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Structural Basis of Protein Kinase C Activation by Diacylglycerols and Tumor Promoters†

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ABSTRACT: Protein kinase C is a ubiquitous and important regulatory enzyme. The enzyme is physiologically activated in a temporary manner by (*S*)-diacylglycerols (DAGs), which are themselves generated by the phospholipase C mediated hydrolysis of polyphosphoinositides. The (*S*)-DAGs specifically bind to the regulatory domain of PKC and cause the activation of the PKC toward substrate. Minor modifications in the DAG result in inactive molecules. On the other hand, the structurally diverse, polycyclic tumor promoters also specifically activate PKC by binding to the same effector site as do the DAGs. The object of this paper is to present a discrete structural model that accounts for the activation of PKC by both the tumor promoters and the DAGs. The unique model presented is based on experimentation rather than on computer-driven hypotheses which, experience has shown, generally produce incorrect structural models when applied to PKC. The model described here begins with a structural analysis of the tumor-promoting debromoaplysiatoxins (DATs). DAT is an ideal starting molecule, because it is conformationally rigid with a known relative and absolute configuration, and it is synthetically manipulable. The pharmacophore of DAT was experimentally determined, and this pharmacophore serves as a template for further analyses. This template is used to predict the active conformer of the acyclic DAGs; this conformer is then used to reveal the pharmacophore of various families of tumor promoters. The overall model presented is consistent with published structure–activity studies on the tumor promoters and makes testable predictions that have proven to be correct thus far.

Protein kinase C (PKC)[1] is an important regulatory enzyme involved in the control of diverse biological phenomena (Nishizuka, 1984, 1986). Under quiescent conditions, the enzyme is located in the cytoplasm, where it remains catalytically inactive (Nishizuka, 1984, 1986). The enzyme is activated in a transient fashion when 1,2-*sn*-(*S*)-DAGs are produced in the membrane by the action of a specific phospholipase C (Berridge, 1987). This latter enzyme cleaves (poly)phosphoinositides to generate DAGs and (poly)-phosphoinositols (Berridge, 1987). The liberated DAG binds to the regulatory domain of PKC and activates the enzyme by increasing its affinity for calcium ions to the steady-state physiological range (Nishizuka, 1984, 1986). PKC is activated transiently, and its activity is terminated when DAG is either phosphorylated by a specific kinase or hydrolyzed at the *sn*-2 position (Nishizuka, 1986).

When assayed in vitro, PKC requires the simultaneous presence of a DAG, calcium ions, and an acidic phospholipid such as phosphatidylserine (Nelsestuen & Bazzi, 1991). Although PKC requires phosphatidylserine and calcium ions for activity, they are not of regulatory interest. DAG is the sole known physiological regulator of PKC, and temporal fluctuations in the concentration of DAG control the activity of the enzyme.

PKC is generally considered to be composed of two domains. The holoenzyme has a molecular mass of approximately 80 kDa (Nelsestuen & Bazzi, 1991). The approximately 50-kDa

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[1] Abbreviations: DAG, diacylglycerol; DAT, debromoaplysiatoxin; PKC, protein kinase C.

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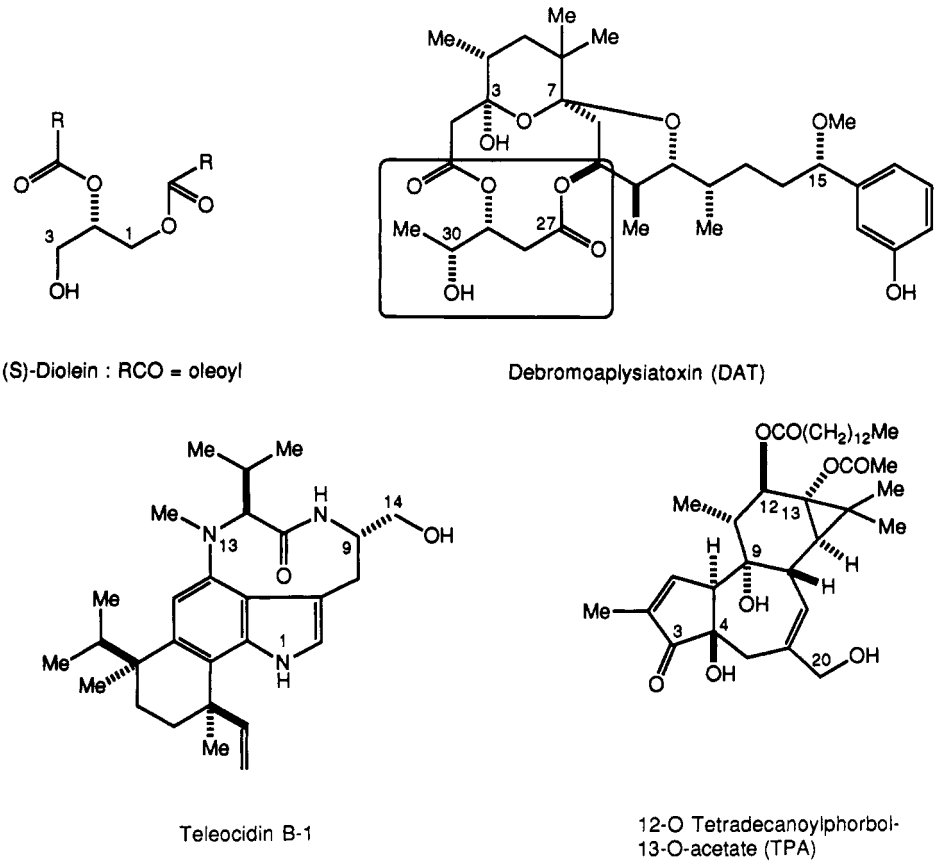


