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Computer-assisted molecular modeling of tumor promoters: Rationale for the activity of phorbol esters, teleocidin B, and aplysiatoxin

(carcinogenesis/tumor promotion/protein kinase C/mezerein/ingenol)

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ABSTRACT **In the two-stage model of skin carcinogenesis, it is believed that initiators bind to DNA and that tumor promoters such as phorbol 12-tetradecanoate 13-acetate (TPA) bind noncovalently to membrane-associated high-affinity receptors, probably protein kinase C. Two other types of potent tumor-promoting substances, aplysiatoxin and teleocidin, appear to act also by binding to and activating protein kinase C, even though their chemical structures are quite different. Therefore, we have undertaken computer modeling of the spacial relationship of various functional groups in these three chemical classes of tumor promoters in an attempt to explain how these diverse structures bind to the same receptor molecule. We propose a stereochemical model in which the oxygens in TPA at C-3, C-4, C-9, and C-20 (O-3, O-4, O-9, and O-20) correspond to the O-11, N-13, N-1, and O-24 positions in teleocidin and the O-27, O-3, O-11, and O-30 oxygens in aplysiatoxin, respectively. In this model all distances with respect to overlap of the corresponding atoms are <1 Å. In addition, all three types of molecules have their hydrophobic moieties oriented in a similar position. This model is further discussed with respect to other compounds showing various degrees of activity as tumor promoters, including mezerein, ingenol, and 4α-TPA. The model explains how chemically diverse structures can have similar biological activity as tumor promoters and provides a basis for designing both agonists and antagonists of tumor promoters.**

A series of compounds having diverse chemical structures have been shown to act as tumor promoters in a two-stage initiation and promotion system (Table 1) (1, 14). Several of these compounds share similar biological and biochemical effects in cell culture systems, including the ability to compete with tritiated phorbol dibutyrate, an active analog of TPA (Fig. 1), in receptor binding assays and to enhance the enzymic activity of the enzyme protein kinase C (2, 3) (Table 1). To explain these findings, we have considered the possibility that similar functionalities in the hydrophilic and hydrophobic moieties of certain tumor-promoting plant diterpenes, the polyacetate compound aplysiatoxin, and the indole alkaloid teleocidin occupy similar spacial positions with respect to cellular receptors.

METHODS AND MATERIALS

The solid-state structures of phorbol (15), teleocidin B (16), mezerein (17), aplysiatoxin (18), and ingenol (19) are known from x-ray crystallographic data. These data were entered in the interactive molecular modeling program (Model 1.2) developed by W. C. Still (Columbia University). These structures were then submitted to force-field calculations (20, 21) (molecular mechanics: Allinger's mm2 version) to obtain the energy of the x-ray conformation. Some molecules were too large to perform these calculations directly. In such cases, the side chains were assumed to be flexible and to impose little conformational changes on the central parts of the molecules, which were considered critical for receptor binding and were not included in the energy calculations. After these calculations, the coordinates of the energy-minimized structures, with the exception of the side chains in which greater rotational freedom might be expected, and the original x-ray coordinates agreed within 0.1 Å. A second approach investigated alternative conformations and was used for those compounds for which x-ray data were not available. In these cases, computer graphic displays of possible conformations of these molecules, based on Dreiding models, were drawn, and these were subsequently refined by using molecular mechanics calculations, as described above, to obtain the energy-minimized structures.

Table 1. Relative biological activities of tumor promoters

Compound	Tumor-promoting activity*	Receptor binding [^{3}H]PDP†	Receptor binding [^{3}H]PDB‡	PKC§
TPA	++++	8.8	8	2.5
TPA 20-acetate	+++	ND	ND	ND
4β-PDD	++++	ND	10	ND
4α-PDD	0	ND	>1000	Inactive
4-OMe-TPA	0	6.4	1500	Inactive
HHPA	ND	ND	4	1
4-deoxy-HHPA	ND	ND	10	ND
HHPA diacetate	ND	ND	>400	ND
PRA	+	8.2	ND	ND
Mezerein	+	7.9	ND	3
Ingenol 3-tetradecanoate	++++	8.8	ND	ND
Teleocidin	++++	8.5	17	18
Aplysiatoxin	++++	ND	19	12
Debromo-aplysiatoxin	+	7.2	200	12

