]

Both synthetic strategies used in the preparation of isoxazole derivatives could, in principle, be applied in obtaining tetrazole analogues. We decided to prepare derivatives from 5-phenyl-1*H*-tetrazole, a commercially available compound that is easily prepared according to literature procedures by a cycloaddition reaction between benzonitrile and sodium azide catalyzed by copper.[40] Considering that the Mitsunobu route was straightforward, we tried the coupling between tetrazole **28** and the known lactone **25**[41] (Scheme 3). Unfortunately, conversion was low even when adding a large excess of PPh_3 and DIAD. The reaction rendered a complex mixture, and several attempts to purify the desired compound were unsuccessful. Compound **27** was always obtained with variable amounts of byproducts. Deprotection under classical conditions for this type of lactones (BCl_3) allowed us to obtain **5** in 17% total yield from compound **25**. Hence, the alternative strategy based on the nucleophilic substitution of a good leaving group, such as iodine, by the phenyltetrazole anion generated *in situ* in the presence of base was attempted. Indeed, compound **27** was obtained in 70% yield from **25** (two steps). Based on two-dimensional NMR experiments, it was concluded that the 2,5-disubstituted regioisomer was obtained as the only product. Finally, benzyl deprotection led to derivative **5**. Unlike the previous case, when we tested the Mitsunobu reaction employing the branched lactone **21** and compound **28**, we could introduce the tetrazole ring into compound **29**, although in a moderate yield. The complete conversion was observed, and the byproducts were removed efficiently by column chromatography. Benzyl deprotection under standard conditions gave the tetrazolic DAG-lactone **6** (Scheme 3).

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Biological Results. *In Vitro Binding of Novel Heterocyclic DAG-Lactones to PKC Isozymes.* In order to determine the affinity of the newly synthesized DAG-lactones, we used an established *in vitro* competition binding assay, with [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu) as a radioligand. The assay was carried out in the presence of 100 μg/mL phosphatidylserine, as previously described (for more details, see the General Procedures section).[42] We first examined the affinity of the compounds for PKCα. Results revealed that DAG-lactone **2**, from now on **10B12**, could compete [^{3}H]PDBu, displaying a K_i = 79.3 nM. The other DAG-lactones only marginally displaced the radioligand at a concentration of 30 μM. For comparison, the DAG-lactone **AJH-836** bound to PKCα, with a K_i = 23.6 nM.[17] We next determined the ability of **10B12** to bind to PKCε. Competition assays revealed that the K_i for PKCε was 2.4-fold lower than that for PKCα (33.6 nM), suggesting a marginal, yet significant preference for the nPKC, at least *in vitro*. For comparison, **AJH-836** displayed a K_i = 1.89 nM for PKCε, therefore showing 12.4-fold lower K_i than that for PKCα.[17] Two of the DAG-lactones with limited binding for PKCα (**4** and **6**) were also examined for their ability to bind PKCε, and they were found to display limited binding for this nPKC using a fixed concentration of 30 μM (Table 1).

Table 1. DAG-Lactones Binding Affinities for PKCα and PKCε *In Vitro*[a]

		K_I (nM) (or % inhibition, if partial)	
compound	ClogP	PKCα	PKCε
AJH-836	5.52	23.6 ± 2.0[17]	1.89 ± 0.20[17]
1	0.78	31.3% at 30 μM	ND
2 (10B12)	4.75	79.3 ± 20.7	33.6 ± 6.8
3	2.61	11.7% at 30 μM	ND
4	6.58	21.3% at 30 μM	17% at 10 μM
5	2.08	23.5% at 30 μM	ND
6	6.05	37.5% at 30 μM	69.2% at 30 μM

[a]Values represent the mean ± SEM of three independent experiments and were calculated from the ID_{50} values determined from the competition curves. ND: nondetermined. ClogP calculated in ChemDraw.

Previous studies with chemical series of DAG-lactones have shown that there is a parabolic relationship between C1 domain binding affinity and logP, with a peak at a logP of approximately 5. A logP close to this value gives compounds adequate lipophilicity required for partitioning into membranes with the lowest possible amount of nonspecific membrane affinity.[10] Of the heterocyclic DAG-lactones, compounds **10B12** and **6** are closest in logP value to the model compound **AJH-836** and closest to this log P "sweet spot", suggesting that these compounds have the ideal balance between the membrane and C1 domain binding ability.

Differential Translocation of PKCα and PKCε by Heterocyclic DAG-Lactones in Cells. While the [^{3}H]PDBu binding assay using reconstituted 100% phosphatidylserine vesicles is a well-accepted approach to determine the *in vitro* binding affinities of C1 domain ligands to PKC isozymes, assessing their effects in cellular models provides more reliable information about their ability to activate these kinases in their physiological environment. Indeed, small differences in K_i's for individual PKCs, as determined *in vitro*, may translate into significant differences in isozyme specificity in cellular-based assays. We, therefore, examined the ability of heterocyclic DAG lactones to translocate PKC isozymes to membranes, a well-established hallmark of enzyme activation in cells. As an experimental strategy, we expressed Green Fluorescence Protein (GFP)-fused PKCα or PKCε in HeLa cervical adenocarcinoma cells and examined the ability of DAG lactones to induce kinase relocalization to the plasma membrane using fluorescence microscopy. Coupling this imaging analysis to the determination of membrane to cytosol fluorescent ratio allows accurate quantification of enzyme translocation and the precise determination of potencies in cellular models. Using this approach, we determined that **AJH-836** displays 43-fold higher potency for translocation of PKCε relative to PKCα.[17] As shown in the images from Figure 4A and subsequent quantification of

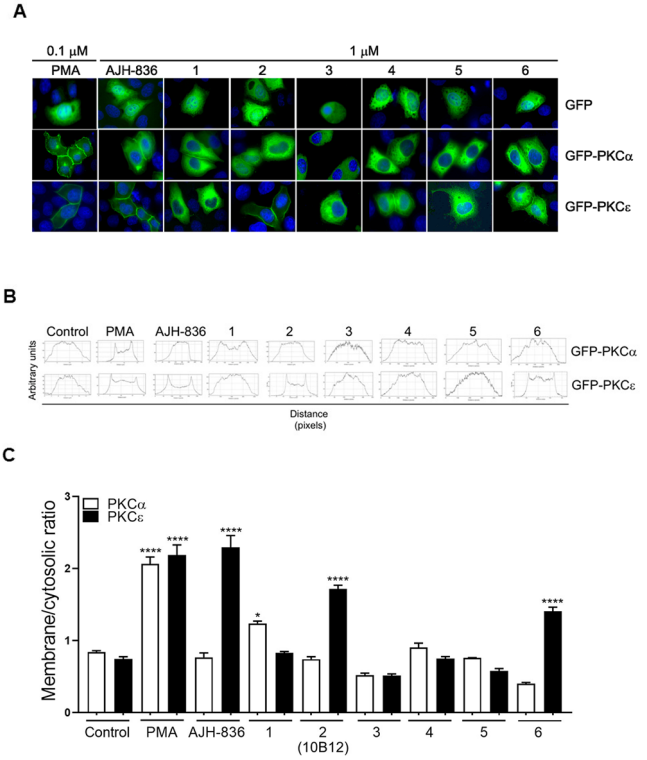

Figure 4. Translocation of PKC isozymes by DAG-lactones. HeLa cells expressing GFP (control), GFP-PKCα, or GFP-PKCε were stimulated for 30 min with the indicated compounds, fixed, counterstained with DAPI, and visualized by fluorescent microscopy. (A) Representative micrographs are shown. (B) Representative graphs obtained using Image G that depicts the quantification of membrane/cytosol ratio. (C) Membrane/cytosol ratio (n = 10−15 cells/group). Results are expressed as mean ± SD *, $p < 0.05$. ****, $p < 0.001$ vs control. Similar results were observed in two additional experiments.

the membrane/cytosol fluorescence ratio in Figure 4B, **10B12** (**2**) induced an evident redistribution of fluorescence in GFP-PKCε expressing HeLa cells. On the other hand, compounds **1**, **3**, **4**, and **5** had essentially no effect on GFP-PKCε redistribution, and fluorescence remained cytoplasmic after treatment. Despite its weak *in vitro* binding affinity, compound **6** also displays some PKCε translocation effect, although smaller than that of **AJH-836**, possibly reflecting other mechanisms contributing to its efficacy in cells such as preferential partition into membranes or unique lipid associations. As controls, we found that the phorbol ester PMA (100 nM), which displays similar activity on cPKCs and nPKCs, fully translocated GFP-PKCα and GFP-PKCε to the plasma membrane, whereas the