FIGURE 1: Chemical structures of a DAG and representative tumor promoters.

domain contains the catalytic subunit which binds MgATP and the substrate. Phosphorylation occurs at serine or threonine residues (Edelman et al., 1987). The approximately 30-kDa cysteine-rich domain contains the regulatory region to which DAGs specifically bind (Huang & Huang, 1986; Lee & Bell, 1986). The holoenzyme can be specifically cleaved by a calcium-dependent kinase to produce the two domains (Kishimoto et al., 1983). Both domains are independently active, but of course, the catalytic domain is no longer susceptible to regulation. It should also be noted that PKC exists as isoforms with considerably different catalytic and regulatory characteristics (Nishizuka, 1989). These isoforms are differently expressed in different tissues, with presumed important physiological consequences (Nishizuka, 1989).

The specificity of the activation of PKC by DAG is considerable. In the simple DAG series, neither 1,3-DAGs nor 2,3-*sn*-(*R*)-DAGs can activate PKC or inhibit its stereospecific activation by 1,2-*sn*-DAGs (Young & Rando, 1984; Boni & Rando, 1985). The binding of the 1,2-*sn*-DAGs is stoichiometric and saturable (Hannun et al., 1985). Most structural modifications of DAG result in the generation of inactive derivatives (Rando, 1988). At the same time, however, the structurally diverse tumor promoters, some of which are shown in Figure 1, also activate PKC (Castagna et al., 1982). PKC is thought to be the most important, if not the sole target for the tumor promoters (Castagna et al., 1982). Generally, these compounds are much more potent than the DAGs, often activating PKC in the subnanomolar range, rather than in the micromolar range. In addition to their increased potencies, the tumor promoters persistently activate the kinase, because there is no natural mechanism to terminate their action in an animal cell.

The question that we address here is what the salient structural features in the PKC agonists are that are recognized by the enzyme. The central problem in PKC modeling is to resolve the paradox of how the same binding site of the regulatory domain of PKC can specifically recognize the chemically diverse tumor promoters and DAGs in selective ways. The key to solving this puzzle is to uncover commonalities in the three-dimensional structures of the various PKC activators. As many of the tumor promoters are relatively rigid molecules, revealing their essential moieties will also yield information on the three-dimensional disposition of interacting amino acids at the active site of the regulatory domain. The model developed begins with a discussion of DAG agonists and the specificity manifest in their binding.

DAG Agonists

Simple DAGs can activate PKC in the micromolar range (Rando, 1988). As mentioned above, the activation is stereospecific, with only the naturally occurring (*S*)-DAGs being active. This is fortunate, because if (*R*)-DAGs could activate PKC, they would be tumor promoters, since there would not be a mechanism in place to terminate their activity enzymatically. The only modifications in DAG structure which routinely produce active molecules are those found in the acyl side chains (Lapetina et al., 1985; Mori et al., 1982; Go et al., 1987). (*S*)-Diolein is a standard DAG used in in vitro assay systems. The all-trans isomer of diolein—dielaidin—is approximately as active as diolein itself (Boni & Rando, 1985). Saturated DAGs are active (Boni & Rando, 1985), as are shorter chain acyl derivatives (Huang & Huang, 1986). For example, 1-oleoyl-2-acetylglycerol and 2-oleoyl-1-acetylglycerol are active PKC activators (Mori et al., 1982). As long as the acyl side chains are sufficiently hydrophobic to partition in the membrane, active DAGS are obtained.

Alterations in other parts of the DAG structure do not generally yield active agonists. Substitution for the 3-hydroxyl

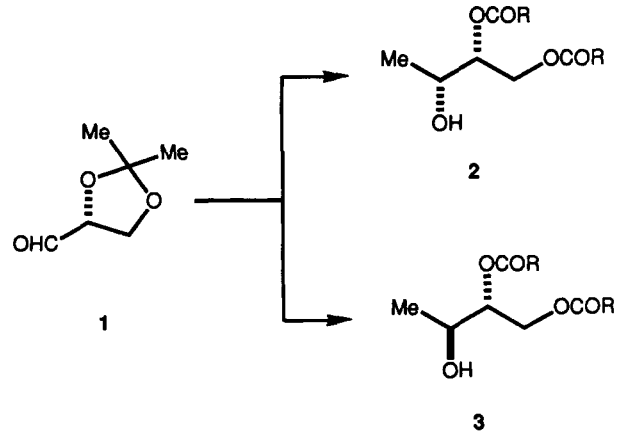


FIGURE 2: C3 methylated DAG analogues.

group by an ether (Ganong et al., 1986; Molleyres & Rando, 1988) or a halogen (Ganong et al., 1986) abolishes activity, as does elimination of one or more of the carbonyl groups of the esters (Rando, 1988; Molleyres & Rando, 1988). These results suggest that the hydrophilic hydroxyl and ester moieties are essential in the interactions of DAGs with PKC.