Data was taken from refs. 1–13. PDD, phorbol 12,13-didecanoate; PRA, phorbol 12-retinoate 13-acetate; PDB, phorbol 12,13-dibutanoate (dibutyrate); PDP, phorbol 12,13-dipropionate; ND, not determined.
*Complete or second-stage tumor-promoting activity on mouse skin.
†$-\log k_1$ for inhibition of tritiated phorbol 12,13-dipropionate.
‡Concentration (nM) that gives 50% inhibition.
§Concentration (nM) that gives 50% of maximum stimulation of protein kinase C.

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Abbreviations: TPA, phorbol 12-tetradecanoate 13-acetate; HHPA, 16-hydroxyphorbol 12-hexadecanoate 13-acetate.

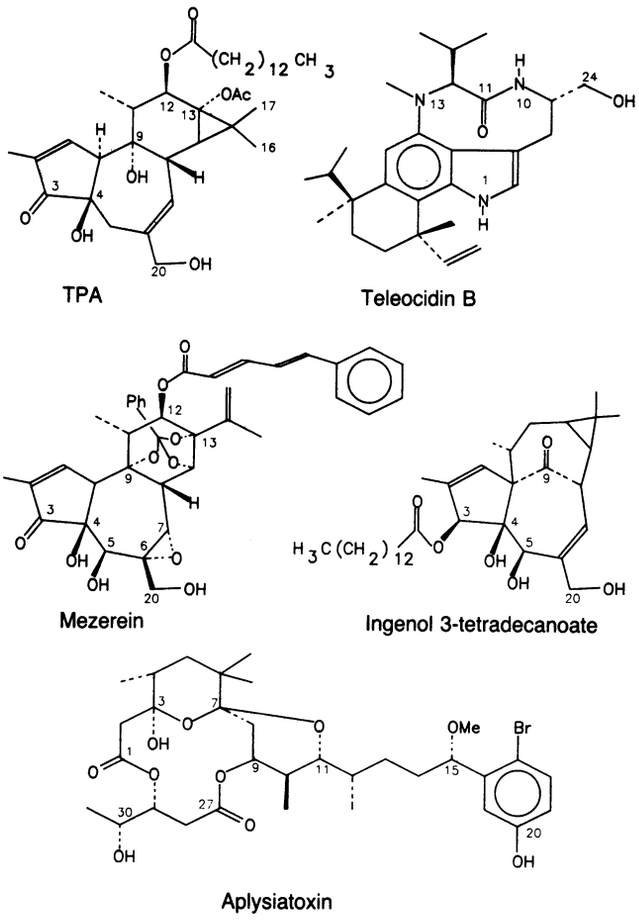

FIG. 1. Chemical structures of various tumor promoters.

RESULTS AND DISCUSSION

Critical Determinants in the Structure of Phorbol Esters. The rigidity of the phorbol skeleton favors the choice of TPA (Fig. 1) and other active phorbol esters as reference points for comparison with the structures of other chemical classes of tumor promoters. In addition, the availability of biological data on a large number of derivatives and analogs of TPA permits correlations between specific atoms or functional domains and biological activity. Certain points with respect to the biological data (Table 1) should be considered. (*i*) In many cases, these compounds were studied at only one concentration or over a limited concentration range; therefore, the data are not strictly quantitative. (*ii*) There is not a direct parallel between the relative potencies of a given compound in the various assays. (*iii*) Not all of the compounds have been studied, or studied in detail, in each of the assays listed. (*iv*) The pleiotropic effects of these compounds in intact cells and tissues may reflect the existence of multiple binding sites on a complex receptor that see somewhat different structural features of the agonist or may reflect receptor heterogeneity. (*v*) The biological effects of these compounds might reflect summation of binding sites—i.e., a compound might be equipotent with TPA because lower binding at one region of its structure is compensated by higher association at another. Therefore, we have correlated structural parameters with only three major biological effects: tumor promotion, inhibition of phorbol ester receptor binding, and activation of protein kinase C, emphasizing only major differences between analogs with respect to their relative potencies.

