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ARTICLES

Structural Basis of Protein Kinase C Activation by Tumor Promoters

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Introduction

Protein kinase C (PKC) is central to diverse signal transduction processes.[1] PKC is a serine/threonine kinase which phosphorylates a variety of protein substrates depending on the cell type. The enzyme is normally quiescent and cytoplasmic, but upon activation it becomes associated with the inner leaflet of plasma membranes. Binding to the plasma membrane is transient, and is importantly regulated by the association of hydrophobic (*S*)-diglycerides (DAGs) with the enzyme. The simultaneous binding of the hydrophobic DAGs to PKC and the lipid bilayer enhances the association of PKC to the membrane by hydrophobic interactions. In a sense, DAG binding to PKC is a reversible posttranslational modification.

Yoshito Kishi was born in Nagoya, Japan, and received both B.S. and Ph.D. degrees from Nagoya University under the supervision of Professors Y. Hirata and T. Goto. During the period from 1966 through 1969 when he was Instructor in the Department of Chemistry at Nagoya University, he took a leave of absence to conduct research at Harvard University as a postdoctoral research fellow with Professor R. B. Woodward. Upon returning to Nagoya, he was promoted to Associate Professor in the Department of Agricultural Chemistry. He held that position from 1969 through 1974. He was invited as Visiting Professor of Chemistry at Harvard for the academic year 1972−73, and appointed as Professor of Chemistry at Harvard in July 1974. He currently holds the title of Morris Loeb Professor of Chemistry.

Robert R. Rando was born in New York City. He received a doctorate in physical organic chemistry with Professor William von E. Doering in 1966 at Yale University. He then studied with Professor Konrad Bloch in the Department of Chemistry, Harvard University, where he investigated the mechanism of action of β-hydroxydecanoyl thioester dehydrase. He also carried out mechanistic studies on the first mechanism-based irreversible enzyme inhibitor, 3-decynoyl-*N*-acetylcysteamine. Dr. Rando has worked on the development of new kinds of mechanism-based inhibitors and also discovered natural products which follow this mechanistic paradigm. For the past 20 years Dr. Rando has worked on the chemical biology of vertebrate vision and signal transduction mechanisms. He has recently also initiated studies on the design of small molecules that specifically inhibit RNA function. Dr. Rando is currently the Gustavas Adolphus Pfeiffer Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School.

The process of PKC activation is of substantial interest for chemists, given that activation of the enzyme is caused not only by (*S*)-DAGs but also by the structurally diverse tumor promoters (Figure 1).[2] Thus, clarification of the mechanism of the small-molecule-mediated activation of PKC is also likely to be of interest with respect to chemical carcinogenesis. The activation of PKC, however, involves more than the simple binding of an (*S*)-DAG or a tumor promoter to the enzyme; it also requires the interactions of Ca^{2+} and an acidic lipid such as phosphatidylserine (PS) with the enzyme.[1,3]

While the three effectors mentioned are essential biochemically for PKC activation, the availability of (*S*)-DAG is of central regulatory significance. DAGs arise via the action of specific phospholipase C2, and less regularly phospholipase D2, on specific membrane phospholipids, the phosphoinositides.[4] These phospholipases are activated and regulated by cell-membrane-receptor-mediated events, and result in the activation of PKC. The information that a cell has been stimulated by a particular agonist is thus translated to PKC activation. The phosphorylation of various proteins by PKC is the next stage in signal transduction after the binding of agonists to cell surface receptors.

The novel mode of activation of PKC has, of course, a mechanistic basis determined by the structure of the protein. Much has been written about this, and many recent reviews can be referred to for detailed accounts on this subject.[5,6] Briefly, the approximately 80 kDa, single polypeptide chain enzyme is composed of a regulatory domain and a catalytic domain connected by a flexible hinge region as shown in Figure 2. The catalytic domain contains ATP- and substrate-binding sites, and the regulatory domain contains binding sites for DAG, Ca^{2+}, and PS, along with a pseudosubstrate sequence. The DAG-binding site is found in a constant region among the isoforms which contain two cysteine rich subdomains (Cys1 and Cys2). While both of these regions are potential binding sites for DAG/tumor promoters, the cloned Cys2 region by itself can bind phorbol esters with the expected structure−activity profile.[7] Moreover, the binding of phorbol esters to the Cys2 region occurs in the absence of added phosphatidylserine, albeit with much lower affinities than in the presence of the lipid.[7] Both X-ray[8] and high-field NMR studies[9] were performed on cloned Cys2 regions with very similar results, and reveal the nature of the amino acids that interact with phorbol ester tumor promoter. Interestingly, the NMR data show that the structure of the Cys2 region is unaffected by lipid micelles. At least 11 isoforms of PKC are known, some of which are either Ca^{2+} independent or, less frequently,

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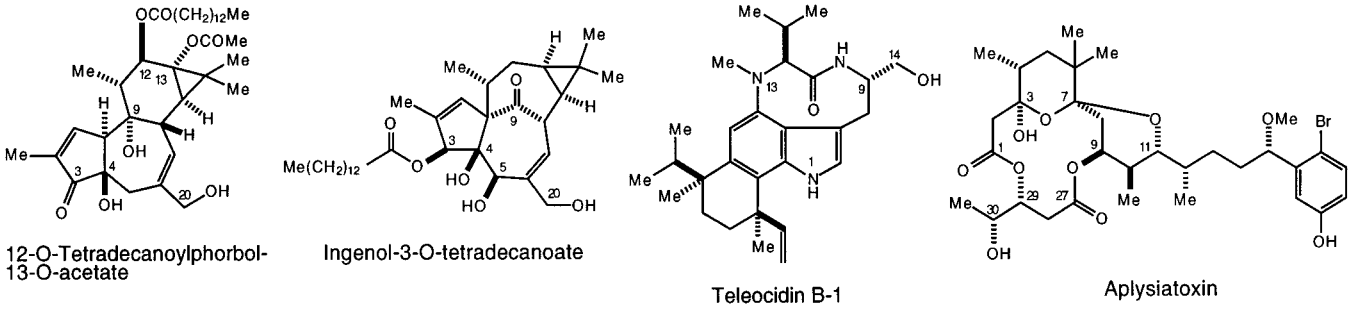


FIGURE 1. Chemical structures of representative tumor promoters.