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PKCε-selective DAG lactone **AJH-836** (1 μM) only translocated GFP-PKCε, as we established in a previous study.[17]

10B12 Induces Major Changes in Cytoskeletal Morphology. In order to pursue a deeper biological characterization of the heterocyclic DAG-lactones, we next examine a cellular function known to be specifically mediated by PKCε. Specifically, PKCε, but not PKCα, is the PKC implicated in the formation of membrane ruffles, actin-rich protrusions involved in cancer cell motility. Indeed, both PMA- and **AJH-836**-induced ruffle formation in A549 lung cancer cells was severely affected upon silencing PKCε by means of RNA interference (RNAi) or pretreatment with a selective PKCε pharmacological inhibitor.[17] To address the effect of the newly synthesized DAG-lactones on ruffle formation, we treated A549 cells with the different compounds (1 μM, 30 min) and then subjected cells to polymerized actin staining using phalloidin. Upon microscopy examination, we observed a prominent stimulation of ruffle formation in response to **10B12** treatment. No effects were observed for the other synthesized DAG-lactones, other than a small response by DAG-lactone **6** (Figure 5A). Quantification of ruffle formation using a densitometric

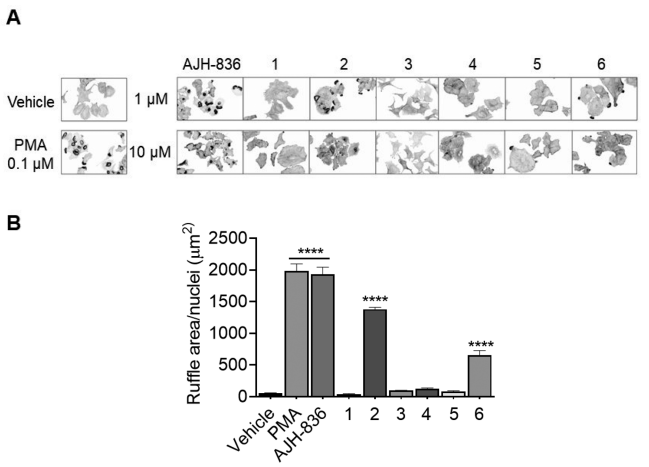

Figure 5. Stimulation of ruffle formation by DAG-lactones. A549 cells were stimulated for 30 min with DAG-lactones (1 μM) or PMA (0.1 μM). Cells were fixed and stained with phalloidin-rhodamine. (A) Representative micrographs. (B) Quantification of ruffle area/cell using ImageJ. ****, $p < 0.001$ vs vehicle.

approach[17] revealed that the effect of **10B12** was near the maximum response caused by 1 μM **AJH-836** and 100 nM PMA (Figure 5B). We also tested a higher dose of the DAG lactones (10 μM), but at this concentration, those compounds that induce ruffles at 1 μM showed significant toxicity, as evidenced by cell detachment and death (data not shown). The rank in potency observed in these experiments essentially matched that observed for GFP-PKCε peripheral translocation.

Finally, in order to confirm the involvement of PKCε in the formation of ruffles induced by **10B12**, we used PKCε RNAi to silence the expression of this kinase in A549 cells. In order to minimize misinterpretations due to the "off-target" effects of RNAi duplexes, we used two different RNAi duplexes (ε1 and ε2). We observed an 80−90% reduction in PKCε mRNA levels (as determined by Q-PCR) (Figure 6A) and ∼70% reduction in PKCε protein expression (as determined by Western blot) 48 h after transfection of either RNAi duplex (Figure 6B). Notably, the formation of ruffles by **10B12** was markedly impaired in PKCε-depleted cells in a magnitude that was consistent with its

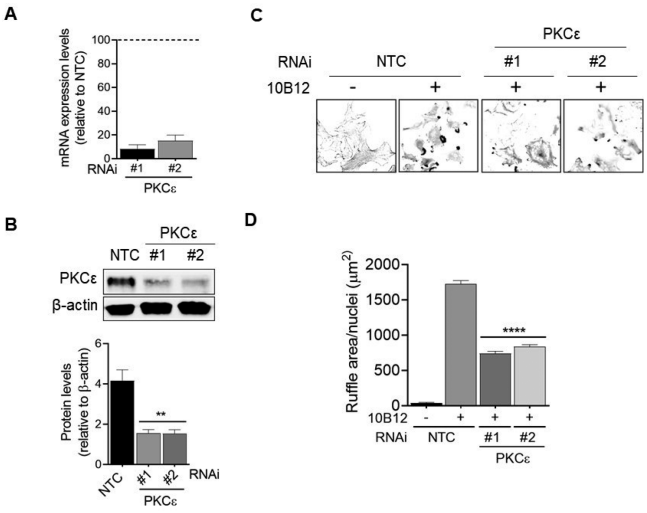

Figure 6. PKCε mediates the ruffle formation response of compound **10B12**. A549 cells were subjected to PKCε RNAi by delivery of specific siRNA duplexes (PKCε1 and PKCε2). As a control, cells were transfected with a nontarget control (NTC) siRNA duplex. Experiments were carried out 48 h later. (A) PKCε mRNA levels relative to NTC (dotted line). (B) PKCε protein levels. Upper panel, representative Western blot. Lower panel, densitometric analysis of PKCε protein levels, expressed as arbitrary units relative to β-actin. (C) A549 cells were stimulated for 30 min with **10B12** (1 μM), fixed, and stained with phalloidin-rhodamine. Upper panel. Representative micrographs. (D) Quantification of ruffle area/cell using ImageJ. ****, $p < 0.001$ vs **10B12**-treated cells, NTC.

depletion (Figure 6C,D), as was previously observed with **AJH-836** using a similar approach.[17] Together with the membrane translocation studies, these actin reorganization experiments establish **10B12** as a *bona fide* PKCε-activating DAG-lactone.

CONCLUSION

Over the past decades, a large number of DAG-lactones analogues with broad structural variation have been designed and synthesized, generating diversity at the *sn*-1 and *sn*-2 positions and covering an extensive chemical space. Most of these compounds have an ester moiety at the *sn*-1 position that is important for receptor interaction, and only a few attempts have been made to replace it. In this study, different types of heterocyclic groups were introduced as substituents for the DAG-lactone structure at the *sn*-1 position. Synthetic strategies were developed for the introduction of isoxazolol, triazole, and tetrazole rings as ester moiety replacements. The isoxazolol ring was introduced in DAG-lactone **1** by a Mitsunobu approach starting from lactone **7**. A change in the protective groups of the starting material, following the Mitsunobu route, led the analogues with a saturated-branched alkyl chain at the *sn*-2 position (DAG-lactone **2**, **10B12**) in low yields. An improvement in the **2** yield was achieved by employing a synthetic alternative based on a substitution reaction. Triazole analogues **3** and **4** were obtained in good yields by methodologies based on the copper(I)-catalyzed variant of the Huisgen 1,3-dipolar cycloaddition between alkynes and azides catalyzed. The first attempts to introduce a tetrazole ring in DAG-lactones **5** and **6** were carried out in Mitsunobu conditions. Compound **6** was straightforwardly obtained by this route, but the strategy did not proceed well for compound **5**, which was obtained using the alternative based on the nucleophilic substitution of a good leaving group by the phenyltetrazole anion.

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Modeling predicted that compound **10B12**, with its higher logP and its ability to adopt the favored *sn*-2 binding mode for DAG-lactones, would have a stronger C1 domain binding ability, and this was in agreement with the *in vitro* binding assay results. However, as has been shown previously with PKCα-binding and RasGRP-binding DAG-lactones,[43] the binding affinity to C1 domains is only one aspect of the biological effects of a DAG-lactone, and compound **6**, with relatively weak C1 domain binding, was also able to induce GFP-PKCε translocation and a small effect on ruffle formation. Agents that belong to the different families of C1 domain ligands, such as phorbol esters, deoxyphorbol esters, and bryostatins, display similar affinities *in vitro* for individual PKC isoforms, yet these ligands have widely diverse biological functions.[44,45] This is epitomized by bryostatins and deoxyphorbol esters, compounds that display antitumor-promoting activities in mouse models rather than the typical tumor promoting effects caused by phorbol esters. Although not fully understood, it may be possible that additional mechanisms, namely, the interactions of ligands with lipid microdomains, the time-dependent concentration of ligand in different subcellular membranes, and/or the formation of specific ligand−protein−membrane ternary complexes, may greatly influence the biological responses of C1 domain-binding compounds. While these mechanisms still remain subject to experimental examination, it is clear that such rich diversity in the effects of C1 domain ligands underscores opportunities for the generation of PKC isozyme-specific ligands. In this regard, our data is in line with previous studies demonstrating that it is possible to achieve remarkable selectivity upon introduction of side chains in cyclized DAG structures, as has been previously demonstrated by means of combinatorial libraries for DAG-lactones with different alkyl-chain and acyl-chain substitutions.[12]