Conformation of Phorbol Compounds. The published x-ray structure of the phorbol nucleus (15) compares very well with the structure we derived by our minimum energy calculations (Figs. 1 and 2). The major differences from the x-ray structure result from rotations (ΔE, 0.9–1.3 kcal) of the 20-hydroxyl group. The energy-minimized structure of HHPA also corresponds well with the x-ray structure of phorbol. The compound 4α-phorbol 12,13-didecanoate is inactive, whereas 4β-phorbol 12,13-didecanoate is active (Table 1) in several biological systems. Therefore, we studied 4α-phorbol in detail. Two possible conformations were considered: one in which the seven-membered ring assumes a boat shape with respect to the C-5 and C-9 atoms (174.4 kcal) and one in which the seven-membered ring has a chair shape (176.3 kcal).

The Role of Specific Functional Groups in Phorbol Esters. The biologically active phorbol esters can be considered as amphiphilic compounds (2), with a hydrophilic domain spanning the C-3 to C-9 region of the molecule and a hydrophobic domain consisting of the acyl substituents on C-12 and C-13 and the cyclohexane- cyclopropane-annellated ring system (Fig. 1). It is important that the C-12 substituent be hydrophobic, but its precise structure can vary considerably without loss of biological activity (4). Consistent with studies using photoaffinity derivatives (22, 23), we assume this region of the molecule is involved in a relatively nonspecific hydrophobic interaction, presumably with lipids. However, the hydrophilic region plays a much more specific role. Reduction of the 1,2 double bond of the phorbol nucleus results in considerable loss of biological activity (3, 4). We found that this does not distort the conformation of the cyclopentane ring significantly, although it does alter the orientation of the 2-methyl group. Computer graphics indicated that other active tumor promoters have a relatively open space in this region of the molecule (Fig. 2). Therefore, steric hindrance by the 2-methyl group may be an important determinant in the loss of biological activity associated with reduction of the 1,2 double bond. When the 3-carbonyl group in TPA or HHPA (Table 1) is reduced to the corresponding β-alcohol, there is a considerable decrease in biological activity. In addition, the 3-deoxy derivative of HHPA (3) has reduced activity, suggesting that the 3-carbonyl oxygen plays an important role in biological activity. The acyl function at the corresponding position of ingenol (Fig. 1) may play an analogous role. The lack of biological activity of 4β-methoxy-TPA suggests that a free hydroxy function at this position is necessary for optimal activity or that increased steric crowding in this region impairs activity. The addition of a β-hydroxy group to TPA at position 5 results in a slight decrease in activity, but the effect is much more pronounced in compounds in which there is a 5,6 double bond and a hydroxyl or carbonyl group at the 7-position (4). The weak (or absence of) biological activity of compounds lacking a free 20-hydroxy function emphasizes the importance of this hydroxyl group (4).

The 6,7 double bond is not essential for biological activity of TPA, since an α- or β-epoxide at this position is equally active (3). Such changes have little effect on the conformation of the ring system and suggest that this region of the molecule is not subjected to steric crowding. However, the 6,7-epoxide of HHPA (3) shows about a 100-fold reduction in activity in terms of inhibition of phorbol dibutyrate-receptor binding (3), the reasons for which are not known. The 100-fold loss in activity in the 6,7-dihydro-HHPA (3) is explicable by a change from sp^2 to sp^3 hybridization of C-6 and C-7 and increased flexibility of the ring system, resulting in changes in the spacial orientation of the 20-hydroxyl group. Unfortunately, the absolute stereochemistry of this reduction is not known.