It is instructive to consider the effects of alterations in the DAG backbone on DAG activity. Extension of the length of the DAG backbone produces decreasingly active molecules, suggesting that the distances between the hydrophilic moieties are important (Ganong et al., 1986; Molleyres & Rando, 1988). Substitutions of the glyceride backbone are also informative. The introduction of a single methyl group at the *sn*-1 position of a DAG strongly decreases agonist activity (Molleyres & Rando, 1988). The 1,3-dimethylated DAGs are virtually without activity (Molleyres & Rando, 1988).

Interestingly, the introduction of a methyl group at the *sn*-3 position of DAG, which creates a new asymmetric center, reveals an unanticipated stereospecificity. The (*R*,*R*) and (*R*,*S*) diastereomers were prepared from D-glyceraldehyde acetonide (Figure 2). The stereochemistry of the products was unambiguously assigned by an independent synthesis using diethyl L-(+)-tartrate as the starting material (Kong et al., 1990). It is shown that only the (*R*,*R*) diastereomer **2**, which has the same absolute stereochemistry at the *sn*-2 position as (*S*)-diolein, is active as a PKC agonist (Kong et al., 1990). Using rat brain PKC as the source of enzyme, the (*R*,*R*)-dioleoyl isomer showed a K_D = 0.2 μM and a V_{max} = 7.7 nmol min^{-1} mg^{-1}, which compares to K_D and V_{max} values of 0.09 μM and 9.36 nmol min^{-1} mg^{-1}, respectively, for (*S*)-diolein (Kong et al., 1990). On the other hand, the (*R*,*S*)-dioleoyl derivative proved to be inactive as a PKC activator (Kong et al., 1990). Thus, this work establishes the stereoselectivity of PKC for a second asymmetric center in the DAGs. The observed stereoselectivity is conserved in certain tumor promoters and, indeed, was important in developing our effector binding model described later.

As mentioned above, 1,3-dimethyl-DAGs are inactive as PKC activators. This latter finding is unfortunate, because it suggests that certain cyclic DAG analogues may not be active as agonists. Cyclic DAG analogues are of interest because they restrict the conformational freedom of the DAGs, making it possible to be more certain of the structure of the agonists at the effector site. It is difficult to predict the low-energy conformer(s) of a freely rotating DAG (Leli et al., 1990). Moreover, even if one could make such a prediction, the assumption that the low-energy form of the agonist is the one that binds to the effector site is not necessarily a valid assumption. As expected, the synthesis of conformationally restricted DAGs came to little, because of their negligible activities (Rando, 1988). Thus, the search for commonalities in the structural features of agonists could not fruitfully utilize DAGs as a template, because of the extreme risk in trying to decide which conformation of the conformationally mobile DAG was actually bound to the effector site of PKC.

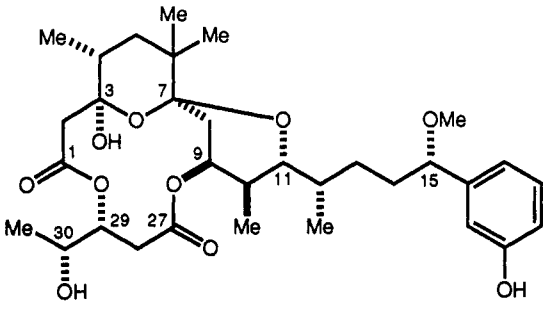
Tumor Promoter Binding to PKC

In trying to accommodate the various tumor promoters and DAGs into a structural model for effector-linked activation of PKC, it is necessary to choose a suitable structural template. It is clear that acyclic DAGs are not suitable, because of their conformational flexibility (Leli et al., 1990). On the other hand, the tumor promoters are generally polycyclic and conformationally rigid. Thus, a conformationally rigid, synthetically manipulable tumor promoter is required as a template so that structural variants can be generated and tested. The debromoaplysiatoxins (DATs) (Figure 1), which are a member of a class of inflammatory agents produced by the blue-green alga *Lyngbya majuscula* (Serdula et al., 1982), are near perfect molecules for this purpose (Park et al., 1987). First, their three-dimensional crystal structures are known (Moore et al., 1984), and their structures in solution can be deduced by high-field NMR measurements (Moore et al., 1984; Nakamura et al., 1989). Second, elements of a DAG-like moiety can be recognized in DAT, so that an initial structural hypothesis can be generated. Finally, although DAT is a complex molecule, it is synthetically manipulable so that a given structural hypothesis can be tested. It is noteworthy that the total synthesis of DAT has been achieved (Park et al., 1987).

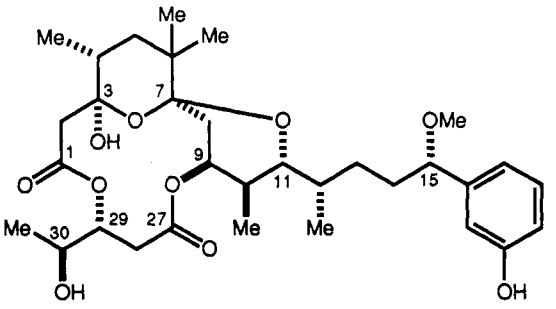
The Debromoaplysiatoxins

Consideration of DAT as a template for the DAGs suggests that the 3,4-dihydroxybutyrate moiety of DAT (enclosed in a box in Figure 1) may be analogous to an (*S*)-DAG. The stereo center at C29 of DAT would be analogous to the *sn*-2 of the DAG. The stereospecificity of PKC for this latter center has already been established as *S* (Young & Rando, 1984; Boni & Rando, 1985). The same absolute configuration is found at C29 of DAT. Moreover, the stereo center at C30 of DAT has the same absolute configuration as the C3 stereo center of the biologically active 3-methyl-DAG (Figures 1 and 2). Thus, by stereochemical analysis, the 3,4-dihydroxybutyrate moiety in DAT requires that stereochemical preferences for PKC activation be observed at the C29 and C30 stereo centers of the tumor promoter.

