CONFORMATIONALLY RESTRICTED DIACYLGLYCEROL ANALOGUES AS ULTRAPOTENT PROTEIN KINASE C LIGANDS

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A. State in general terms the purpose and object of the research

Protein kinase C (PK-C) represents a central element transducing signals generated by a broad range of pathways which produce the lipid second messenger *sn*-1,2-diacylglycerol (DAG) directly or indirectly. Many dominant oncogenes have proven to function, at least in part, through this pathway. Likewise, this pathway is involved in expression of other aspects of the transformed phenotype, such as tumor invasion or multidrug resistance. As expected from its broad role in cell signaling, PK-C is also important in a range of other physiological and pathophysiological processes, including inflammation, differentiation, and nerve function.

We know that ligands binding to the diacylglycerol site of PK-C can function either as activators or as partial antagonists. Examples of the former are phorbol 12-myristate 13-acetate and phorbol 12,13-dibutyrate. Examples of the latter are bryostatin 1, 12-deoxyphorbol13-acetate, and 12-deoxyphorbol,13-phenylacetate. Agents acting as inhibitors at the catalytic site include UCN-01, staurosporine, and Ro-31-8830. All of these classes of agents have proven to have therapeutic potential, whether in cultured cell model systems, in animal models, or in a human clinical setting. For example, bryostatin 1 is in clinical trials with promising results in melanoma, supporting earlier findings of anti-melanoma activity in a mouse model. The 12-deoxyphorbol13-acetate inhibited mouse skin tumor promotion by 97%. Staurosporine has proven to have potent activity against grafted tumor lines in a mouse model. Ro-31-8830 was orally active in a rat model of inflammation and in cultured human T-cells.

Of the two strategies for designing modulators of the PK-C pathway, those targeted to the diacylglycerol site have the particular advantage of selectivity. Whereas the binding domain for diacylglycerol is found only in PK-C and two closely related proteins, the catalytic sites of the approximately 1000 kinases in the cell all share general homology and cross-reactivity is a major problem. For ligands targeted to the diacylglycerol site, a general problem is that most of the potent classes of ligands, namely the phorbol esters, the bryostatins, and the ingenols,

are structurally extremely complex natural products with multiple chiral centers. Their practical synthesis for selective medicinal chemical modifications is therefore extremely problematic. In the case of the indole alkaloids, the remaining group of high affinity natural products, their structural homology to the other classes of ligands is difficult to assess, so that rational drug design has proven elusive. Although the diacylglycerols themselves are synthetically accessible, their low potency precludes their suitability as drugs.

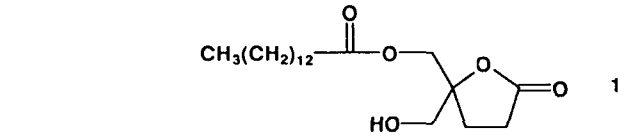
The present invention consists of a template, which embodies relevant hydrophilic pharmacophoric groups derived from diacylglycerol, embedded in a lactone ring system and with appropriated hydrophobic regions for proper orientation and partitioning in the membrane as well as for appropriate interaction with the receptor itself. It is noteworthy that the invented compounds embody with them the inactive or *sn*-2,3-configuration of diacylglycerol rather than the active or *sn*-1,2-configuration. The recognition of the appropriateness of this configuration within the structure is just one aspect of the conceptual novelty of this approach. Current examples of this class of invented compounds possess binding affinities for PK-C alpha approaching 10 nM. This affinity is comparable to that of phorbol-12,13-diacetate, for example. Claimed along with this template are additional modifications, as specified in more detail below, which would be readily apparent to one skilled in the art. We predict that these modifications will generate congeners with sub-nanomolar affinity. Along with these claimed compositions of matter are pharmaceutical compositions and method of use.

B. Description of the closest known technology

The closest known technology which pertains to this invention was generated in our own laboratory.[1-14] The manuscripts listed describe earlier work that produced other conformationally restricted lactone templates that were effective only in the low micromolar range. The present invention represents a significant leap forward due to the magnitude in potency achieved with the new templates.

In previous work from our laboratory we have ascertained the capacity of 4-bis(hydroxymethyl)-4-butanolides to function as DAG analogues (compounds **1-3**).[14] This capacity was measured by the ability of the compounds to function as inhibitors of the binding of [^{3}H]-phorbol-

12,13-dibutyrate ([^{3}H]-PDBU) to a protein kinase C (PK-C) preparation consisting either of a mixture of isozymes or pure PK-C alpha. The corresponding order of potency, ranked according to the value of the K_i, was 135.7 nM for the myristate (**1**), 95.92 nM for the oleate (**2**), and 47.40 nM for the arachidonate (**3**).



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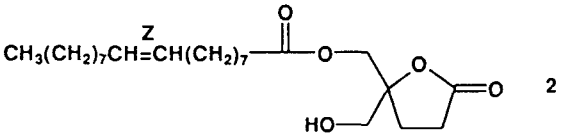

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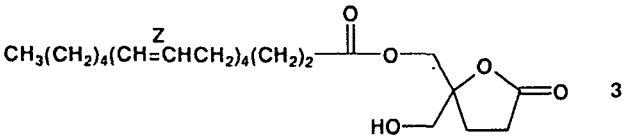

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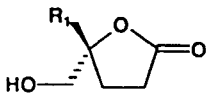
C. Description of the research

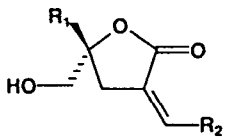
This invention relates to the preparation of stable lactone analogues that function as conformationally restricted surrogates of the physiological PK-C agonist, DAG. More specifically, it relates to the synthesis and use of new chiral 4-butanolides built on templates **I** and **II** as very potent PK-C agonists with binding affinities for the enzyme in the nanomolar range. Templates **I** and **II** were selected as skeletons for the construction of the new compounds which are the subject of the present invention.