Taken together, the above findings suggest that the C-3 and C-4 oxygens and the C-20 hydroxyl of the phorbol esters play critical roles in biological activity. We have also included the

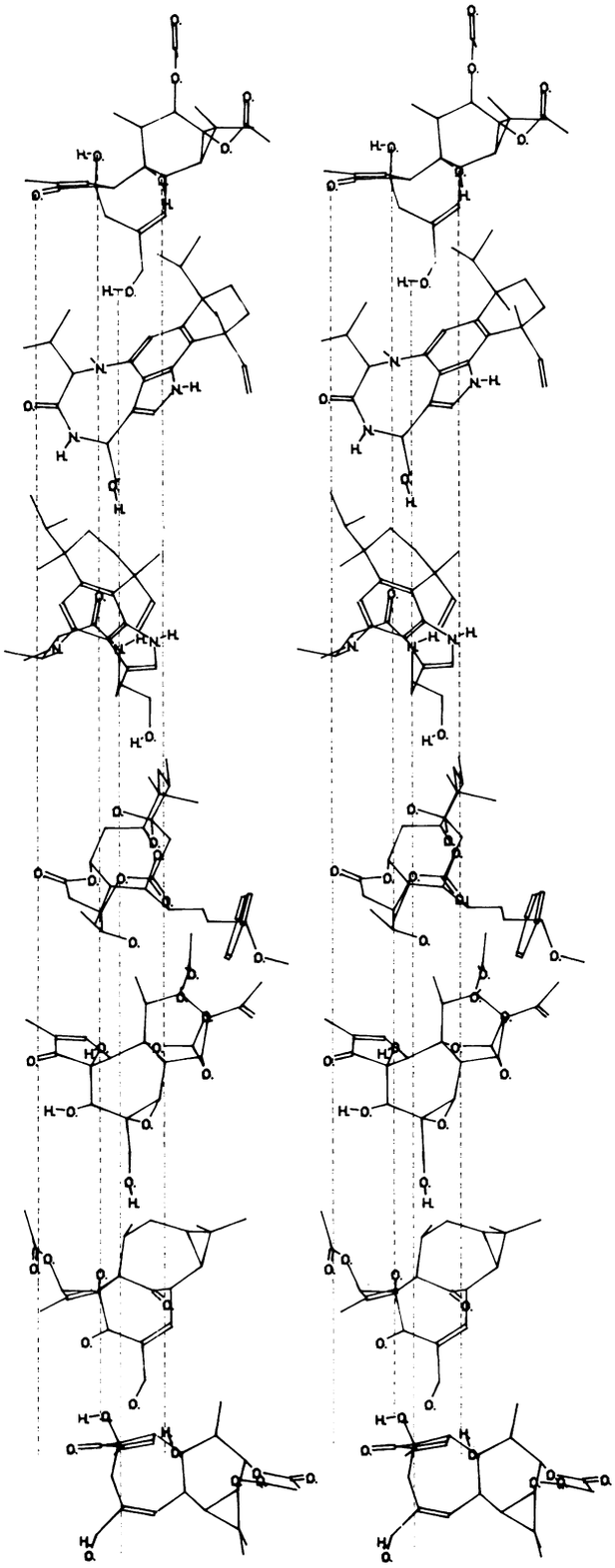

FIG. 2. Stereoscopic computer-generated views of various tumor promoters indicating their similarities. The dotted lines connect residues that occupy corresponding positions in space. The structures are arranged in the following order (from top to bottom): TPA, teleocidin (*RR*-conformation), teleocidin (*SS*-conformation), mezerein, ingenol, and 4α-TPA. The following modifications were made for purposes of clarity: in TPA, ingenol, and 4α-TPA, the tetradecanoate side chain is replaced by acetate; in mezerein, the phenyl group of the *ortho* ester is omitted, and the ester function at the O-12 position is substituted by an acetate in mezerein. Images should be observed with a stereoscopic viewer.

C-9 oxygen as a critical atom in our model, even though it has not yet been evaluated with respect to biological activity, because it corresponds to residues present in teleocidin and aplysiatoxin (see below).

The coincidence in space of the above four oxygens of TPA at C-3, C-4, C-9, and C-20 (O-3, O-4, O-9, and O-20) with a corresponding set of functionalities in various other tumor promoters was then investigated by superimposition, using computer graphics. Because of possible rotation around the bond between C-6 and C-20, the C-20 hydroxyl of TPA was a less-specific reference point than were the other three oxygens and was not generally used. Many of the models tested resulted in poor overlap of the putative corresponding centers, with distances greater than 1 Å, and were discarded. The models worthy of further consideration are discussed below.