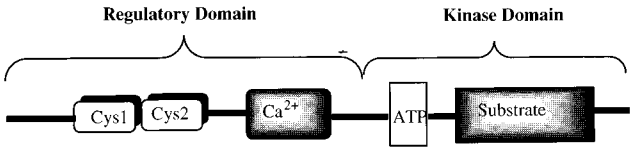


FIGURE 2. Primary structure of a conventional PKC.

DAG independent. In this Account, the ubiquitous and conventional DAG-activated PKCs will be under consideration.

It is generally considered that cytoplasmic PKC is catalytically inactive because the pseudosubstrate domain of the regulatory subunit binds to the catalytic subunit, preventing access of endogenous substrate molecules. The enzyme weakly associates with membranes by means of interactions mediated by Ca^{2+}.[10] The binding of DAGs at the regulatory domain has several important consequences. It increases the affinity of PKC for both Ca^{2+} and PS, and most importantly, it causes the translocation of PKC to the plasma membrane from the cytoplasm.[1] The hydrophobic character of the acyl chains of DAGs is essential in the process by interacting with membrane phospholipids. Short-chain DAGs do not lead to the activation of PKC.[11,12] An important question is then whether there are further aspects of structural specificity, aside from hydrophobicity, inherent in the interactions of activating DAGs with PKCs. This is a central issue to be dealt with in this Account.

Inquiries into the structural basis of PKC activation were enlightened and broadened by the discovery that the structurally diverse tumor promoters (Figure 1) can also activate PKCs (Figure 2). Thus, PKCs can be activated by molecules which superficially do not resemble each other. Assuming that the mechanism of activation of PKCs is specific, it is then of interest to be concerned with whether there is a common shared structural core (pharmacophore) among the various activators of PKCs, and, further, to determine the structure of the pharmacophore. These issues often arise at the intersection of natural product chemistry and biochemistry. It is not uncommon in biology for a particular receptor to be activated or inhibited by structurally diverse natural products in which common structural elements are not obvious. For example, serine/threonine phosphatases PP1 and PP2 inhibitors such as okadaic acid,[13] the microcystins,[14] calyculin A,[15] tautomycin,[16] and others[17] do not obviously share structure commonalities, yet these molecules are all thought to bind to the same phosphatase-binding site.[18] Structural analysis of this problem is complicated by the fact that an endogenous ligand has not yet been identified which inhibits phosphatase at the same binding site. Thus, one does not know what structural moiety the various natural product inhibitors are supposed to mimic. In the case of PKC, analysis is vastly simplified because the endogenous activators of PKC have clearly been identified as (*S*)-DAGs. The activation of PKC by the tumor promoters must refer back to (*S*)-DAGs in structural terms, making the structural analysis, in principle, more straightforward. However, before the analysis can proceed, the question as to whether the mode of PKC activation by DAGs and tumor promoters is inherently specific in nature must first be addressed. To begin to investigate this issue, structure−activity studies were performed on simple DAGs to reveal the inherent specificity in the activation process.

Specificity of DAG Binding to PKC

The first issue at hand was to determine if the mode of activation by DAGs is inherently stereospecific in nature. In fact, activation of PKC is entirely stereospecific with respect to DAGs, with only the naturally occurring (*S*)-DAGs exhibiting activity.[19] The stereospecificity of PKC activation by DAGs was further explored by determining the activity of 3-methyl-DAGs. An unanticipated stereospecificity in PKC activation was observed in studies of the 3-methyl-DAGs (Figure 3). Interestingly, only the (*R*,*R*)-stereoisomer is active as a PKC activator.[20] Thus, stereospecificity in the activation process is apparent at two chiral centers in DAGs, which becomes the basis of a stereochemical theme with variations observed with many non-DAG PKC activators, as will be described later.

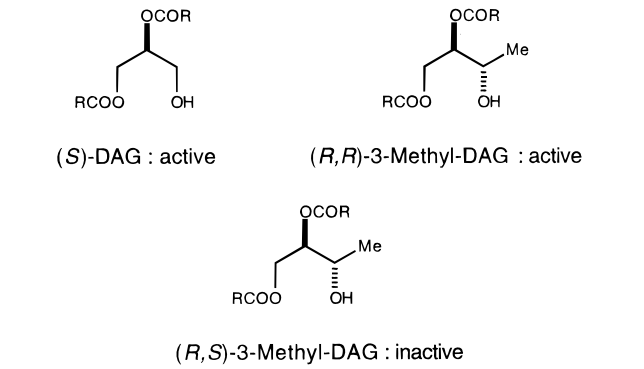


FIGURE 3. Stereospecificity at a second chiral center revealed through studies on 3-methyl-DAGs.

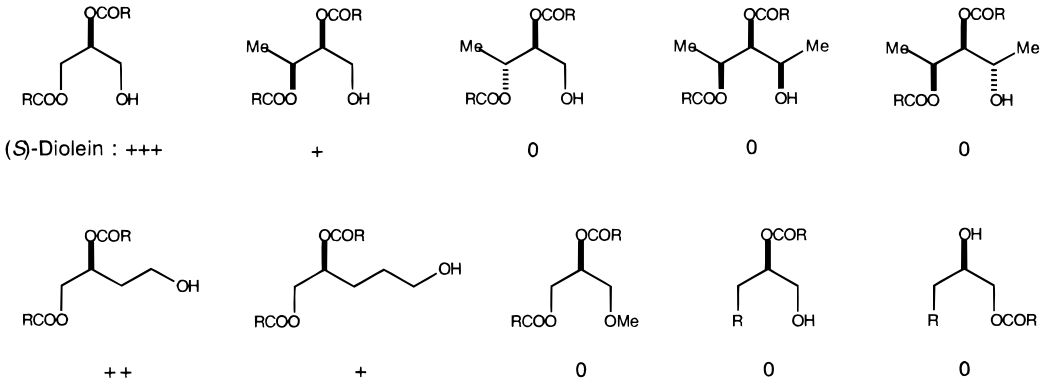
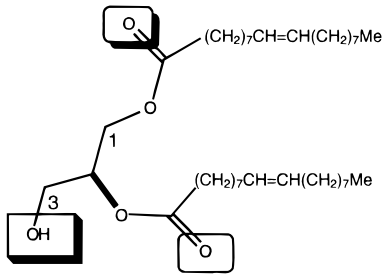


FIGURE 4. Structural specificity in the DAG-mediated activation of PKC. Relative activators indicated by very active (+++) (relative to (*S*)-diolein) and inactive (0).