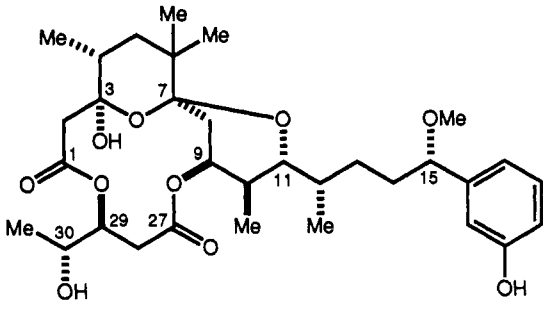
To establish the importance of the C29 and C30 stereo centers of DAT, the epimeric compounds at these centers were synthesized and tested as PKC activators (Nakamura et al., 1989). The structures of the analogues are shown in Figure 3. Importantly, 29-epi-DAT proved to be inactive as an activator of PKC, establishing the link with the inactive (*R*)-DAG (Nakamura et al., 1989). As noted above, the absolute configuration at the C30 stereo center is the same as the absolute configuration established for the second asymmetric center found in 3-methyl-DAG (Kong et al., 1990). 30-epi-DAT proved to be only marginally active, and 29,30-bisepi-DAT is without activity as a PKC activator (Nakamura et al., 1989). These studies establish that the stereochemistry at the C29 and C30 centers matches the stereochemistry at the *sn*-2 and *sn*-3 positions of 3-methyl-DAG and render it highly likely that the 3,4-dihydroxybutyrate moiety of DAT matches the 2 and 3 positions of DAG (Nakamura et al., 1989). The 3,4-dihydroxybutyrate moiety is structurally similar to DAG, with the only difference being



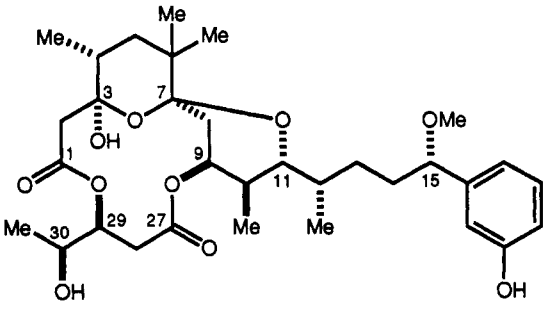
debromoaplysatoxin (DAT)



30-epi-DAT

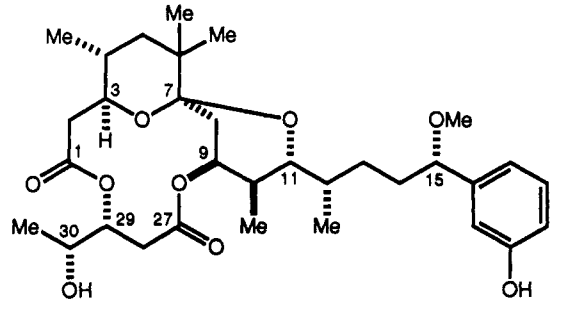


29-epi-DAT

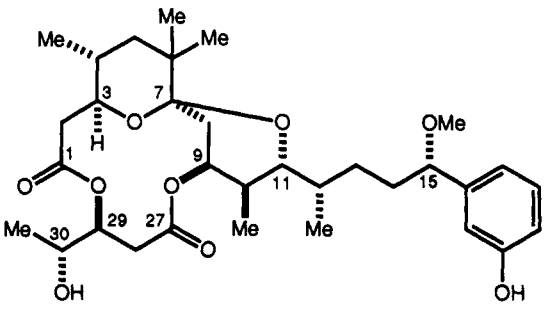


29,30-bisepi-DAT

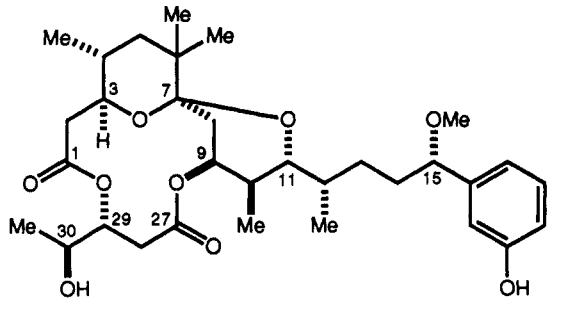
FIGURE 3: DAT and its C29 and C30 stereoisomers.