The present results highlight the feasibility of generating C1 domain ligands with preferential selectivity toward specific members of the PKC family, a highly complex task due to the remarkable similarity among C1 domains. In previous studies, we demonstrated significant selectivity toward PKCε for the DAG-lactone **AJH-836**. This derivative represents, to date, the best-characterized example for a cyclized DAG with preferential selectivity for an nPKC relative to cPKCs.[16−18] Similar to **AJH-836**, the DAG-lactone **10B12** synthesized in the present study, in which the ester moiety at the *sn*-1 position has been replaced by an isoxazole ring, displays a significant degree of specificity for PKCε relative to PKCα, particularly in a cellular context. Indeed, although the [^{3}H]PDBu competition binding assays revealed a lower PKCε/PKCα selectivity index for **10B12** compared to **AJH-836**, it shows a preferred ability to translocate PKCε relative to PKCα. This selectivity is reflected in its ability to induce the reorganization of the cellular actin cytoskeleton into peripheral ruffles, a well-described PKCε-mediated effect. We carefully validated this conclusion by means of PKCε RNAi silencing, which was able to abrogate the activity of the new *sn*-1 derivative to form ruffles in lung cancer cells. Thus, a stable isoxazole ring can function as an ester surrogate to allow binding to C1 domain and dictate specificity toward PKCε as a receptor in a cellular environment. Since PKCε is very important for tumor growth and metastasis and has been implicated in cancer cell motility and invasiveness,[46,47] novel *sn*-1 derivatives may have significant utility for the study of this kinase in cancer progression. Considering the limited availability of isozyme-specific PKC activators, this class of C1 domain ligands may represent a novel tool that promotes PKC isozyme biological response patterns.

In summary, our study provides a proof of principle for the generation of DAG-lactones with replacements in the labile ester group at the *sn*-1 position having significant potency for PKC activation. DAG-lactones with a stable isoxazole ring that can function as an ester surrogate represent a novel class of PKC activators, thus expanding the diversity of C1 domain ligands with PKC isozyme selectivity. Given the simplicity of the DAG-lactone structure for chemical modifications, the search for additional substitutions could help in the design of additional potent nPKC selective agents for experimentally assessing the functional involvement of these kinases in human diseases.

EXPERIMENTAL SECTION

General Procedures. All chemical compounds were purchased from commercial sources or synthesized. Reaction monitoring was performed on Merck silica gel 254F TLC plates. Column chromatography was performed on a Teledyne Isco CombiFlash Rf+ instrument under gradient elution conditions with RediSep disposable flash columns. All melting points were determined on an Electrothermal IA9000 series digital melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were carried out on a Bruker Fourier 300, 300 MHz or Bruker Avance DPX 400, 400 MHz spectrometer using $CDCl_3$, MeOD, or DMSO-d_6 as the solvent and TMS as the internal standard. HRMS was performed at CIBION (Bioanalytical Mass Spectrometry Group). High-resolution positive ion electrospray ionization MS was conducted on a Xevo G2S Q-TOF spectrometer (Waters Corporation, Manchester, UK) operated at both positive and negative modes. Data processing was carried out using MassLynx v4.1 software (Waters Corp.). Data was corrected during acquisition using a reference compound (LockSpray). The purity was determined by high-performance liquid chromatography (HPLC). The purity of all final compounds was 95% or higher. HPLC analysis was performed on a Shimadzu SPD-M20A UV−vis photo diode array (PDA) detector, HPLC system (Shimadzu, Tokyo, Japan). The column used was a Zorbax Eclipse XDB C8 (5 μm, 150 mm × 4.60 mm) at a temperature of 40 °C, using a linear gradient of 0−100% H_2O (0.1% HAcO)/acetonitrile at a flow rate of 1.0 mL/min over 20 min. All title compounds were examined for PAINS and passed.[48]

Docking Studies. Compounds were docked into the crystal structure of the C1B domain of PKC δ[14] (PDB ID: 1PTR) using the program GOLD, version 5.2.2.[49,50] We used standard default genetic algorithm (GA) settings, with the binding site defined by atoms within a 10.0 Å radius of the Nε atom of residue Q257. The scoring function was GoldScore, and we added a template similarity constraint to the hydrogen bond acceptor atoms in bound phorbol-13-*O*-acetate from the crystal structure. Twenty GA runs were performed for each compound. After docking, the docked poses were refined with 500 steps of conjugate gradient energy minimization using the Embrace facility in MacroModel.[51] The ligand and residues 240−242, 250−253, and 257 in the C1 domain were free to move, residues 237−239, 243, and 254−256 were restrained with a force constant of 100 kJ/mol Å^2, and all other protein residues were fixed in place.

Chemistry. *General Procedure A: Aldol Condensation Followed by Olefination. Standard Alkylation Procedure.* A solution of lactone **7** or **19** (1 equiv) in THF (5 mL/mmol) at −78 °C was treated dropwise with LiHMDS (1.5−2.0 equiv, 1 M in THF). After the mixture was stirred at −78 °C for 1 h, a solution of R_2CHO (1.5 equiv) or acetone (5 equiv) in THF (1 mL/mmol) was added, and the stirring continued for 2−3 h at −78 °C. The reaction was quenched by the slow addition of a saturated aqueous solution of NH_4Cl and allowed to warm to room temperature. The layers were separated, and the aqueous layer was extracted with Et_2O (3×). The combined organic phases were washed with H_2O (1×) and brine (1×), dried over $MgSO_4$, and concentrated *in vacuo*. Purification by silica gel flash column chromatography (gradient 0−20% EtOAc/hexanes) gave a mixture of alcohol diastereomers, which were used directly in the next step.

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Standard Mesylation–Olefination Procedure. A solution of the alkylation product in dichloromethane (10 mL/mmol) at 0 °C was treated with methanesulfonyl chloride (2 equiv) and triethylamine (4 equiv) and then stirred at room temperature for 2–5 h. DBU (3–5 equiv) was added at 0 °C, and the resulting solution was stirred overnight at room temperature. The reaction mixture was treated with a saturated solution of NH_4Cl (10 mL/mmol) and extracted with CH_2Cl_2 (3×). The combined organics were washed with H_2O (2×) and brine (1×), dried (Na_2SO_4), and concentrated *in vacuo*. Purification by silica gel flash column chromatography (gradient 0–20% EtOAc/hexanes) gave **14**, **20**, and **24**.

General Procedure B: BnO Deprotection. A solution of **14**, **22**, **27**, or **29** (1 equiv) in anhydrous dichloromethane (20 mL/mmol) at −78 °C was treated dropwise with boron trichloride (1 M in CH_2Cl_2, 2 equiv). The reaction was monitored by TLC and quenched upon completion (0.5–1 h) by the slow addition of $NaHCO_3$, and the aqueous phase was extracted with dichloromethane (2 × 15 mL). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. Purification by silica gel flash column chromatography (gradient 0–20% EtOAc/hexanes) gave **15**, **2**, **5**, and **6**.

General Procedure C: PMP Deprotection. Ceric ammonium nitrate (3 equiv) was added to a solution of **20** or **24** (1 equiv) in 80% CH_3CN/H_2O (v/v, 10 mL/mmol) at 0 °C. The reaction mixture was stirred for 15 min. A solution of 5% $Na_2S_2O_3$ was added at room temperature, and the aqueous phase was extracted with CH_2Cl_2 (3×). The organic phase was dried (Na_2SO_4) and concentrated *in vacuo*. Purification by silica gel flash column chromatography (gradient 0–30% EtOAc/hexanes) gave **21** and **25**.

*5-(((tert-Butyldiphenylsilyl)oxy)methyl)-5-(((5-methylisoxazol-3-yl)oxy)methyl)-3-(propan-2-ylidene)dihydrofuran-2(3H)-one (**10**).* A solution of triphenylphosphine (557 mg, 2.0 mmol), lactone **9**[31] (638 mg, 1.5 mmol), and 5-methylisoxazol-3-ol[32] (119 mg, 1.2 mmol) in anhydrous tetrahydrofuran (6 mL) was treated with diisopropyl azodicarboxylate (410 μL, 2.0 mmol) and stirred at room temperature overnight. The reaction mixture was evaporated, and the residue was purified by flash chromatography using hexane/ethyl acetate (8:2) as an eluent to afford **10** as a white solid (314 mg, 52%). Mp: 93–94 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67–7.65 (m, 4H), 7.42–7.40 (m, 6H), 5.62 (s, 1H), 4.38–4.32 (m, 2H), 3.78 (s, 2H), 2.90 (AB d, *J* = 16.4 Hz, 1H), 2.78 (AB d, *J* = 16.4 Hz, 1H), 2.34 (s, 3H), 2.28 (s, 3H), 1.88 (s, 3H), 1.05 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.6, 170.6, 169.1, 150.2, 135.6, 135.6, 132.7, 132.5, 129.9, 127.8, 127.8, 119.5, 92.9, 81.2, 70.5, 66.0, 35.3, 26.6, 24.5, 19.8, 19.1, 12.9. HRMS (ESI): *m*/*z* calcd for $C_{29}H_{35}NO_5Si$ $[M + H]^+$, 506.2363; found, 506.2364.