(*S*)-Diolein, a representative DAG

FIGURE 5. The indicated hydrophilic atoms are essential for the activation of PKC.

Further structural studies in the DAG series also exhibited substantial specificity of action. For example, the hydroxylmethyl group of DAGs was essential, as were the diacyl moieties.[21,22] These conclusions are based on studies on a large number of analogues, including those shown in Figure 4. Interestingly, 1- and 2-methyl-DAGs and 1,2-dimethyl-DAGs showed virtually no activity. Homologous analogues of (*S*)-DAGs showed some activity, but the activities dropped off as the number of carbon atoms increased.

While specificity was significant with respect to the DAG backbone, the nature of the acyl chains is less relevant as long as a minimal overall hydrophobicity is met.[11,12] This is consistent with the idea that the main function of the acyl chains is to ensure stable interactions with the phosopholipid bilayer of membranes. Overall, structural studies on DAGs led to an understanding that the hydrophilic atoms identified in Figure 5 serve as the basis set for activation of PKC.[21,22] The requirement for hydrophilic atoms suggests that H-bonds formed between these atoms and specific amino acid residues at the DAG-binding site of PKC may be important in the activation process. Of course, the relative orientations of these atoms cannot be known from simple structure−activity studies with conformationally flexible DAGs for at least two reasons. First, DAGs are likely to possess many low-energy conformers within a few kilocalories of each other, and hence, a preferred solution conformation would be difficult to identify. Second, even if there were a single low-energy conformer in solution, there is no reason to believe that this particular conformer is the one able to bind to and activate PKC.

In summary, there is a great deal of structural and stereochemical specificity inherent in the activation of PKCs by DAGs. This poses an interesting question with respect to the structurally diverse tumor-promoting non-DAG activators of PKCs (Figure 1). Because all of these analogues appear to compete for an identical binding site on PKCs,[23] it is important to explain how these molecules are structurally related in the sense of sharing the same pharmacophore. Moreover, because many of the tumor promoters are conformationally rigid, an experimentally based understanding of which hydrophilic atoms match the key related DAG atoms should allow for a description of the active conformation of DAG when it binds to PKC.

Mechanism of Activation of PKC by the Aplysiatoxin Class of Tumor Promoters

In beginning to explore the relationship between DAG-mediated PKC activation and tumor-promoter-mediated PKC activation, three necessary preconditions must be met. First, it should be possible to frame a structural hypothesis in which a DAG core can be recognized in the tumor promoter. Second, the tumor promoter should be conformationally rigid so that the precise geometric distances between key hydrophilic atoms in the pharmacophore can be readily determined. Third, the tumor promoter in question, and more importantly its analogues, should be available by organic synthesis for providing unambiguous experimental evidence for establishment of the structural correlation between the tumor promoter and DAG. These preconditions are admirably met with the aplysiatoxin class of tumor promoters.

Aplysiatoxin and debromoaplysiatoxin were first isolated from the digestive gland of the sea hare *Stylocheilus longicauda* by Scheuer and Kato. On the basis of elegant spectroscopic and chemical degradation studies, they elucidated the gross structures of these molecules.[24] Moore and co-workers isolated aplysiatoxins and the structurally closely related oscillatoxins from the marine blue-green *Lyngbya majuscula*, and succeeded in establishing the complete structure of aplysiatoxins, including the absolute stereochemistry and the preferred solution

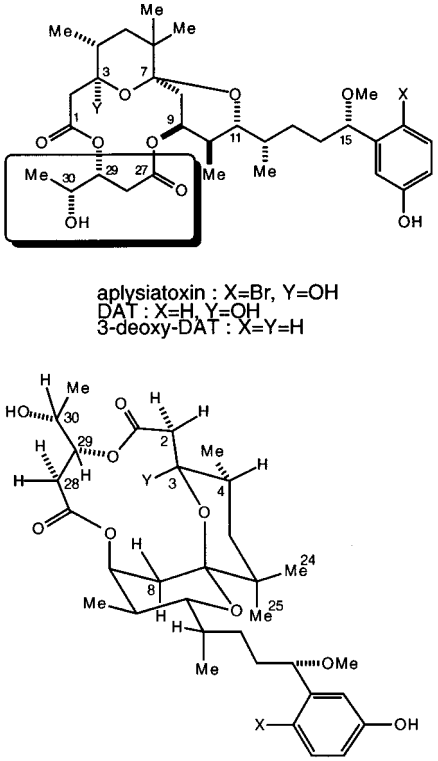


FIGURE 6. Structure and preferred solution conformation of aplysiatoxin, DAT, and 3-deoxy-DAT. The outlined area in the top structure identifies the putative DAG mimic.

conformation (Figure 6).[25] The total synthesis was completed in 1987.[26] Since both aplysiatoxin and debromoaplysiatoxin (DAT) are known to exhibit the same biological characteristics, we chose to use DAT and its analogues for our studies.

There are two extremely interesting aspects in the structure of DAT which make it ideal as a starting point for investigating the three-dimensional structural requirements of a PKC agonist. First, a DAG-like structure can easily be identified in DAT as a substituted dihydroxyvaleryl moiety (Figure 6). Second, the two chiral stereocenters of DAT in the dihydroxyvaleryl moiety are of the same absolute stereochemistries as found in the active 3-methyl-DAGs.