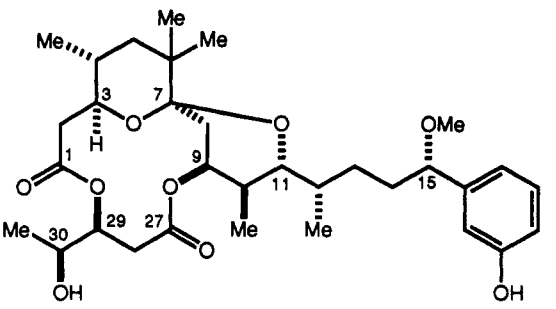
3-deoxy-DAT



3-deoxy-29-epi-DAT



3-deoxy-30-epi-DAT



3-deoxy-29,30-bisepi-DAT

FIGURE 4: 3-Deoxy-DAT and its C29 and C30 stereoisomers.

the transposition of the carbonyl group for an oxygen at the *sn*-1 position. The hypothesis that the 3,4-dihydroxybutyrate moiety of DAT is the pharmacophore suggests that other oxygen atoms of DAT are not essential for activity.

To examine the effect of the C3 oxygen on biological activity, 3-deoxy-DAT and its C29 and C30 stereoisomers (Figure 4) were studied (Nakamura et al., 1989). It should be noted that, in model-building exercises by others, attention has been erroneously drawn to the C3 hydroxyl group of DAT as being important in binding to the PKC regulatory domain (Jeffrey & Liskamp, 1986; Wender et al., 1986; Itai et al., 1988; Wender et al., 1988).

However, before studying the 3-deoxy series, it was important to verify that the preferred solution conformations in the 3-deoxy-DAT are the same as that of DAT itself, or else conclusions drawn from studies in the 3-deoxy series will not

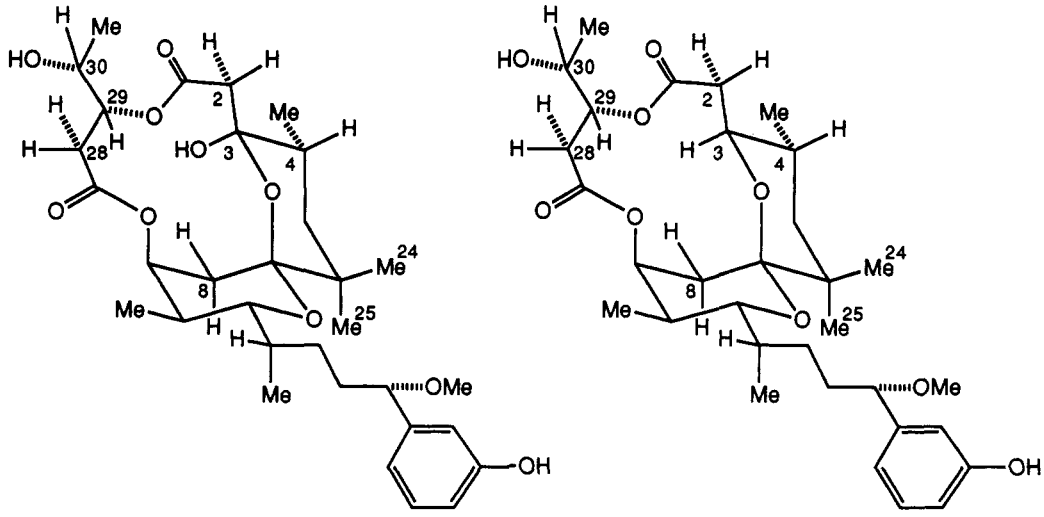


FIGURE 5: Preferred solution conformation of DAT and 3-deoxy-DAT.

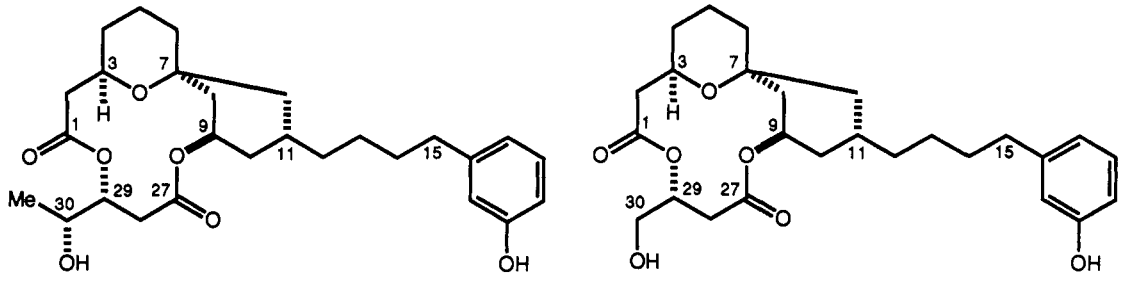


FIGURE 6: C-Substituted 3-deoxy-DAT analogues.

be relevant to a general discussion of the DATs. Extensive ^{1}H NMR studies established that the preferred solution conformation of 3-deoxy-DAT (Figure 5) is virtually superimposable on that of DAT (Nakamura et al., 1989). Given this result, the 3-deoxy-DATs shown in Figure 4 were synthesized and studied as PKC activators (Nakamura et al., 1989).

Most interestingly, 3-deoxy-DAT proved to be approximately equipotent with DAT, eliminating the C3 OH from consideration as an obligate part of the pharmacophore (Nakamura et al., 1989). In addition, as expected, 3-deoxy-29-epi-DAT was virtually without PKC agonist activity, 3-deoxy-30-epi-DAT had very little agonist activity, and 3-deoxy-29,30-bisepi-DAT was inert (Nakamura et al., 1989). Thus, the 3-deoxy-DATs ran true to form with respect to stereochemical expectations. These results support our conclusions with respect to the C29 and C30 stereo centers of DAT and remove from consideration any proposed structural model which ascribes an important role to the C3 oxygen of DAT.