*5-(Hydroxymethyl)-5-(((5-methylisoxazol-3-yl)oxy)methyl)-3-(propan-2-ylidene)dihydrofuran-2(3H)-one (**1**).* To a stirred solution of **10** (160 mg, 0.32 mmol) in anhydrous THF (3 mL) was added tetrabutylammonium fluoride (1.0 M in THF, 320 μL, 0.32 mmol) at 0 °C. After the mixture was stirred for 1 h, the reaction was evaporated. The residue was purified by column chromatography over silica gel with hexane/ethyl acetate (6:4) as an eluent to afford **1** (68 mg, 80%) as a white solid. Mp: 139–140 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.63 (s, 1H), 4.33–4.27 (AB q, *J* = 11.1 Hz, 2H), 3.69 (m, 2H), 3.15 (t, *J* = 6.6 Hz, 1H), 2.85 (AB d, *J* = 16.5 Hz, 1H), 2.73 (AB d, *J* = 16.5 Hz, 1H), 2.30 (s, 3H), 2.23 (s, 3H), 1.86 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.8, 170.9, 169.1, 151.9, 118.7, 92.9, 81.3, 70.5, 64.3, 31.9, 24.5, 19.9, 12.8. HRMS (ESI): *m*/*z* calcd for $C_{13}H_{18}NO_5$ $[M + H]^+$, 268.1185; found, 268.1190.

*(2-(((tert-Butyldiphenylsilyl)oxy)methyl)-5-oxo-4-(propan-2-ylidene)tetrahydrofuran-2-yl)methyl 4-methylbenzenesulfonate (**11**).* To a solution of compound **9** (127 mg, 0.30 mmol) in CH_2Cl_2 (2 mL) in a sealed vial were added triethylamine (606 mg, 6 mmol) and *p*-toluenesulfonyl chloride (114 mg, 0.6 mmol). The reaction mixture was heated at 40 °C overnight and treated with 1 N HCl (5 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 5 mL). The combined organic layers were washed with $NaHCO_3$ (sat. solution, 5 mL) and NaCl (sat. solution, 5 mL), dried over Na_2SO_4, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (9:1) to afford **11** as a white solid (148 mg, 88%). Mp: 44–46 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.75 (d, *J* = 8.3 Hz, 2H), 7.63–7.56 (m, 4 H), 7.47–7.35 (m, 6H), 7.31 (d, *J* = 8.0 Hz, 1H), 4.12 (d, *J* = 10.1 Hz, 1H), 4.05 (d, *J* = 10.1 Hz, 1H), 3.60 (s, 2H), 2.79–2.65 (m, 2H), 2.43 (s, 3H), 2.21 (t, *J* = 2.0 Hz, 3H), 1.82 (s, 3H), 0.98 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.5, 151.1, 145.2, 135.5, 132.4, 132.3, 132.1, 129.9, 128.0, 127.8, 127.8, 118.7, 80.3, 70.2, 66.0, 32.0, 26.5, 24.5, 21.6, 19.8, 19.1. HRMS (ESI): *m*/*z* calcd for $C_{32}H_{39}O_6SSi$ $[M + H]^+$, 579.2237; found, 579.2231.

*5-(Azidomethyl)-5-((tert-butyldiphenylsilyloxy)methyl)-3-(propan-2-ylidene)dihydrofuran-2(3H)-one (**12**).* To a solution of tosylate **11** (140 mg, 0.245 mmol) in DMF (3 mL) in a sealed vial was added sodium azide (319 mg, 4.9 mmol). The reaction mixture was heated at 120 °C for 4 h, then cooled to room temperature, and evaporated. The residue was dissolved in CH_2Cl_2 and washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (95:5) to afford **12** (105 mg, 96%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.67–7.62 (m, 4H), 7.47–7.36 (m, 6H), 3.65 (AB m, 2H), 3.52 (d, *J* = 12.8 Hz, 1H), 3.46 (d, *J* = 12.8 Hz, 1H), 2.73 (AB m, 2H), 2.25 (s, 3H), 1.85 (s, 3H), 1.04 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.7, 150.7, 135.6, 135.6, 132.6, 132.5, 129.9, 127.8, 119.3, 81.9, 66.8, 55.1, 32.8, 26.6, 24.5, 19.9, 19.2. HRMS (ESI): *m*/*z* calcd for $C_{25}H_{32}N_3O_3Si$ $[M + H]^+$, 450.2213; found, 450.2218.

*5-((tert-Butyldiphenylsilyloxy)methyl)-3-(propan-2-ylidene)-5-((4-p-tolyl-1H-1,2,3-triazol-1-yl)methyl)dihydrofuran-2(3H)-one (**13**).* 4-Ethynyltoluene (25 mg, 26 μL, 0.21 mmol) and compound **12** (95 mg, 0.21 mmol) were dissolved in 5 mL of a 1:1 *tert*-BuOH/H_2O mixture. While the mixture was being stirred, sodium ascorbate (1 M in H_2O, 189 μL, 0.189 mmol) was added, followed by copper(II) sulfate pentahydrate (0.3 M, 280 μL, 0.084 mmol). The resulting mixture was stirred at room temperature for 18 h before dilution with ethyl acetate (10 mL) and saturation with EDTA solution (0.4 mL) and water (10 mL). The aqueous layer was extracted with ethyl acetate (10 mL × 3). The combined organic layers were dried over Na_2SO_4, filtered through a short silica gel column, and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (9:1) to afford **13** (89 mg, 75%) as a white solid. Mp: 59–62 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (s, 1H), 7.68 (d, *J* = 8.3 Hz, 2H), 7.66–7.63 (m, 4H), 7.46–7.36 (m, 6H), 7.23 (d, *J* = 8.0 Hz, 2H), 4.72 (AB d, *J* = 14.5 Hz, 1H), 4.61 (AB d, *J* = 14.5 Hz, 1H), 3.72 (AB d, *J* = 10.9 Hz, 1H), 3.65 (AB d, *J* = 10.8 Hz, 1H), 2.75 (AB q, *J* = 16.6 Hz, 2H), 2.38 (s, 3H), 2.06 (s, 3H), 1.74 (s, 3H), 1.08 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.5, 151.8, 148.3, 138.1, 135.6, 135.5, 132.3, 132.2, 130.1, 129.5, 127.9, 127.4, 125.7, 121.0, 118.3, 81.5, 67.2, 54.0, 32.3, 26.7, 24.5, 21.3, 19.9, 19.2. HRMS (ESI): *m*/*z* calcd for $C_{34}H_{40}N_3O_3Si$ $[M + H]^+$, 566.2839; found, 566.2847.

*5-(Hydroxymethyl)-3-(propan-2-ylidene)-5-((4-p-tolyl-1H-1,2,3-triazol-1-yl)methyl) dihydrofuran-2(3H)-one (**3**).* To a stirred solution of **13** (60 mg, 0.106 mmol) in anhydrous THF (3 mL) was added tetrabutylammonium fluoride (TBAF, 1.0 M solution in THF, 106 μL, 0.106 mmol) at 0 °C. After the mixture was stirred for 1 h, the reaction was evaporated. The residue was purified by column chromatography over silica gel with hexane/ethyl acetate (2:8) as an eluent to give the alcohol **3** (28 mg, 80%) as a white solid. Mp: 167–168 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (s, 1H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.23 (d, *J* = 8.0 Hz, 2H), 4.69 (AB q, *J* = 14.6 Hz, 2H), 3.61 (dq, *J* = 12.2 Hz, 6.5 Hz, 2H), 3.02 (t, *J* = 6.6 Hz, 1H), 2.84 (AB q, *J* = 16.5 Hz, 2H), 2.37 (s, 3H), 2.10 (s, 3H), 1.80 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.5, 153.5, 148.3, 138.3, 129.5, 127.3, 125.7, 121.1, 117.6, 81.4, 65.3, 53.9, 32.5, 24.6, 21.3, 20.0. HRMS (ESI): *m*/*z* calcd for $C_{18}H_{22}N_3O_3$ $[M + H]^+$, 328.1661; found, 328.1665.