To verify that the dihydroxyvaleryl moiety of DAT indeed contains the biochemically functional portion (pharmacophore) of the aplysiatoxins, all the possible C-29 and C-30 diastereomers of DAT and 3-deoxydebromoaplysiatoxin (3-deoxy-DAT) were synthesized and tested for their effects on PKC. 3-Deoxy-DAT and its C-29 and C-30 diastereomers were important for two reasons. First, all the hypotheses advanced, prior to our own studies, for the structural correlation of aplysiatoxins with DAG or phorbol esters included the C-3 hydroxy oxygen of aplysiatoxin.[27−30] Second, the removal of the C-3 hydroxyl group eliminates a potential complication due to the possible tautomeric structures in the DAT series, cf. **A** (DAT), **B**, and **C** (Figure 7), thus validating the use of DAT as a template for probing the DAG conformation recognized by PKC. On the other hand, an inherent risk associated with the study of the 3-deoxy series is that the removal of the C-3 hydroxyl group might significantly affect the conformational characteristics of DAT. Importantly, however, extensive NMR studies demonstrated (1) DAT and 3-deoxy-DAT, especially the cyclic portions, are conformationally rigid and (2) the preferred solution conformation of 3-deoxy-DAT is virtually identical with that of DAT.[31] Furthermore, except for the side chain portion beyond the C-13 position, these preferred solution conformations are very close to the conformation found in the crystals of 19,21-dibromoaplysiatoxin (Figure 11).[25]

The PKC activation studies on all the C-29 and C-30 diastereomers of DAT and 3-deoxy-DAT (Figure 8) yielded interesting results. First, 3-deoxy-DAT is as active as DAT itself, firmly establishing that the C-3 hydroxyl group is *not* required for PKC activation. Second, the PKC-activating capacity of both DAT and 3-deoxy-DAT series uniquely depends on the stereochemistry at C-29 and C-30, and identical biological profiles are found for both series. Namely, DAT and 3-deoxy-DAT proved to be highly active as PKC activators, while 30-*epi*-DAT and 30-*epi*-3-deoxy-DAT showed only marginal activity.[31] The 29-*epi* and 29,30-bis-*epi* diastereomers of both DAT and 3-deoxy-DAT were inert. The biological profile thus obtained from these experiments is consistent with the stereospecificity observed for DAGs and 3-methyl-DAGs, making a convincing case that the pharmacophore of DAT resides in its dihydroxyvaleryl moiety.

To verify the pharmacophore correlation thus established between DATs and DAGs, simple dihydroxyvaleryl esters were synthesized and tested as PKC activators. Importantly, they were shown to activate PKC with the same absolute stereochemical requirements as found in the 3-methyl-DAG series (Figure 9).[32] Furthermore, these analogues were approximately as active as DAGs with respect to their abilities to activate PKC.

The experimental approach applied here defined the essential pharmacophore of a tumor promoter for the first time.[31,33] The essential moieties in the structure of DAT responsible for the activation of PKC and their relationship to a DAG are shown in Figure 10.

The fact that DAT is a conformationally rigid molecule allows us to assign the precise geometrical relationship between the hydrophilic atoms essential for PKC activation. This, of course, was not possible with the conformationally flexible acyclic DAGs. Moreover, the conformationally rigid DAT can be used as a template to determine the active conformation of DAG responsible for the activation of PKC; the DAG is superimposed on an X-ray structure of 19,21-dibromoaplysiatoxin,[25] yielding the approximate conformation of DAG recognized by PKC (Figure 11).[31,33]

The active conformation of DAGs deduced from the experimental work outlined can be used to find stereochemical matches in other tumor promoters (Figure 12). The corresponding distribution of three hydrophilic atoms and a hydrophobic locking core can be identified in all of the tumor promoters studied. This model is clearly consistent with published structure−activity studies. For example, the C-20 hydroxyl group of phorbol ester is

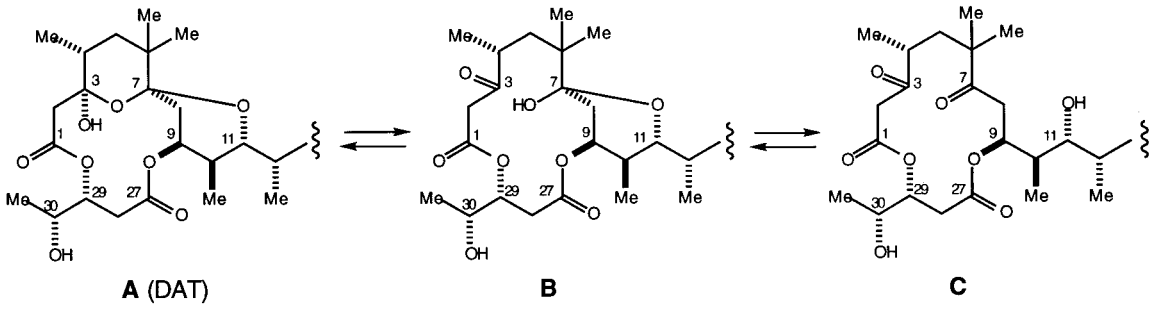
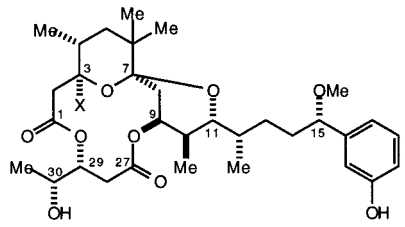
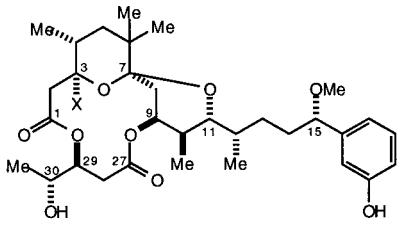
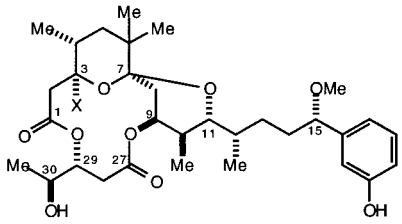


FIGURE 7. Possible tautomers involving the C-3 hydroxyl group of DAT.