TEMPLATE I

$R_1 = CH_2CH_2COOR_2$

$R_1 = CH{=}CHCOOR_2$



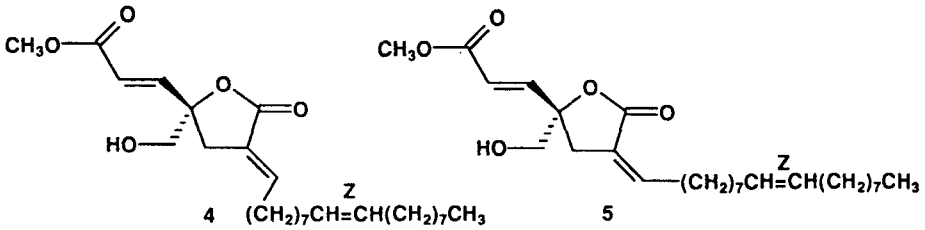
TEMPLATE II

$R_1 = CH{=}CHCOOCH_3$

$R_2 = CH_3(CH_2)_{12}$-

$R_2 = CH_3(CH_2)_7CH{=}CH(CH_2)_7$-

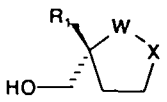
The limitation associated with the use of compounds **1-3** described in Part B is related to the fact that these compounds were racemic and by their chemical nature the isolation of the single enantiomer responsible for the biological activity was very difficulty. This difficulty stems from the facile migration of acyl groups from one hydroxyl group to the other under extremely mild conditions. Such a rapid acyl migration causes racemization and the concomitant loss of biological potency. The use of the new templates **I** and **II** circumvent this problem and permit the preparation of single and stable enantiomers with very high affinity towards PK-C. Specific examples of this invention are compounds **4** and **5**.



These compounds represent examples of the use of template **II** for the construction of rigid DAG analogues. Compound **4** has a K_i of 20.1 nM and compound **5**, which represents our most potent compound to date, has a K_i of just 11.37 nM.

The biological activity of compound **5** was evaluated further. First, we sought to determine whether the binding of compond **5** to PK-C alpha led to activation of the enzyme or not. We found that it stimulated phosphorylation of the alpha pseudosubstrate peptide (a standard substrate for assay of the enzyme) with an ED_{50} of 163.3 ± 16.7 nM. This finding demostrates that **5** would function in the intact cell or organism as either an agonist or as a partial antagonist. In intact cells, compound **5** caused inhibition of binding of epidermal growth factor with an ED_{50} of 1 μM. Inhibition of epidermal growth factor binding is a typical response to PK-C activation. Finally, we examined the ability of compound **5** to down regulate PK-C alpha and PK-C delta in primary mouse keratinocytes treated for 24 hours. We found that it down regulated the levels of PK-C alpha and delta with an ED_{50} of 1 μM, whereas it did not down regulate the levels of PK-C epsilon or PK-C eta. It is known to those skilled in the art that the pattern of down regulation is different for PK-C ligands with different patterns of induced biological response. The pattern of down regulation of compound **5** thus distinguishes it from the typical phorbol ester phorbol 12-myristate,13-acetate, which has greater potency for down regulation of PK-C delta than alpha (0.8 nM versus 40 nM at 6 hrs). The results are of further interest in that activity of phorbol 12-myristate,13-acetate is lost by 24 hrs, reflecting metabolism. This is not found for compound **5**. We conclude that compound **5** has a different pattern of behavior from the typical phorbol ester and may thus have unique utility.

The present invention is not limited to compounds **4** and **5**. These compounds and related ones are considered to be the first generation of stable, chiral, and rigid diacylglycerol analogues. Isosteric replacement of functional groups and the imposition of additional rotational constrains on templates **I** and **II** are expected to give more potent compounds based on similar principles. The scope of this invention is, therefore, beyond the specific compounds listed here and new analogues should be incorporated later as part of the same invention. A synopsis of the range of this invention is shown below.

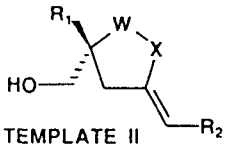


TEMPLATE I

R_1 = CH=CHSO$_2$R$_2$
R_1 = CH=CHSO$_2$NHR$_2$
R_1 = CH=CHCONHR$_2$
R_1 = CH=CHCON(OH)R$_2$

W = O, NH

X = C=O, X = SO_2



TEMPLATE II

R_1 = CH=CHSO$_2$CH$_3$
R_1 = CH=CHSO$_2$NHCH$_3$
R_1 = CH=CHCONHCH$_3$
R_1 = CH=CHCON(OH)CH$_3$
R_1 = CH$_2$CH(OH)COOCH$_3$
R_1 = CH$_2$CH(OH)SO$_2$CH$_3$
R_1 = CH$_2$CH(OH)SO$_2$NHCH$_3$
R_1 = CH$_2$CH(OH)CONHCH$_3$

R_2 = any alkyl, alkenyl, aryl, or heteroaryl chain

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