When the two conformations of 4α-TPA were compared with TPA, there was reasonable overlap between the O-3, O-4, and O-9 atoms. However, the 20-hydroxy group of 4α-TPA did not overlap well with that of TPA even if rotated in various ways. In addition, the hydrophobic moiety of 4α-TPA occupied a completely different region in space from that of TPA. When we attempted to force an overlap of the 20-hydroxyl group of 4α-TPA with that of TPA, there was poor overlap of the other three oxygens (Fig. 2 and Table 2), using either of the energy-minimized conformations of 4α-TPA. Thus, based on our model, the inactivity of 4α-phorbol didecanoate is not surprising.

Analogies to Ingenol and Mezerein. The conformation of mezerein obtained by energy minimization shows an excellent overlap with TPA (Fig. 2, Table 2). It is not possible currently to explain why TPA is a complete tumor promoter whereas mezerein functions only in the second stage (24). The addition of a 5-hydroxy function to TPA and, as mentioned above, epoxidation of the 6,7 double bond in HHPA decrease their respective activities (Table 1). Both of these functionalities are present in mezerein. In addition, mezerein contains a carboxylic acid chain with unsaturated bonds and an aromatic nucleus in the region of the molecule corresponding to the C-12 substituent of TPA. If the saturated fatty acid C-12 in TPA is replaced by a polyunsaturated carboxylic acid (e.g., retinoic acid), the resulting compound (e.g., phorbol 12-retinoate 13-acetate) (Table 1) also functions only in the second stage of tumor promotion (25). However, further studies are required to determine why these structural features might influence the abilities of compounds to act at specific stages of tumor promotion. It should be noted that mezerein exerts a number of cell culture effects similar to those of TPA and also is a potent activator of protein kinase C (5).

Table 2. Interatomic distances (in parentheses) between corresponding atoms in various compounds

Compound	Atom and interatomic distance in Å relative to specific oxygen atoms in TPA		
	O-3 of TPA	O-4 of TPA	O-9 of TPA
Teleocidin B, *RR*	O-11 (0.53)	N-13 (0.10)	N-1 (0.53)
Teleocidin B, *SS*	N-13 (0.94)	O-11 (0.95)	N-1 (0.60)
Aplysiatoxin	O-27 (0.65)	O-3 (0.90)	O-11 (0.43)
Mezerein	O-3 (0.03)	O-4 (0.04)	O-9 (0.02)
Ingenol 3-tetradecanoate	O-3 (0.58)	O-4 (0.58)	O-9 (0.50)
4α-TPA*	O-3 (1.35)	O-4 (1.80)	O-9 (1.04)

*In order to obtain overlap of 4α-TPA with TPA, the C-20 of 4α-TPA and TPA (0.54 Å) had to be included, in addition to the overlap of the oxygen atoms at C-3, C-4, and C-9 (O-3, O-4, and O-9).

A comparison of the x-ray structure of ingenol with that of TPA shows that the best overlap can be achieved by assuming that the carbonyl oxygen of the C-3 acetate of the ingenol derivative corresponds with the O-3 atom of TPA (Fig. 2, Table 2). The large hydrophobic side chain of ingenol 3-tetradecanoate is oriented differently from that of the hydrophobic region of the other tumor promoters we have considered. It is conceivable, however, that this long alkyl side chain may fold over the molecule in order to assume an analogous position to the tetradecanoate side chain in TPA (Fig. 2).