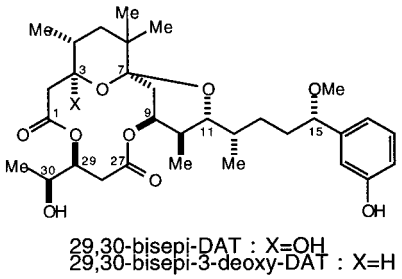


FIGURE 8. Structures of DAT, 3-deoxy-DAT, and their C-29- and C-30-diastereomers.

critical for tumor-promoting activity, whereas the C-4 hydroxyl group is not.[34]

A recent X-ray crystallographic study on cocrystals of the Cys2 PKC region and a phorbol ester show H-bonds formed between the oxygens of C-3 keto and C-20 hydroxyl groups and active-site amino acids.[8] A possible H-bond may also be found involving the C-4 hydroxyl group of phorbol esters. However, this latter result is not

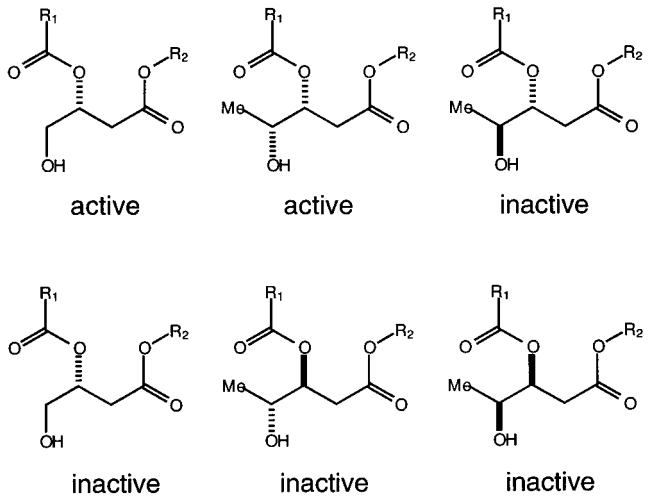


FIGURE 9. Dihydroxyvaleryl esters activate PKC stereospecifically.

consistent with structure−activity studies and may reflect the fact that the X-ray structural studies were performed on a small domain of PKC in the absence of phospholipid using a relatively weakly binding hydrophilic phorbol ester. Moreover, the position that the C-4 hydroxyl group of phorbol esters is *not* involved in essential interactions with PKC is also consistent with recent molecular modeling studies on PKC−phorbol ester recognition.[35]

Structure−activity studies on other tumor promoters also support our model. For example, the stereochemistry of the methylated hydroxylmethyl moiety of bryostatin is important for activity.[36] The same is true of the indolactams.[37] Thus, the same stereochemical requirements at this site are thus found in the 3-methyl-DAGs, in the DATs, in the dihydroxylvalerates, in the bryostatins, and in the indolactams. There is, of course, a second stereochemical requirement found in the DAGs, DATs, and dihydroxyvalerates, namely, the chiral center corresponding to the 2-position of DAGs. It was of interest to further explore and further amplify the apparent stereochemical requirement at this chiral center.

Pharmacophore of Teleocidins/Indolactams

As an explicit test of the PKC activator binding-site model developed (Figure 12), structure−activity studies were performed in the teleocidin/indolactam series. Importantly, a clear prediction was made as to the pharmacophore of this series (teleocidin B-1 in Figure 12) that can be experimentally tested. In this regard it is important

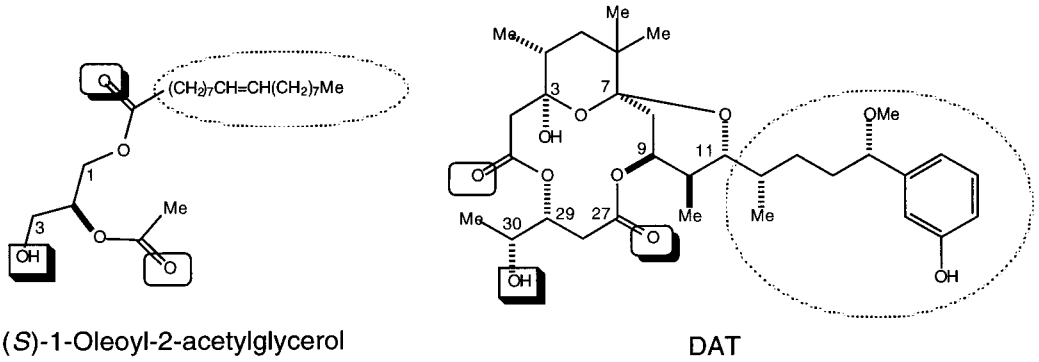


FIGURE 10. The pharmacophore of DAT and its relationship to an (*S*)-DAG. Corresponding moieties are indicated by the same outlining feature.