*(E)-5-((Benzyloxy)methyl)-5-(((tert-butyldiphenylsilyl)oxy)methyl)-3-(3-isobutyl-5-methylhexylidene)dihydrofuran-2(3H)-one (**14**).* Starting from lactone 7[30] (1.654 g, 3.46 mmol) and 3-isobutyl-5-methylhexanal[13] (940 mg, 5.45 mmol), and following general procedure A, the reaction product was isolated as a 1:1 mixture of *E*- and *Z*-isomers. Two fractions were isolated after purification by silica gel flash column chromatography with hexane/ethyl acetate (98:2) as an eluent. The first fraction corresponded to the *Z*-isomer (724 mg, 34%), and the second fraction was the *E*-isomer **14** (763 mg, 35%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64 (d, *J* = 6.9 Hz, 4H), 7.46–7.22 (m,

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11H), 6.74 (m, 1H), 4.53 (s, 2H), 3.73 (AB q, J = 10.7 Hz, 2H), 3.59 (d, J = 10.1 Hz, 1H), 3.53 (d, J = 10.1 Hz, 1H), 2.82−2.69 (AB m, 2H), 2.10 (t, J = 6.2 Hz, 2H), 1.64−1.60 (m, 3H), 1.14−1.06 (m, 4H), 1.02 (s, 9H), 0.85 (d, J = 6.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.3, 139.2, 137.7, 135.6, 135.6, 132.9, 132.6, 129.8, 129.8, 128.4, 127.9, 127.8, 127.7, 127.6, 84.4, 73.7, 71.9, 66.2, 43.9, 43.8, 34.7, 32.8, 30.3, 26.7, 25.2, 23.0, 22.9, 22.7, 22.6, 19.2. HRMS (ESI): m/z calcd for $C_{40}H_{55}O_4Si$ $[M + H]^+$, 627.3870; found, 627.3879.

*(E)-5-((tert-Butyldiphenylsilyloxy)methyl)-5-(hydroxymethyl)-3-(3-isobutyl-5-methylhexylidene)dihydrofuran-2(3H)-one (**15**).* Starting from **14** (644 mg, 1.03 mmol) and following general procedure B, **15** (490 mg, 89%) was obtained as a colorless oil after purification by silica gel flash column chromatography with hexane/ethyl acetate (9:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.64−7.62 (m, 4H), 7.48−7.35 (m, 6H), 6.76 (m, 1H), 3.77−3.62 (m, 4H), 2.87−2.65 (AB m, 2H), 2.12 (t, J = 6.7 Hz, 2H), 1.93 (t, J = 6.8 Hz, 1H), 1.75−1.56 (m, 3H), 1.14−1.07 (m, 4H), 1.03 (s, 9H), 0.86 (d, J = 6.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 170.2, 140.0, 135.6, 135.6, 132.7, 132.5, 130.0, 129.9, 127.8, 127.7, 85.1, 66.0, 65.3, 43.9, 43.8, 34.8, 32.8, 29.7, 26.7, 25.2, 23.0, 22.9, 22.7, 22.6, 19.2. HRMS (ESI): m/z calcd for $C_{33}H_{48}O_4SiNa$ $[M + Na]^+$, 559.3220; found, 559.3224.

*(E)-(2-((tert-Butyldiphenylsilyloxy)methyl)-4-(3-isobutyl-5-methylhexylidene)-5-oxotetra hydro furan-2-yl)methyl 4-methylbenzenesulfonate (**16**).* To a solution of **15** (474 mg, 0.88 mmol) in pyridine (4 mL) was added p-toluenesulfonyl chloride (421 mg, 2.2 mmol). The reaction mixture was heated at 90 °C overnight and treated with 1 N HCl (8 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were washed with $NaHCO_3$ (sat. solution, 10 mL) and NaCl (sat. solution, 10 mL), dried over Na_2SO_4, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography with hexane/ethyl acetate (98:2) to afford **16** (423 mg, 90%) as a white solid. Mp: 135−136 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.74 (d, J = 8.1 Hz, 2H), 7.63−7.55 (m, 4 H), 7.48−7.34 (m, 6H), 7.31 (d, J = 8.1 Hz, 1H), 6.74 (m, 1H), 4.14 (d, J = 10.2 Hz, 1H), 4.06 (d, J = 10.2 Hz, 1H), 3.62 (s, 2H), 2.79−2.65 (m, 2H), 2.43 (s, 3H), 2.08 (t, J = 6.4 Hz, 2H), 1.73−1.55 (m, 3H), 1.15−1.02 (m, 4H), 0.98 (s, 9H), 0.86 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.2, 145.2, 140.9, 135.5, 132.3, 132.2, 130.0, 130.0, 128.0, 127.9, 126.3, 82.1, 70.1, 65.9, 43.9, 43.8, 34.9, 32.8, 30.0, 26.6, 25.2, 22.9, 22.9, 22.6, 22.6, 21.6, 19.2. HRMS (ESI): m/z calcd for $C_{40}H_{55}O_6SSi$ $[M + H]^+$, 691.3489; found, 691.3475.

*(E)-5-(Azidomethyl)-5-((tert-butyldiphenylsilyloxy)methyl)-3-(3-isobutyl-5-methylhexylidene)dihydrofuran-2(3H)-one (**17**).* To a solution of tosylate **16** (400 mg, 0.58 mmol) in DMF (3 mL) was added sodium azide (754 mg, 11.6 mmol). The reaction mixture was heated at 90 °C for 72 h, then cooled to room temperature, and evaporated. The residue was dissolved in CH_2Cl_2, washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (99:1) to afford **17** (235 mg, 72%) as a colorless syrup. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.66−7.61 (m, 4H), 7.54−7.40 (m, 6H), 6.78 (m, 1H), 3.75−3.67 (mAB, 2H), 3.54 (AB d, J = 12.8 Hz, 1H), 3.47 (AB d, J = 12.8 Hz, 1H), 2.70 (AB m, 2H), 2.11 (t, J = 6.7 Hz, 2H), 1.75−1.56 (m, 3H), 1.17−1.04 (m, 4H), 1.04 (s, 9H),0.86 (m, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.5, 140.5, 135.6, 135.6, 132.5, 132.3, 130.0, 127.9, 126.9, 83.7, 66.5, 55.1, 43.9, 43.8, 34.8, 32.8, 30.7, 26.7, 25.2, 23.0, 22.9, 22.7, 22.6, 19.2. HRMS (ESI): m/z calcd for $C_{33}H_{47}N_3O_3SiNa$ $[M + Na]^+$, 584.3284; found, 584.3290.

*(E)-5-((tert-Butyldiphenylsilyloxy)methyl)-3-(3-isobutyl-5-methylhexylidene)-5-((4-p-tolyl-1H-1,2,3-triazol-1-yl)methyl)-dihydrofuran-2(3H)-one (**18**).* 4-Ethynyltoluene (23 mg, 25 μL, 0.20 mmol) and **17** (102 mg, 0.18 mmol) were dissolved in 4 mL of a 1:1 *tert*-BuOH/H_2O mixture. While the mixture was being stirred, sodium ascorbate (0.3 M in H_2O, 480 μL, 0.144 mmol) was added, followed by copper(II) sulfate pentahydrate (0.3 M, 240 μL, 0.072 mmol). The resulting mixture was stirred at room temperature for 2 h before dilution with ethyl acetate (10 mL) and saturation with EDTA solution (0.4 mL) and water (10 mL). The aqueous layer was extracted with ethyl acetate (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, filtered through a short silica gel column, and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (93:7) to afford **18** (95 mg, 77%) as a colorless syrup. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.85 (s, 1H), 7.67 (d, J = 8.1 Hz, 2H), 7.66−7.61 (m, 4H), 7.49−7.34 (m, 6H), 7.22 (d, J = 8.0 Hz, 2H), 6.62 (m, 1H), 4.74 (AB d, J = 14.5 Hz, 1H), 4.64 (AB d, J = 14.5 Hz, 1H), 3.73 (AB d, J = 11 Hz, 1H), 3.65 (AB q, J = 11 Hz, 1H), 2.82 (AB q, J = 17.6 Hz, 1H), 2.71 (AB q, J = 17.6 Hz, 1H), 2.38 (s, 3H), 1.99 (t, J = 6.6 Hz, 2H), 1.67−1.46 (m, 3H), 1.13−0.93 (m, 4H), 1.08 (s, 9H),0.81 (d, J = 6.5 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 169.2, 148.4, 141.5, 138.1, 135.6, 135.5, 132.2, 132.1, 130.1, 129.5, 127.9, 127.4, 125.8, 125.7, 120.8, 83.1, 66.9, 53.9, 43.8, 34.8, 32.7, 30.2, 29.7, 26.7, 25.1, 22.9, 22.6, 22.6, 21.3, 19.2. HRMS (ESI): m/z calcd for $C_{42}H_{56}N_3O_3Si$ $[M + H]^+$, 678.4091; found, 678.4121.