In addition to the 3-deoxy-DAT series, a series of DAT-like analogues containing a C substituted for the C11 ether oxygen (Figure 6) were also synthesized and tested for PKC agonist activity. The two oscillatoxin analogues shown in Figure 6 were active, but their 29-epi isomers were not (Jirousek, Kishi, Perez-Sala, and Rando, unpublished experiments). These experiments remove the C11 ether oxygen from further consideration as a possible hydrophilic contact point in the action of DAT-like activators.

The Pharmacophore of DAT

The structural and stereochemical analysis in the DAT series described above is consistent with the hypothesis that the pharmacophore of DAT is embedded in the 3,4-dihydroxybutyrate moiety of the molecule. To prove that the 3,4-dihydroxybutyrate moiety of DAT is an essential part of the pharmacophore, derivatives of this moiety were synthesized and studied as activators of PKC (Kong et al., 1991). The dioleoyl esters of the chiral dihydroxybutyrates and the chiral 4-methyl-3,4-dihydroxybutyrates (dihydroxyvalerates) were prepared and studied as potential activators of PKC (Figure 7). The K_D values for these compounds are also shown in Figure 7. On the basis of previous stereochemical considerations, only analogues **4** and **5** should be active. This is exactly what was observed. Analogue **4** has the same absolute stereochemistry as (*S*)-diolein, and **5** has the same absolute stereochemistry as both the 3,4-dihydroxybutyrate moiety of DAT and the active 3-methyl-DAG. These results strongly support our hypothesis on the active moiety of DAT.

It is instructive to compare the potency of DAT and its active pharmacophore, the 3,4-dihydroxyvalerate derivative **5**. The K_D for DAT binding to PKC is approximately 2 nM, whereas it is 660 nM for **5** (Kong et al., 1991). Therefore, DAT is over 300-fold more active than **5**. The substantially increased potency of DAT over that of **5** may suggest that additional favorable contacts occur between DAT and PKC beyond those possible between simple 3,4-dihydroxyvalerate and PKC.

Debromoaplysiatoxin as a Template for the Diglycerides

The experiments on DAT, taken together, strongly support our hypothesis in which the 3,4-dihydroxyvalerate moiety of DAT contains the essentials of the pharmacophore that interacts with PKC. This experimentally developed model then allows us to use the relatively rigid DAT structure to fix the essential interacting atoms of the effector in space. This in turn allows us to use the DAT structure as a template for the other tumor promoters and DAGs. On the basis of these considerations and the known stereochemical preference of PKC for (*S*)-DAGs, we argue that the active (*S*)-DAGs must

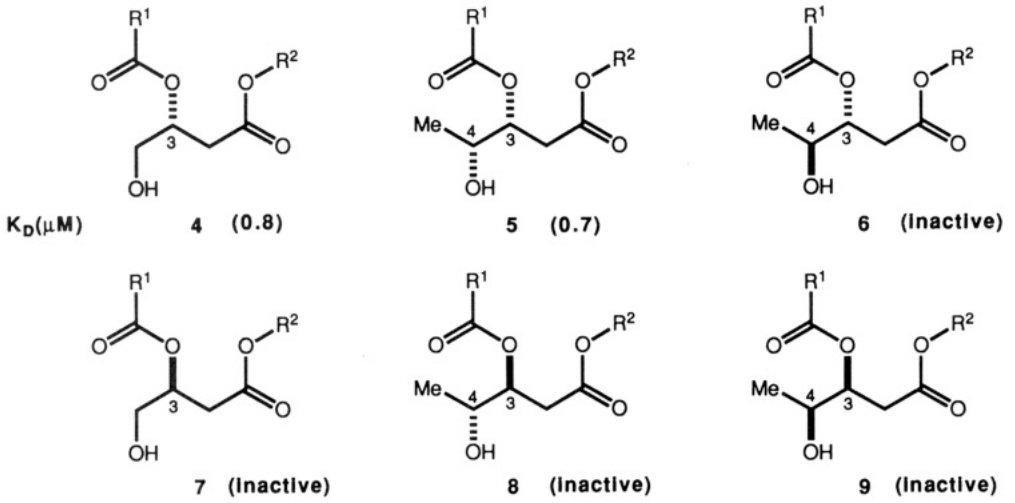


FIGURE 7: 3,4-Dihydroxybutyrate DAT analogues.