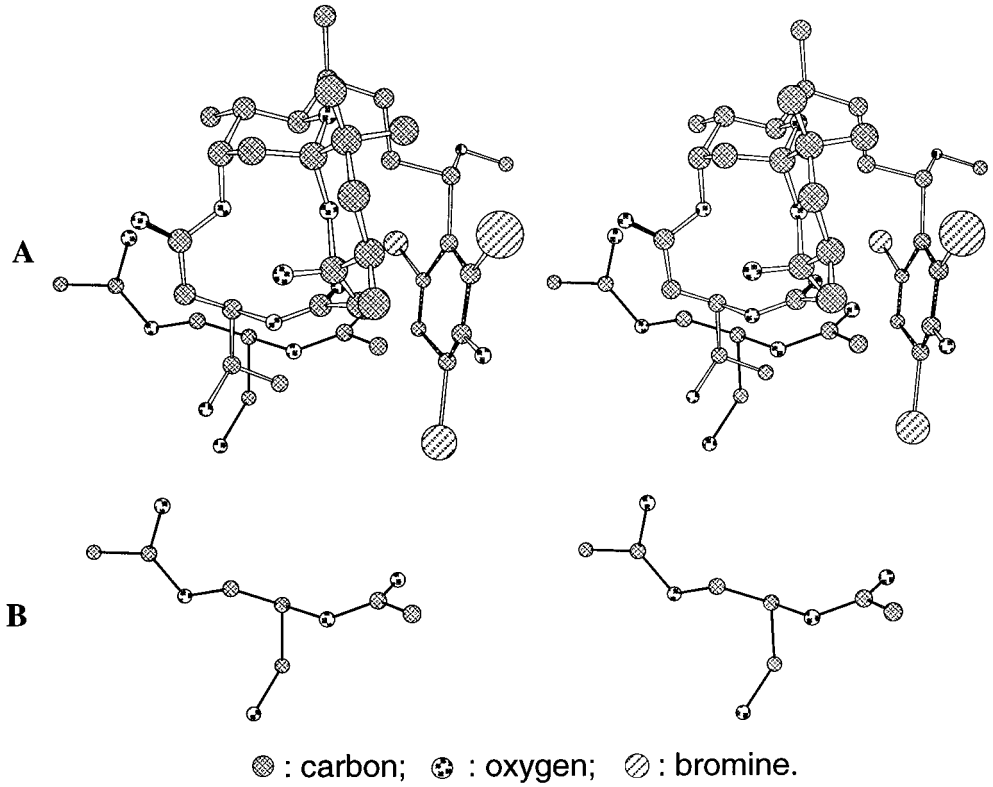


FIGURE 11. Overlay of (*S*)-DAG onto the conformationally rigid DAT (panel A) allows one to deduce the active DAG conformer (panel B).

to note that naturally occurring (−)-indolactam, which contains the active pharmacophore embedded in teleocidin B-1, has already been synthesized.[38] According to our structural hypothesis, the indicated atoms of (−)-indolactam (Figure 12) are essential for PKC activation. As previously mentioned, very strong stereochemical preferences have been found in the DAG- and aplysiatoxin-mediated activation of PKC. Simple indolactams possess two asymmetric centers, one at the C-9 carbon bearing the putatively critical hydroxylmethyl group and the other at the C-12 carbon bearing the isopropyl group. While it is not a priori possible to predict what affect inversion of the C-12 stereocenter will have on PKC activation, inversion of the C-9 stereocenter should be deleterious. The four stereoisomeric indolactams were prepared and tested as putative PKC activators (Figure 13).[39] The naturally occurring (−)-indolactam is the most active molecule in the series, having a $K_D = 2.4\ \mu M$ and a $V_{max} = 585$ (nmol/min)/mg. (−)-Indolactam proved to be both a more potent and a more efficacious PKC activator than is (*S*)-diolein under the conditions of the experimental measurements. As predicted (+)-indolactam is virtually inactive. As perhaps would be expected, there was not a great deal of stereospecificity directed toward the isopropyl group, although the natural stereoisomer is a better activator than its C-12-diastereomer.

At least one other modeling effort on the tumor promoters suggested that the N-13 amino group of the indolactams is essential in the activation process.[28,29] Therefore, the four stereoisomers possible for the MeN → CH_2 substituted indolactam analogue (*C*-indolactam) were also synthesized and characterized (Figure 13).[39] Importantly, this experiment showed that the *C*-indolactam analogues are still active, with a strong stereochemical preference at the C-9 position. While an explicit study on the importance of the indole nitrogen was not

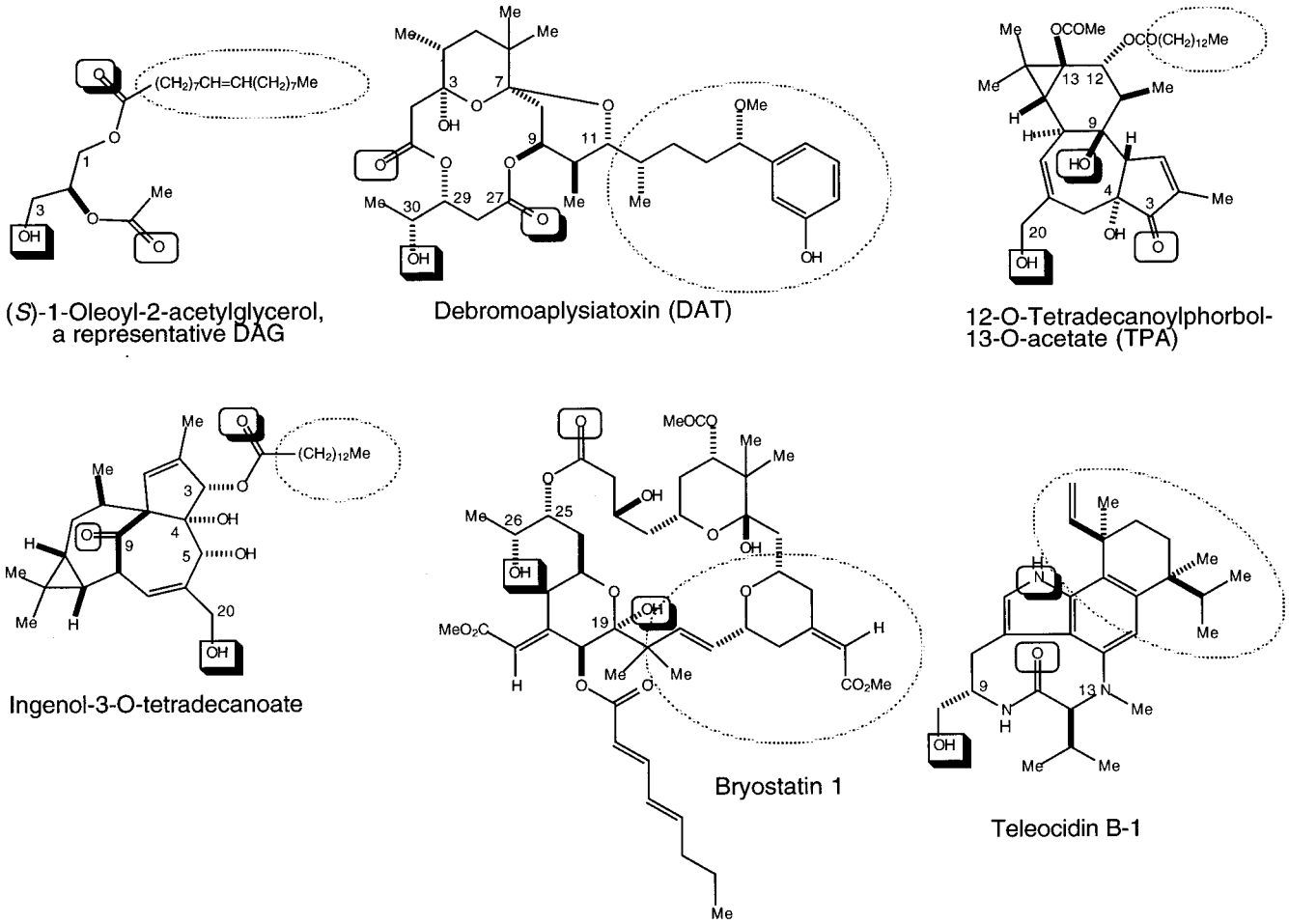


FIGURE 12. Pharmacophores of various tumor promoters. Corresponding moieties are indicated by the same outlining feature.