*(E)-5-(Hydroxymethyl)-3-(3-isobutyl-5-methylhexylidene)-5-((4-p-tolyl-1H-1,2,3-triazol-1-yl)methyl)dihydrofuran-2(3H)-one (**4**).* To a stirred solution of **18** (79 mg, 0.12 mmol) in anhydrous THF (3 mL) was added tetrabutylammonium fluoride (1.0 M solution in THF, 120 μL, 0.12 mmol) at 0 °C. After the mixture was stirred for 1 h, the reaction was evaporated. The residue was purified by column chromatography over silica gel with hexane/ethyl acetate (75:25) as an eluent to afford **4** (31 mg, 60%) as a white solid. Mp: 155−156 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (s, 1H), 7.69 (d, J = 7.7 Hz, 2H), 7.23 (d, J = 7.7 Hz, 2H), 6.72 (m, 1H), 4.79 (AB d, J = 14.6 Hz, 1H), 4.63 (AB d, J = 14.6 Hz, 1H), 3.70−3.45 (m, 2H), 2.93 (t, J = 6.3 Hz, 1H), 2.83 (s, 2H), 2.37 (s, 3H), 2.07 (m, 2H), 1.71−1.49 (m, 3H), 1.15−0.93 (m, 4H), 0.83 (d, J = 5.9 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.1, 148.4, 142.7, 138.3, 129.5, 127.2, 125.7, 125.3, 121.0, 83.10, 65.0, 53.7, 43.9, 34.9, 32.8, 30.6, 25.2, 22.9, 22.9, 22.6, 22.5, 21.3. HRMS (ESI): m/z calcd for $C_{26}H_{38}N_3O_3$ $[M + H]^+$, 440.2913; found, 440.2926.

*(E)-5-((Benzyloxy)methyl)-5-(hydroxymethyl)-3-(3-isobutyl-5-methylhexylidene)dihydrofuran-2(3H)-one (**21**).* Compound **21** was synthesized following a procedure described elsewhere.[37]

Starting from lactone **19**[36] (2.52 g, 7.4 mmol) and 3-isobutyl-5-methylhexanal[13] (1.9 g, 11.41 mmol) and following general procedure A, the reaction product was isolated as a 1:1 mixture of *E*- and *Z*-isomers. Two fractions were isolated after purification by silica gel flash column chromatography with hexane/ethyl acetate (98:2) as an eluent. The first fraction corresponded to the *Z*-isomer (1.23 g, 34%) and the second fraction corresponded to the *E*-isomer **20** (1.27 g, 35%). Recorded spectra perfectly matched the published results. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.26 (m, 5 H), 6.81 (s, 4 H), 6.82−6.76 (m, 1 H), 4.58 (AB q, J = 12.3 Hz, 2H), 4.03 (AB q, J = 9.8, 26.6 Hz, 2H), 3.76 (s, 3H), 3.66 (AB q, J = 10.3, 23.7 Hz, 2H), 2.92−2.77 (AB m, 2H), 2.13 (t, J = 11.6 Hz, 2H), 1.76−1.58 (m, 3H), 1.14−1.06 (m, 4H), 0.85 (m, 12H).

Starting from *E*-isomer **20** (1.26 g, 2.55 mmol) and following general procedure C, **21** was obtained as a colorless oil (746 mg, 75% yield). Its spectrum data perfectly matched with published results. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40−7.26 (m, 5H), 6.82−6.73 (m, 1H), 4.56 (m, 2 H), 3.84−3.64 (m, 2H), 3.57 (AB q, J = 9.9, 28.0 Hz, 2H), 2.83−2.65 (m, 2H), 2.19−2.07 (m, 3H), 1.77−1.55 (m, 3H), 1.15−1.05 (m, 4H), 0.84−0.86 (m, 12H).

*(E)-5-((Benzyloxy)methyl)-5-(iodomethyl)-3-(3-isobutyl-5-methylhexylidene)dihydrofuran-2(3H)-one (**22**).* A solution of **21**[37] (128 mg, 0.33 mmol) in toluene (6 mL) was treated with triphenylphosphine (173 mg, 0.66 mmol), imidazole (67 mg, 0.99 mmol), and iodine (126 mg, 0.5 mmol). The mixture was heated at 90 °C for 1 h and, after reaching room temperature, was poured into a saturated solution of $NaHCO_3$. Excess of triphenylphosphine was destroyed by the addition of iodine until the iodine coloration persisted in the organic layer. The organic layer was washed with $Na_2S_2O_3$ (10%, 5 mL) and brine, dried over ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was purified on silica gel (hexanes:ethyl acetate, 98:2) to give **22** (146 mg, 89%) as a colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38−7.27(m, 5H), 6.82−6.75 (m, 1H), 4.58 (s, 2H), 3.68 (AB d, J = 10.0 Hz, 1H), 3.63 (AB d, J = 10.0 Hz, 1H), 3.49 (AB d, J = 10.8 Hz, 1H), 3.43 (AB d, J = 10.8 Hz, 1H), 2.92−2.72 (AB m, 2H), 2.15−2.08 (m, 2H), 1.76−1.67 (m, 1H), 1.66−1.58 (m, 2H), 1.17−

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1.02 (m, 4H), 0.85 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 169.3, 140.8, 137.3, 128.5, 127.9, 127.7, 127.1, 81.5, 73.8, 73.1, 43.9, 43.9, 34.8, 34.1, 32.8, 25.2, 23.0, 22.9, 22.6, 11.0. HRMS (ESI): m/z calcd for $C_{24}H_{36}IO_3$ $[M + H]^+$, 499.1709; found, 499.1703.

*(E)-5-((Benzyloxy)methyl)-3-(3-isobutyl-5-methylhexylidene)-5-(((5-methylisoxazol-3-yl)oxy)methyl)dihydrofuran-2(3H)-one (**23**).* From **21**, a solution of triphenylphosphine (325 mg, 1.24 mmol), **21** (160 mg, 0.41 mmol), and 5-methylisoxazol-3-ol (62 mg, 0.62 mmol) in anhydrous tetrahydrofuran (5 mL) was treated with diisopropyl azodicarboxylate (243 μL, 1.24 mmol) and stirred at room temperature overnight. The reaction mixture was evaporated, and the residue was purified by flash chromatography using hexane/ethyl acetate (95:5) as an eluent to afford **23** (70 mg, 37%) as a colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.37−7.25 (m, 5H), 6.83−6.73 (m, 1H), 5.59 (s, 1H), 4.57 (s, 2H), 4.41−4.32 (AB q, 2H), 3.65 (AB d, J = 13.4 Hz, 1H), 3.57 (AB d, J = 13.4 Hz, 1H), 2.92−2.70 (AB m, 2H), 2.32 (s, 3H), 2.11 (t, J = 8.0 Hz, 2H), 1.74−1.54 (m, 3H), 1.15−1.02 (m, 4H), 0.85 (d, J = 6.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.6, 170.7, 169.7, 140.3, 137.4, 128.4, 127.8, 127.6, 126.8, 92.9, 82.2, 73.7, 71.5, 70.8, 43.7, 34.7, 32.7, 30.5, 25.2, 22.9, 22.6, 12.9. HRMS (ESI): m/z calcd for $C_{28}H_{39}NO_5Na$ $[M + Na]^+$, 492.2726; found, 492.2742.

From **22**, a solution of iodine **22** (50 mg, 0.10 mmol), K_2CO_3 (140 mg, 1 mmol), and 5-methylisoxazol-3-ol (99 mg, 1 mmol) in DMF (0.5 mL) was stirred at 140 °C for 4 h in a sealed vial. Afterward, the reaction was cooled to room temperature, diluted with CH_2Cl_2 (3 mL), and washed with NaOH (5%) and brine. The organic phase was dried over anhydrous Na_2SO_4, filtered, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (95:5) to afford **23** (29 mg, 62%).