Comparisons with Teleocidin. The x-ray structure of teleocidin B (16) provides the relative but not the absolute configuration of the two chiral centers in the lactam portion of the molecule. Using the x-ray conformation, we found the best overlap with our model of TPA using the *RR* isomer of teleocidin (Fig. 2 and Table 2). Our model indicates further that the 24-hydroxyl group of teleocidin is a critical substituent, a prediction consistent with recent data on biological activity of teleocidin analogs (26). Both teleocidin B (Fig. 1) and teleocidin A (also termed lyngbyatoxin) (26) show similar biologic activities (Table 1). The hydrophobic moieties in teleocidins A and B could overlap with the tetradecanoate side chain of TPA. Analogs of teleocidin, which do not contain this hydrophobic moiety or a moiety of comparable hydrophobicity, have a marked decrease in biological activity (27). Thus, the hydrophobic region of teleocidin, by analogy with the phorbol esters, plays an important role in biological activity.

Although the above described model of teleocidin B is based on the *RR* isomer, during the course of our studies, other investigators synthesized all four stereoisomers of the lactam moiety of teleocidin B and concluded, on the basis of comparison of CD spectra, that teleocidin B is the *SS* isomer (28, 29). However, the CD spectrum of the synthetic compound did not precisely match that of teleocidin B. Furthermore, there are two additional chiral centers close to the tryptophane ring that could perturb the CD spectrum of teleocidin, making simple comparisons difficult. Therefore, we included the possibility that teleocidin B may have *RR* absolute stereochemistries with respect to its amino acid chiral centers. We have considered, however, several possible conformations of the *SS* enantiomer.

NMR data suggested that the nine-membered ring of teleocidin B may be in conformational equilibrium (28). Our calculations indicated that the published x-ray structure (16) showed the lowest minimized energy (46.4 kcal) of all of the conformations we considered. In this conformation the amide bond is antiparallel with respect to the indole plane, thus causing a twisted conformation such that the isopropyl and hydroxymethyl groups of the lactam ring are pointing in opposite directions and are on opposite sides of the indole plane (Figs. 1 and 2). Attempts to model a conformation with the alternative twist were unsuccessful, probably because of steric hindrance generated when either the *N*-methyl or isopropyl group were forced towards the center of the nine-membered ring. Inspections of Dreiding models suggested four other possible conformations of teleocidin, all containing a *cis*-amide bond: two boat and two chair conformations, with the lactam ring either above or below the indole plane. Minimized-energy calculations gave values for the two boat conformations of 56.7 and 49.7 kcal, respectively. Of the two chair conformations, the below-plane conformation relative to the orientation shown in Fig. 1 could not be modeled, probably because of steric hindrance when the isopropyl group was oriented towards the center of the ring system. The above-plane chair conformation had an energy of 51.2 kcal.

To determine whether any of the calculated comformations might resemble the solution conformations, we used the generalized Karplus equation (30). Unfortunately, the coupling constants derived from the calculated conformations using this equation (x-ray structure, 3.3 and 5.7 Hz; boat above plane, 1.7 and 11.8 Hz; boat below the plane, 1.2 and 7.4 Hz) differ significantly from the experimental values [3.3 and 5.5 Hz (31)]. This could be partially explained by assuming an equilibrium of conformation in solution (28). Unfortunately, no coupling constants are available for the minor form observed in the NMR spectra (28).

A conformer with a *trans*-amide bond could be modeled with the molecular modeling program. This conformer had a relatively low energy (50.5 kcal), which compares favorably with the conformations discussed above. In the x-ray structure (16), however, teleocidin apparently contains a *cis*-amide bond. If we take into account the high activation energy to rotate the amide bond from *cis* to *trans* (15–23 kcal) (32), it seems unlikely that this conformation will be present as a major component at equilibrium despite its low energy. By using Dreiding models and assuming that the nitrogen and carbons of the amide bond were sp^2-hybridized, it was not possible to construct models in which the stereochemistry of the amide bond was *trans*.

Based on the above considerations, we obtained a reasonable overlap between teleocidin B and TPA using the *SS* stereoisomer of teleocidin in which (*i*) the lactam ring system has a boat structure, (*ii*) there is a *cis*-amide bond, and (*iii*) the ring system is below the indole plane with respect to the orientation shown in Fig. 1 (Fig. 2). The latter structure also had a low minimized energy (49.7 kcal). Other possible conformations of the *SS* stereoisomer of teleocidin gave a poor overlap with TPA and/or a high minimized energy.