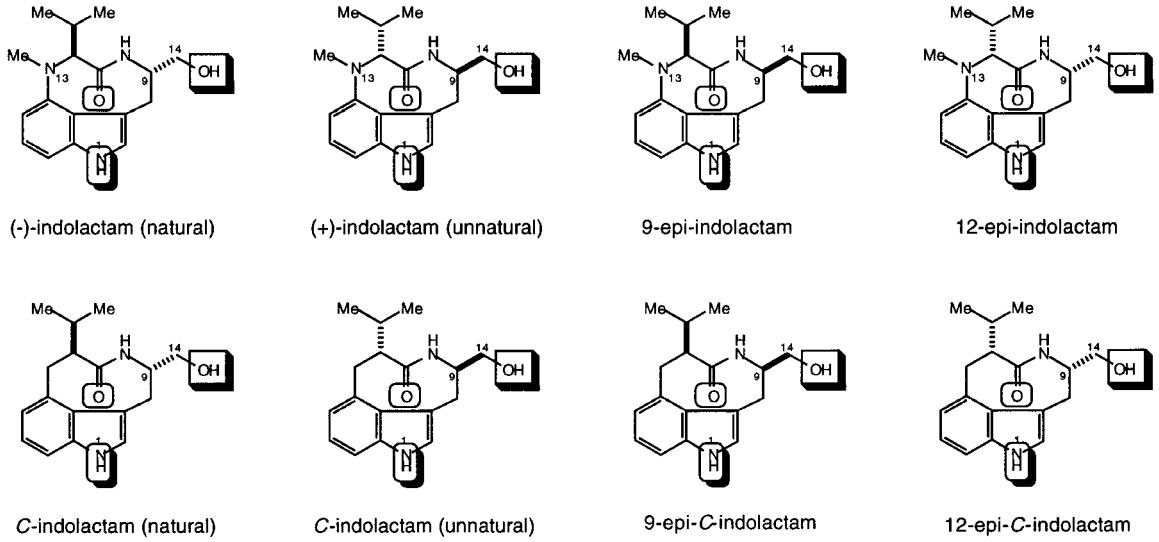


FIGURE 13. Indolactam and MeN → CH_2 substituted indolactam analogues synthesized for probing the stereospecificity and the nonrequirement of the N-13 nitrogen. Corresponding moieties are indicated by the same outlining feature.

performed, previously reported experiments at least suggest the importance of a hydrophilic atom at that position.[40] For example, while the benzopyrrole analogues shown in Figure 14 are active as PKC activators, the benzo derivative is not. These experimental results summarized in this section support the aforementioned pharmacophore correlation between DAGs and teleocidins.

Related to the teleocidin class of tumor promoters, the recent report on cytoblastin, a low molecular weight immunomodulator produced by *Streptoverticillium eurocidium*, is interesting.[41] Structurally, the upper half of cytoblastin apparently corresponds to (−)-indolactam V. Intriguingly, when the aforementioned pharmacophore correlation is adopted and an imaginary acyl group is

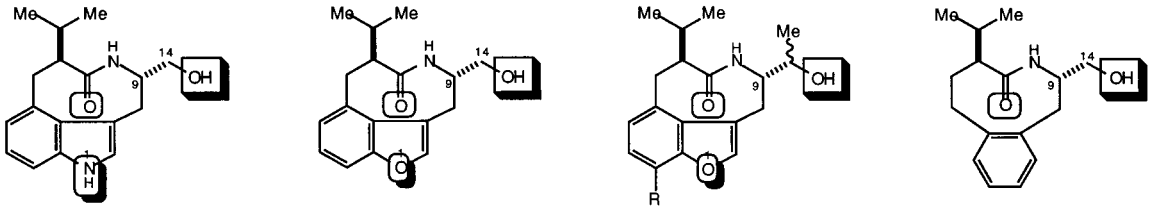


FIGURE 14. Indolactam analogues which probe the requirement of the indole (N-1) nitrogen. Corresponding moieties are indicated by the same outlining feature.

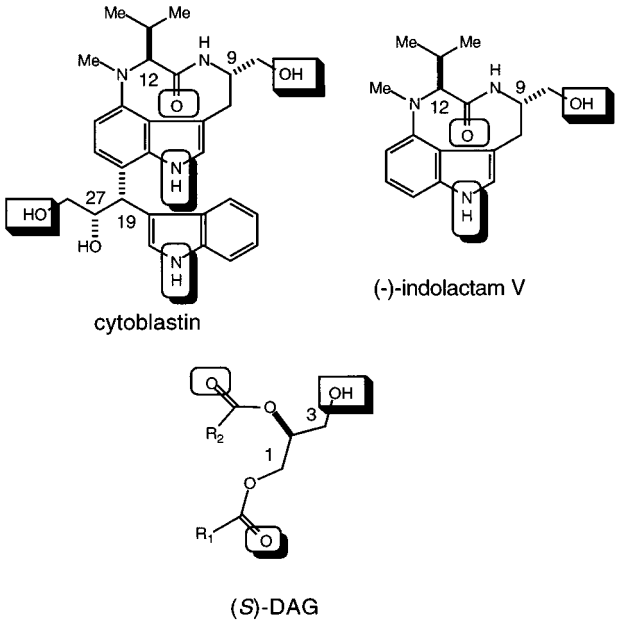


FIGURE 15. Possible pharmacophore correlation among cytoblastin, (−)-indolactam V, and an (S)-DAG. Identical symbols indicate equivalent atoms.