*(E)-5-(Hydroxymethyl)-3-(3-isobutyl-5-methylhexylidene)-5-(((5-methylisoxazol-3-yl)oxy)methyl)dihydrofuran-2(3H)-one (**2**, **10B12**).* Starting from **22** (26 mg, 0.055 mmol) and following general procedure B, compound **2** (**10B12**) was obtained as a white solid (14 mg, 67%). Mp: 81−82 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.80 (m, 1H), 5.63 (s, 1H), 4.40 (AB d, J = 11.3 Hz, 1H), 4.32 (AB d, J = 11.3 Hz, 1H), 3.78−3.67 (m, 2H), 2.85 (AB d, J = 17.1 Hz, 1H), 2.73 (AB d, J = 17.1 Hz, 1H), 2.71 (t, J = 6.9 Hz, 1H), 2.32 (s, 3H), 2.13 (t, J = 6.5 Hz, 2H), 1.76−1.55 (m, 3H), 1.15−1.03 (m, 4H), 0.85 (d, J = 6.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 171.8, 171.0, 169.7, 141.2, 126.5, 92.9, 83.1, 70.5, 64.4, 43.8, 34.8, 32.8, 30.0, 25.2, 25.2, 22.9, 22.6, 12.9. HRMS (ESI): m/z calcd for $C_{21}H_{34}NO_5$ $[M + H]^+$, 380.2437; found, 380.2451.

*5-((Benzyloxy)methyl)-5-(hydroxymethyl)-3-(propan-2-ylidene)-dihydrofuran-2(3H)-one (**25**).* Compound **25** was synthesized following a procedure described elsehwere.[41]

Starting from lactone **19** (3.51 g, 10.24 mmol) and acetone (1.78 g, 30.72 mmol) and following general procedure A, **24** (3.5 g, 90%) was obtained as a white solid. Mp: 77−79 °C. Its spectrum data perfectly matched with published results. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.37−7.26 (m, 5H), 6.87 (s, 4H), 4.65 (AB q, J = 12.3 Hz, 2H), 4.11 (AB q, J = 9.6, 2H), 3.82 (s, 3H),3.71 (AB q, J = 10.2, 2H), 2.97−2.84 (AB m, 2H), 2.33 (t, J = 2.1 Hz, 2H), 1.92 (s, 3H).

Starting from **24** (890 mg, 2.32 mmol) and following general procedure C, **25** was obtained as a colorless oil (586 mg, 92%). Its spectrum data perfectly matched with published results.^{1}H NMR (400 MHz, $CDCl_3$): δ 7.40−7.26 (m, 5H), 4.56 (AB q, J = 12.1, 2H), 3.70 (AB dq, J = 6.6, 12.1, 34.5 Hz, 2H), 3.55 (AB q, J = 9.9, 27.6 Hz, 2H), 2.82−2.67 (m, 2H), 2.25 (m, 4H), 1.86 (s, 3H).

*5-((Benzyloxy)methyl)-5-(iodomethyl)-3-(propan-2-ylidene)-dihydrofuran-2(3H)-one (**26**).* A solution of **25** (200 mg, 0.73 mmol) in toluene (13 mL) was treated with triphenylphosphine (380 mg, 1.45 mmol), imidazole (148 mg, 2.18 mmol), and iodine (277 mg, 1.1 mmol). The mixture was heated at 90 °C for 2 h and, after reaching room temperature, was poured into a saturated solution of $NaHCO_3$. Excess triphenylphosphine was destroyed by the addition of iodine until the iodine coloration persisted in the organic layer. The organic layer was washed with $Na_2S_2O_3$ (10%, 5 mL) and brine, dried over ($MgSO_4$), filtered, and concentrated *in vacuo*. The residue was purified on silica gel (hexanes/ethyl acetate, 9:1) to give **26** (266 mg, 95%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.38−7.27 (m, 5H), 4.64−4.53 (AB m, 2H), 3.68−3.60 (AB m, 2H), 3.49 (AB d, J = 14.2 Hz, 1H), 3.40 (AB d, J = 14.2 Hz, 1H), 2.91−2.73 (AB m, 2H), 2.26 (t, J = 2.8 Hz, 3H), 1.86 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.6, 151.6, 137.4, 128.4, 127.8, 127.7, 119.3, 79.7, 73.7, 73.2, 35.9, 24.6, 20.0, 11.4. HRMS (ESI): m/z calcd for $C_{16}H_{20}IO_3$ $[M + H]^+$, 387.0457; found, 387.0468.

*5-((Benzyloxy)methyl)-5-((5-phenyl-2H-tetrazol-2-yl)methyl)-3-(propan-2-ylidene)dihydrofuran-2(3H)-one (**27**).* A solution of **26** (111 mg, 0.28 mmol), K_2CO_3 (386 mg, 2.8 mmol), and 5-phenyl-1*H*-tetrazole[40] (420 mg, 2.8 mmol) in DMF (1 mL) was stirred at 120 °C overnight in a sealed vial, then cooled to room temperature, diluted with CH_2Cl_2 (3 mL), and filtered. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered, and concentrated under a vacuum. The residue was purified by silica gel flash column chromatography hexane/ethyl acetate (92:8) to afford **27** (84 mg, 74%) as a white solid. Mp: 87−88 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15−8.08 (m, 2H), 7.52−7.45 (m, 3H), 7.38−7.27 (m, 5H), 5.00 (AB d, J = 14.0 Hz, 1H), 4.92 (AB d, J = 14.0 Hz, 1H), 4.67 (AB d, J = 11.9 Hz, 2H), 4.61 (AB d, J = 11.9 Hz, 1H), 3.68 (AB d, J = 10.1 Hz, 1H), 3.60 (AB d, J = 10.1 Hz, 1H), 2.95 (d, J = 16.6 Hz, 1H), 2.75 (d, J = 16.6 Hz, 1H), 2.04 (s, 3H), 1.72 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.0, 165.4, 152.0, 137.2, 130.5, 128.9, 128.5, 128.0, 127.8, 127.0, 126.9, 117.6, 79.9, 73.8, 72.7, 56.9, 33.4, 24.4, 19.8.HRMS (ESI): m/z calcd for $C_{23}H_{25}N_4O_3$ $[M + H]^+$, 405.1927; found, 405.1936.

*5-(Hydroxymethyl)-5-((5-phenyl-2H-tetrazol-2-yl)methyl)-3-(propan-2-ylidene)dihydrofuran-2(3H)-one (**5**).* Starting from **27** (78 mg, 0.194 mmol) and following general procedure B, **5** was obtained as a white solid (55 mg, 90%). Mp: 154−155 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.15−8.08 (m, 2H), 7.52−7.45 (m, 3H), 4.96 (s, 2H), 3.79 (d, J = 6.4 Hz, 2H), 2.95 (d, J = 16.4 Hz, 1H), 2.73 (d, J = 16.4 Hz, 1H), 2.74−2.68 (m, 1H), 2.07 (s, 3H), 1.76 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.1, 165.5, 152.9, 130.6, 128.9, 126.9, 126.8, 117.5, 81.0, 65.8, 56.3, 32.4, 24.5, 19.9. HRMS (ESI): m/z calcd for $C_{16}H_{19}N_4O_3$ $[M + H]^+$, 315.1457; found, 315.1467.

*(E)-5-((Benzyloxy)methyl)-3-(3-isobutyl-5-methylhexylidene)-5-((5-phenyl-2H-tetrazol-2-yl)methyl)dihydrofuran-2(3H)-one (**29**).* A solution of triphenylphosphine (226 mg, 0.85 mmol), lactone **21** (110 mg, 0.29 mmol), and 5-phenyl-1*H*-tetrazole (124 mg, 0.85 mmol) in anhydrous THF (6 mL) was treated with DIAD (168 μL, 0.85 mmol) and stirred at room temperature overnight. The reaction mixture was evaporated, and the residue was purified by flash chromatography using hexane/ethyl acetate (92:8) as an eluent to afford **29** as a white solid (78 mg, 53%). Mp: 85−86 °C.^{1}H NMR (400 MHz, $CDCl_3$): δ 8.14−8.09 (m, 2H), 7.50−7.45 (m, 3H), 7.38−7.28 (m, 5H), 6.68−6.61 (m, 1H), 5.03 (d, J = 14.1 Hz, 1H), 4.93 (d, J = 14.1 Hz, 1H), 4.66 (d, J = 11.9 Hz, 1H), 4.60 (d, J = 11.9 Hz, 1H), 3.68 (d, J = 10.1 Hz, 1H), 3.59 (d, J = 10.1 Hz, 1H), 2.93 (d, J = 17.0 Hz, 1H), 2.76 (d, J = 17.0 Hz, 1H),1.99 (t, J = 6.2 Hz, 2H), 1.66−1.58 (m, 1H), 1.58−1.47 (m, 2H), 1.08−0.91 (m, 4H), 0.82−0.78 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.8, 165.4, 141.4, 137.0, 130.5, 128.9, 128.5, 128.0, 127.8, 126.9, 125.3, 81.5, 73.8, 72.4, 56.6, 43.7, 43.7, 34.7, 32.6, 31.2, 25.1, 22.9, 22.8, 22.6, 22.5. HRMS (ESI): m/z calcd for $C_{31}H_{41}N_4O_3$ $[M + H]^+$, 517.3179; found, 517.3182.