As can be seen in Fig. 2, in the *RR* isomer the N-1, O-11, N-13, and O-24 atoms of teleocidin overlap in three-dimensional space with the O-9, O-3, O-4, and O-20 atoms of TPA. In the case of the *SS* isomer with a boat conformation (Fig. 2), the corresponding atoms of teleocidin B are N-1, N-13, O-11, and O-24, respectively. Furthermore, the hydrophobic cyclohexyl ring systems of both the *RR* and *SS* isomers of teleocidin B occupy potentially similar positions in space to that of the C-12 residues of the phorbol esters.

Comparison with Aplysiatoxin. The spirotetrahydropyran system is quite rigid, and all of the structures that we considered gave the same conformations when energy-minimized. By contrast, the macrocyclic ring system was quite flexible, and a number of conformations could be modeled. The structure with the lowest calculated energy that we obtained also resembled the x-ray structure (18). No predictions could be made with respect to the conformation of the phenolic side chain, although it is reasonable to assume that it is quite flexible. In the x-ray structure (18), it folds back towards the hydrophilic surface of the molecule (Fig. 2).

The x-ray conformation of aplysiatoxin overlaps well with our models of TPA and teleocidin (Fig. 2). Rotation of the 30-hydroxyl group and slight conformational changes in the macrocyclic ring system of aplysiatoxin further improve the overlap (Table 2). TPA derivatives lacking a free β-hydroxyl group at the 4-position are inactive (4). The 3-hydroxyl group of aplysiatoxin, which we assume is analogous to the 4-hydroxyl group of TPA, appears to play an important role, since the 3-anhydro analog of aplysiatoxin is significantly less active than the parent compound (6) despite the similarity in conformation of the rest of the molecule. These findings are consistent with our model (Fig. 2). An interesting feature of aplysiatoxin is the role of the phenolic side chain. Loss of the bromine from aplysiatoxin appears to reduce biological activity, but there are some inconsistencies in the data obtained (7, 33) (Table 1). Loss of the bromine could alter the pKa of the phenolic group, although the change would be small based upon comparisons of the pKa of phenol (9.89) and 4-chlorophenol (9.18). Alternatively, the bromine atom may induce a conformational change because of steric

hindrance between itself and the adjacent methoxy group. In our model, the dimethyl tetrahydropyran ring of aplysiatoxin occupies a position in space similar to that of the C-12 residues of the phorbol esters (Fig. 2), but it is also conceivable that the hydrophobic brominated phenolic side chain plays a role analogous to that of the C-12 residue of TPA.

Potential Applications of This Model. Our model predicts that the critical structural features for certain tumor promoters for mouse skin and possibly other tissues include: (*i*) polar functional groups occupying positions in space approximating those of O-3, O-4, O-9, O-20 of TPA; (*ii*) the C-20 hydroxyl group must be free; (*iii*) the area above and behind the five-membered ring should be free from steric crowding; and (*iv*) there should be a hydrophobic moiety in the region corresponding to the 12-position of phorbol. Functional groups equivalent to the 5-hydroxyl group of ingenol and the brominated phenolic moiety in aplysiatoxin may also play an important role. Based on these relatively simple structural features, it should be possible to design and synthesize entirely new chemical classes of tumor promoters or to predict the potential tumor-promoting activities of existing chemicals. This model might also lead to the design of potential inhibitors of tumor promotion and to a better understanding of the mechanism by which certain tumor promoters can bind to and activate the enzyme protein kinase C. The natural ligand for the phorbol ester is not known, although it has been suggested that it is a diacylglycerol (34, 35). We have emphasized, however, that TPA, teleocidin, and aplysiatoxin are about 10^4 times more potent than are diacylglycerols in activating protein kinase C (5). This is consistent with the fact that in our modeling studies, simple diacylglycerols do not show significant overlap with the model displayed in Fig. 2. In particular, they lack the specific hydrophilic region emphasized in our model. It may prove worthwhile, therefore, to search in eukaryotic systems for naturally occurring compounds with the structural features characteristic of our proposed model, such as complex lipids or conceivably specific polypeptides.

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