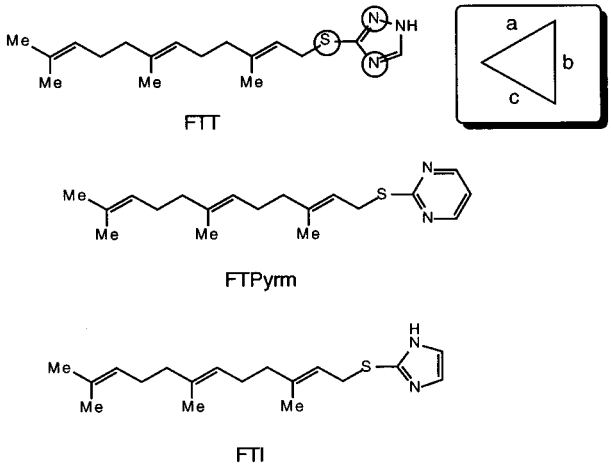


FIGURE 16. Minimal activators of PKC. The circled atoms of FTT indicate those critical for activation. The triangle indicates minimal distances between essential atoms. The mean distances between the vertices are as follows: (a) 2.74 ± 0.04 Å, (b) 2.28 ± 0.09 Å, and (c) 2.81 ± 0.03 Å.

placed on the C-27 alcohol, the lower half of cytoblastin also corresponds to the pharmacophore of DAG. Thus, cytoblastin can be viewed as a pseudodimeric form of DAG (Figure 15). Clearly, the validity of this hypothesis hinges on the stereochemistry of cytoblastin. Through the synthetic work, the stereochemistry in question was indeed demonstrated to match the absolute stereochemistry of (−)-indolactam V and DAGs, respectively.[42] It is interesting and intriguing to note that cytoblastin was originally reported to exhibit a complete lack of activity toward PKC.[41] However, assays of both natural and synthetic cytoblastin have shown an activity toward PKC that is roughly equivalent to that of (−)-indolactam V.[42]

Minimum Three-Point Model

All of the data thus far summarized may be taken as compelling that the stereochemical model developed via the experimental work accurately describes the essential features of molecules that can activate PKCs. In effect, this model argues that molecules with three hydrophilic atoms separated by approximately 6 Å (Figure 11) can activate PKCs. In addition, a hydrophobic moiety is also required for membrane docking. The pharmacophores of the various tumor promoters can all be reduced to the three-point model, where the hydrophilic points are arranged at the vertices of an appropriate equilateral triangle. A question that arises is whether this is the only arrangement of hydrophilic atoms which a PKC activator can possess. Studies on hydrophilic triazole analogues show that this is not the case.

Studies on planar farnesylthiotriazole analogues in neutrophiles showed that FTT (Figure 16) is a more potent agonist of PKC than is (*S*)-diolein, a representative DAG activator.[43] Further studies demonstrated that the three circled atoms of FTT are essential in the activation process.[44] Indeed, very simple molecules, such as FTPyrm and FTI (Figure 16) are the simplest activators of PKC currently known. Removal of one of the nitrogen atoms or the sulfur atom abolishes activity. This is further powerful evidence that the PKC activation process involves three essential hydrophilic atoms which presumably can form H-bonds with essential amino acid residues at the DAG/tumor-promoter-binding site. However, the internuclear distances measured between the hydrophilic atoms of the activators shown in Figure 16 are substantially less than the distances deduced from the tumor promoter studies. The three hydrophilic atoms are at the vertexes of an approximate equilateral triangle with mean distances of 2.74 ± 0.04, 2.28 ± 0.09, and 2.81 ± 0.03 Å, respectively. The distances between the atoms are substantially shorter than those measured for DAT, in which the mean distances measured between the hydrophilic

atoms are 5.4, 4.6, and 5.5 Å, respectively.[45] In fact, using the published general pharmacophore model as a guide, average distances between the hydrophilic triads of the various tumor promoters are 6.00 ± 0.03, 5.70 ± 0.60, and 6.40 ± 0.60 Å, respectively. Moreover, a recent X-ray structural study on the binding of a phorbol ester to the PKC regulatory domain definitely shows that the C-3 keto and C-20 hydroxyl groups of the phorbol ester are involved in hydrogen bonding to the peptide backbone of PKC.[8] The distance between the C-3 keto and C-20 hydroxyl groups of the phorbol ester is anywhere between 4.88 and 6.40 Å, depending on the conformation of the hydroxymethyl group.[45] Clearly, where distance measurements between important hydrophilic moieties have been made on tumor promoters, the distances in question are substantially longer than is evident in these planar heterocyclic molecules.

Several possiblities could explain the activity of molecules such as FTT. First, it is, of course, quite possible that the DAG/tumor-promoter-binding site(s) may be distinct from the minimal-activator-binding site. Simple kinetic analysis does distinguish between one or two binding sites. However, given the very strict structural demands observed in structure−activity studies,[18] it seems unlikely that there are multiple activator sites in PKC. Second, FTT might bind to the DAG/tumor promoter site in a different way than do the familiar activators. Third, FTT might bind to the DAG/tumor promoter site and make the same critical hydrogen bonds with amino acid residues as do the diglycerides and tumor promoters. If the different classes of activators interact with the same binding site on PKC, these results suggest that there must be flexibility at this binding site, allowing for the different classes of activator molecules to function. Given the relatively strict spatial requirements found in the case of the tumor promoters, how is this to be understood? It is reasonable to assume that the distances between hydrophilic atoms derived from the tumor promoters may actually define the maximal distances allowed between these atoms, rather than the exact distances. The scaffolding of the structurally diverse tumor promoters is such that the hydrophilic atoms are not close to each other. Therefore, studying the tumor promoters may not allow one to sample structures in which the three essential hydrophilic atoms are as close to one another as in these planar, heterocyclic activator molecules. It is intriguing to consider that the information available with respect to PKC activation, in a complex molecule such as bryostatin, can be reduced to the simple planar aromatic molecules shown in Figure 16. Finally, the discovery of specific antagonists of PKC should be facilitated by the finding that very simple molecules can activate PKC.

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