*(E)-5-(Hydroxymethyl)-3-(3-isobutyl-5-methylhexylidene)-5-((5-phenyl-2H-tetrazol-2-yl)methyl)dihydrofuran-2(3H)-one (**6**).* Starting from **29** (50 mg, 0.097 mmol) and following general procedure B, **6** was obtained as a white solid (32 mg, 78%). Mp: 110−112 °C.^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20−8.15 (m, 2H), 7.51−7.49 (m, 3H), 6.70 (m, 1H), 5.10−4.94 (AB m, 2H), 3.79−3.76 (m, 2H), 2.94 (d, J = 17.2 Hz, 1H), 2.73 (d, J = 17.2 Hz, 1H), 2.57 (t, J = 6.4 Hz, 1H), 2.03 (t, J = 6.3 Hz, 2H), 1.67−1.60 (m, 1H), 1.60−1.48 (m, 2H), 1.11−0.93 (m, 4H), 0.82 (d, J = 6.5 Hz, 12H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 168.8, 165.6, 142.2, 130.6, 128.9, 126.9, 126.8, 125.3, 82.5, 65.7, 56.0, 43.8, 43.8, 34.8, 32.7, 30.4, 25.1, 22.9, 22.8, 22.6, 22.5. HRMS (ESI): m/z calcd for $C_{24}H_{35}N_4O_3$ $[M + H]^+$, 427.2709; found, 427.2733.

Determination of *In Vitro* Binding Affinities for PKC Isozymes. Recombinant human PKCα and PKCε isozymes were obtained from ThermoFisher Scientific (Waltham, MA). Compounds were assayed *in vitro* by competition for the binding of [^{3}H]PDBu (9 Ci/mmol; prepared as a custom synthesis by Quotient Bioresearch, Cardiff, United Kingdom). Porcine brain L-α-phosphatidylserine (PS)

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was from Avanti Polar Lipids (Alabaster, AL). Immunoglobulin G was purchased from Sigma-Aldrich (St. Louis, MO). Nonradioactive phorbol 12,13-dibutyrate (PDBu) was purchased from LC Laboratories (Woburn, MA). Polyethylene glycol (PEG) 6000 was obtained from EMD Millipore Corporation (Billerica, MA). Briefly, the assay mixture had a final volume of 250 μL and contained the following components: 50 mM Tris-HCl, pH 7.4; 100 μg/mL L-α-phosphatidylserine; 0.1 mM Ca^{2+} for PKCα assay and 1 mM EGTA for PKCε assay; 5 mg/mL bovine immunoglobulin G and 0.003% Tx-100, 10 μM of nonradioactive phorbol 12,13-dibutyrate (PDBu), 2 nM of [^{3}H]PDBu and increasing concentrations of the competing ligand. The assay tubes were incubated at 37 °C for 5 min and then chilled on ice for 10 min, after which 200 μL of 35% polyethylene glycol (PEG) was added. The tubes were vortexed and chilled for an additional 10 min. The tubes were then centrifuged at 12,200 rpm for 15 min at 4 °C. A 200 μL aliquot of each supernatant was transferred to a scintillation vial for the determination of the free concentration of [^{3}H]PDBu. The assay pellets were dried, cut off, and placed in a separate scintillation vial for the determination of bound [^{3}H]PDBu. The supernatant and pellet vials were immersed in 3 mL of scintillation cocktail (CytoScintEco-Safe, MP Biomedicals, Santa Ana, CA). In each experiment, triplicate measurements at each concentration of ligand were performed. MS Excel was used to process data, and graphing was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Final concentrations for both PKC isozymes assays (fixed): 10 μM cold PDBu (nonspecific), 2 nM [^{3}H]PDBu, 20 ng/tube enzyme, 100 μg/mL L-α-phosphatidylserine (porcine brain). In each experiment, at least triplicate measurements at each concentration of ligand were performed. K_i values were calculated from ID_{50} values determined from the competition curves. MS Excel was used to process data, and graphing was done using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

Translocation of GFP-Fused PKCs. Experiments were carried out essentially as previously described.[17] Briefly, HeLa cells (5×10^4) were transfected with 1 μg of either pEGFP-N1-PKCα or pEGFP-N1-PKCε using Lipofectamine 3000 and plated on cover slides in 24-well plates. After 24 h, cells were serum-starved for 24 h and then stimulated with the different DAG-lactones or PMA for 30 min. Cells were then fixed in methanol, mounted on a glass slide, and visualized with a Nikon TE2000-U fluorescence microscope. Cells were stained with DAPI for nucleus visualization. Translocation was quantitated using ImageJ. A line was traced across the cytoplasm of individual cells (~10−15 cells/group), and the signal intensities and profiles were obtained using the Plot profile tool of the program. The operator was blinded to treatment assignment.

Western Blotting. A549 cells were harvested in lysis buffer containing 50 mM Tris-HCl, pH 6.8, 10% glycerol, and 2% β-mercaptoethanol, and lysates were subjected to SDS-PAGE. After transferring to polyvinylidene difluoride membranes (Millipore Corp., Burlington, MA) and blockade for 1 h with 5% milk in TBS and 0.1% Tween 20, membranes were incubated overnight with either anti-PKCε (1:1000 dilution, Cell Signaling Technology, Danvers, MA) or β-actin (1:500 000, Sigma-Aldrich, St. Louis, MO) antibodies. After extensive washing, membranes were incubated for 1 h with either antimouse (1:1000 dilution) or antirabbit (1:3000 dilution) secondary antibodies conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA). Bands were visualized and subjected to densitometric analysis using an Odyssey Fc system (LI-COR Biosciences, Lincoln, NE).

Quantitative PCR (Q-PCR). Total RNA from cultured cells was extracted using the RNeasy kit as directed by the manufacturer (Qiagen, Valencia, CA). One μg of mRNA template was added to the reverse-transcription master mix (Taq-Man Reverse Transcription kit, Applied Biosystems). The cDNA samples were then diluted with 90 μL of RNase-free water and stored at −20 °C. Q-PCR primers for PKCε and UBC (housekeeping gene for normalization) were purchased from Applied Biosystems. PCR amplifications were performed using an ABI PRISM 7300 Detection System in a total volume of 20 μL containing Taqman Universal PCR Master Mix (Applied Biosystems). PCR product formation was continuously monitored using the Sequence Detection System software version 1.7 (Applied Biosystems).

Phalloidin Staining for Analysis of Cell Ruffle Formation. A549 lung cancer cells growing on glass coverslides at low confluence were serum-starved for 24 h and stimulated with the different compounds at the concentrations indicated. Following fixation with 4% formaldehyde, F-actin was stained with phalloidin-rhodamine, and nuclei were counterstained with DAPI. Slides were visualized by fluorescence microscopy, and five random fields were scored for ruffle formation. Ruffle area was measured by thresholding for signal intensity using ImageJ.

RNAi Silencing. A549 cells were transfected with validated siRNA duplexes for PKCε or a nontarget control (NTC) siRNA duplex (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen), as previously described.[17] siRNAi's for PKCε were as follows: J-004653−06 (ε1) and J-004653−08 (ε2). Experiments were carried out 48 h after transfection.

Statistical Analysis. We used GraphPad Prism software built-in analysis tools for ANOVA. A p value <0.05 was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00739.

Data for ^{1}H, ^{13}C NMR spectra and HPLC traces for the title compounds (PDF)

Molecular formula strings (CSV)

Structure representation of isoxazole_sn1 (PDB)

Structure representation of isoxazole_sn2 (PDB)

Structure representation of tetrazole_sn1 (PDB)

Structure representation of triazole_sn1 (PDB)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CAN, ceric ammonium nitrate; DAG, diacylglycerol; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DIAD, diisopropyl azodicarboxylate; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; GEFs, guanine nucleotide exchange factors; LiHMDS, lithium hexamethyldisilazide; mp, melting point; MRCKs, myotonic dystrophy kinase-related Cdc42-binding kinases (MRCKs); PDBu, [20−3H]phorbol 12,13-dibutyrate; PKC, protein kinase C; PKD, protein kinase D; PMP, *p*-methoxyphenyl; pyr, pyridine; RasGRP, Ras guanine nucleotide-releasing protein; TBAF, *tetra-n*-butylammonium fluoride; TBDPS, *tert*-butyldiphenylsilyl; *t*-BuOH, *tert*-butylalcohol; THF, tetrahydrofuran

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