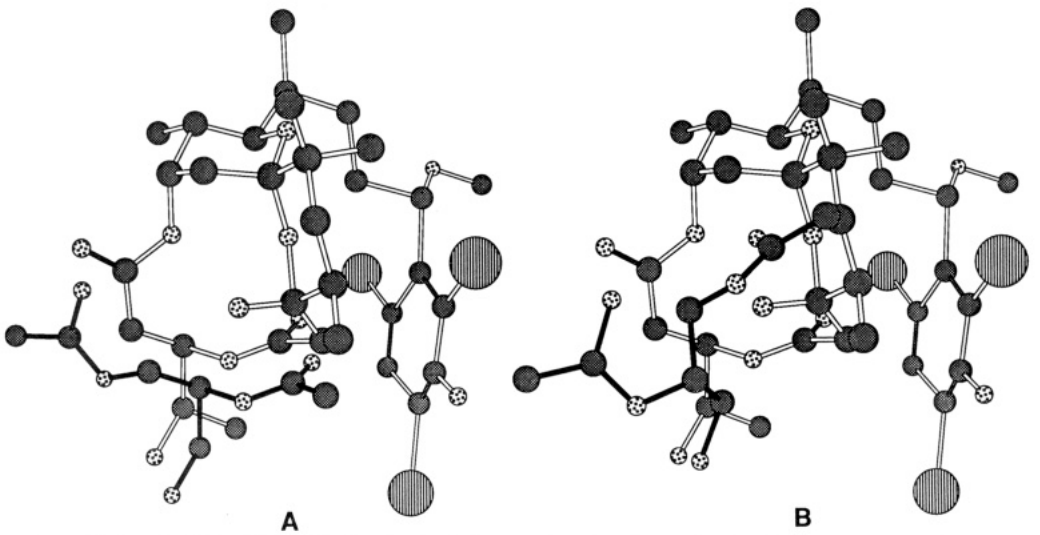


FIGURE 8: Structural correlation of DAT with DAG. Finely stippled, coarsely stippled, and hatched balls represent carbon, oxygen, and bromine atoms, respectively.

be bound to the active site of PKC as shown in A (Figure 8); the DAG is superimposed on an X-ray structure of 19,21-DAT, yielding the approximate conformation of DAG recognized by PKC (Nakamura et al., 1989). There is an alternative mode of structural correlation B (Figure 8), in which the C30 alcohol oxygen, the C27 carbonyl oxygen, and the C3 ether oxygen or the C7 ether oxygen of aplysiatoxins match with the C3 alcohol oxygen, the C2 carbonyl oxygen, and the C3 carbonyl oxygen of DAG, respectively. We can exclude the latter matching at this time, for three reasons. First, the stereochemical fit in A is far better than in B. Second, our structure–activity studies shown in Figure 6 eliminate the ether oxygen bridging the C7 and C11 carbons from consideration. Finally, the active 3,4-dihydroxybutyrate analogues shown in Figure 7 activate PKC even though they do not possess moieties analogous to the C3 or C7 oxygen atoms of DAT. Therefore, in the further analysis described in this paper, we used the conformation of DAG obtained from the correlation shown in A in Figure 8 to further the search for structural commonalities among the PKC activators.

The Generalized Tumor Promoter Activator Model

The conformation of DAG shown in Figure 9 allows us to use it as a template to search for pharmacophores in other tumor promoters. The proposed structural correlations be-

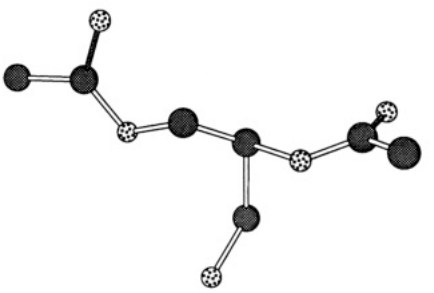


FIGURE 9: DAG template derived from DAT. Finely stippled and coarsely stippled balls represent carbon and oxygen atoms, respectively.

tween PKC activators and an (*S*)-DAG, with the relevant hydrophilic moieties indicated, are summarized in Figure 10; the oxygen atoms in shaded unrounded rectangles correspond, as do those indicated by shaded rounded rectangles and those indicated by unshaded rounded rectangles (Nakamura et al., 1989). The hydroxyl groups in the shaded unrounded rectangles are thought to be hydrogen-bond donors, and the hydrophilic atoms in the shaded rounded rectangles and those in the unshaded rounded rectangles are by necessity hydrogen-bond acceptors. On minimalist grounds and on the basis of structure–activity studies, it is assumed that the interaction of only three hydrophilic atoms is required, as indicated in Figure 10. It is possible that secondary interactions with other

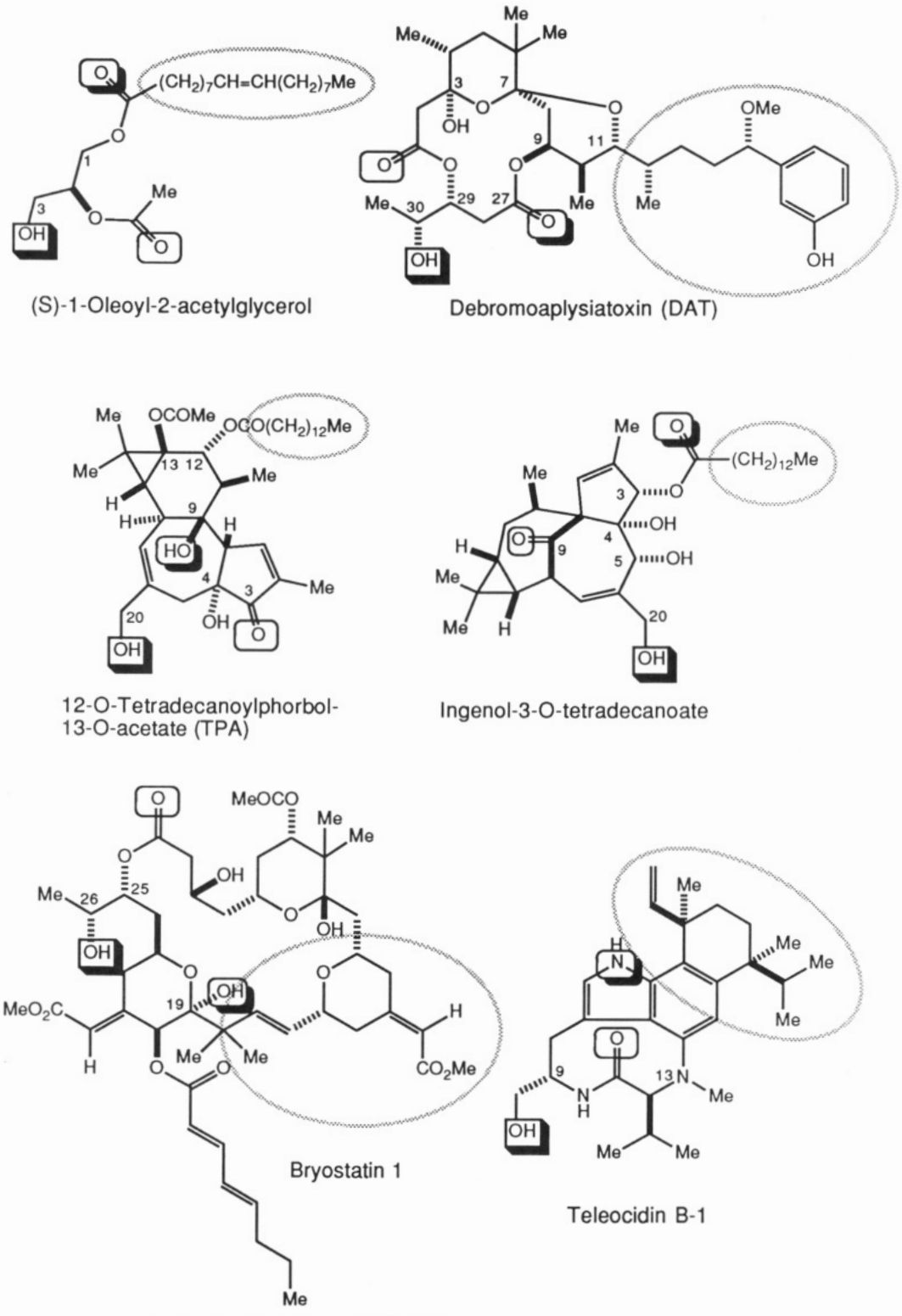


FIGURE 10: Structural correlation between DAG and tumor promoters.

hydrophilic atoms may occur, but we view these interactions as being of minor importance. The interaction of PKC with the three indicated hydrophilic moieties, with the indicated stereochemistries, is viewed as the sine qua non of PKC activation. In addition, the spatially corresponding hydrophobic moieties, which are enclosed in ovals in Figure 10, are obviously required, if only to ensure that the activator is membrane soluble. The model presented is quite different from others that have been proposed in the literature but is consistent with all the published structure–activity observations on the DAGs and the tumor promoters.

The structural correlation shown in Figure 10 is focused on DAG and the phorbol esters, the ingenols, the bryostatins, and the teleocidins. Before arriving at the structural correlation shown, other possibilities has to be discarded for each tumor promoter. Although a good match might be seen between the indicated conformation of DAG and the C9, C12, and C13 oxygens of phorbol esters, the possibility that this is the correct match was negated by the observation that 13-*O*-tetradecanoyl-12-deoxyphorbol shows high tumor-promoting activity (Hecker, 1978). However, using the conformation of DAG discussed above, an excellent match consistent with all the

published structure–activity observations can be identified between phorbol esters and DAG (Figure 10). Here the C3, C9, and C20 oxygens of the phorbol esters are matched with the corresponding DAG oxygens.

Among several possibilities for a structural correlation between the functional groups of ingenols and this conformation of DAG, the match shown in Figure 10 seems to be best. Although ingenols appear structurally similar to phorbol esters, this mode of structural correlation is significantly different from the one given for the correlation between phorbol esters and DAG. Clearly, experimental studies must be performed to verify that this correlation is the correct one for the ingenols.

It is rather straightforward to recognize the structural commonalities between this conformation of the DAG and bryostatins (Figure 10). This structural correlation is the same as the one proposed by Wender et al. (1988). Evidence for this correlation comes from the experimental finding that 26-epibryostatin 4 binds much more weakly to PKC than the natural epimer shown in Figure 10 (Lewin et al., 1991). Thus, the stereochemical selection at the C25 and C26 positions of bryostatin is identical to that found in the DATs and 3-methyl-DAGs. These results further establish the relevance of the model shown in Figure 10.

Because of the conformational flexibility of the teleocidins (Endo et al., 1986), the structural correlation of teleocidins with this conformation of DAG is difficult to establish. Nevertheless, one reasonable structural match seems most likely to be correct, as indicated in Figure 10.

Conclusion

An experimentally derived model for the activation of PKC by DAGs and tumor promoters is presented. This structural hypothesis uses the conformationally rigid tumor promoter DAT as a template. From this template, the effector-site bound conformation of DAG was deduced and utilized to reveal the pharmacophores of several other tumor promoters, including the phorbol esters. The deduced pharmacophores were all consistent with the known structure–activity studies of the various tumor promoters. This work should make the rational design of PKC antagonists possible.

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