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A CONFORMATIONAL STUDY OF THE TOPOGRAPHICAL REQUIREMENTS OF A PHYTOTROPIN RECOGNITION SITE ON THE NAPHTHYLPHTHALAMIC ACID RECEPTOR

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Key Word Index—Phytotropins; conformational study; receptor recognition; NPA receptor; electrostatic potential.

Abstract—Phytotropins are a group of chemicals which have the ability to abolish the gravitropic response in plants by inhibiting the polar movement of auxin in the plant. They have other physiological properties such as inhibiting the phototropic response of stems. They bind to the naphthylphthalamic acid receptor and may elicit their physiological responses by this means. Most phytotropins consist of a benzoic acid moiety substituted at the *ortho* position by a bridging group connected to a second aryl group. Conformational energy calculations were performed on a subset of phytotropins. The calculations yielded a single, low energy conformation common to each molecule and thus identified three dimensional requirements for binding to the receptor. Electrostatic potential calculations, in the vicinity of the benzoic acid moiety, identified recognition and binding requirements for this group. Similar calculations for the second aryl group indicated that some similarity exists between the electrostatic potentials of molecules which bind most tightly to the receptor. The revised binding model was assessed by consideration of a second series of molecules showing phytotropic activity. The model was consistent with the biological activities of these molecules.

INTRODUCTION

Phytotropins were first correlated as a group of chemicals through their ability to abolish the root gravitropic response in cress [1]. They can also inhibit the gravi-and phototropic responses in stems, as well as other physiological properties [2]. At a more mechanistic level, they inhibit the polar movement of auxin in the plant [3, 4] although the precise relationship between this property and the ability to affect the tropic responses remains to be established [5]. They also bind to the naphthylphthalamic acid (NPA) receptor, and such binding has been related to their auxin transport–inhibitory activity, in that, such binding has been shown to prevent auxin egrees from the cell [6, 7]. The NPA receptor is therefore of some physiological importance. It may be, for example, that this receptor is directly involved in the mechanisms of the photo-and gravitropic responses, which mechanisms, despite many attempts at explaining them, remain substantially unknown [8].

Determining the characteristics of the phytotropin recognition site on the NPA receptor is therefore of some interest. Recognition involves electrostatic, conformational and stereochemical requirements to be fulfilled by the interacting molecule, and such factors have been shown to be present in structure-activity correlations [1, 4, 5]. Crystal structures of phytotropins also display configurational differences [9–11].

In order to further define these factors, a recognition site model was constructed by correlation of receptor binding of candidate molecules [12]. Conformational and electronic factors which may be required for phytotropin–receptor interaction are assessed on the basis of this model.

RESULTS AND DISCUSSION

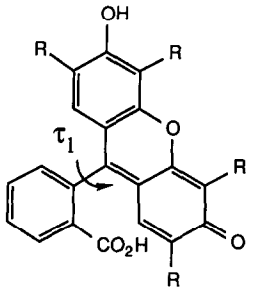
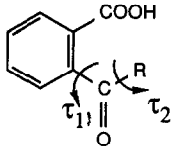
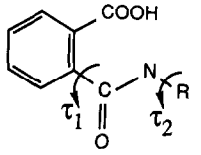
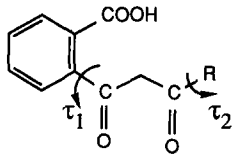
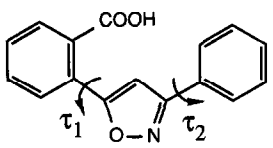
Assessment of the phytotropin recognition site model

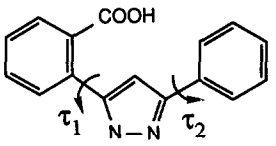
Katekar *et al.* [12] have proposed a simple model for phytotropin binding consisting of two orthogonal aryl binding regions Ar_1 (associated with the benzoic acid moiety) and Ar_2. An additional requirement for the phenylphthalamic acids is that the amide and Ar_2 aromatic ring be coplanar.

Conformational calculations were performed on a subset of the phytotropins for which relatively reliable binding and biological activity data were available, namely types 2, 3 and 5, (see Table 1) to assess the validity of the model. The requirement for coplanarity of the amide group and Ar_2 aromatic rings in type 3 compounds, together with the necessity for the 'binding' conformations of the molecules in the subset to superimpose, implies that $\tau_1 = 90°$ and $\tau_2 = 0°$ or 180° for all molecules (i.e. Ar_2 is perpendicular to Ar_1 [12]). The energy of the hypothetical binding conformation for each molecule relative to its global energy minimum is given in Table 2.

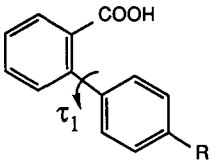
As the Table 2 shows, molecule 2-3 and the most active molecule, 2-5, have binding conformations which are same 250 kJ/mol above their respective global minima. Even allowing for some structural relaxation or resonance stabilization, which the calculations do not account for, it is extremely unlikely that in order to bind tightly the molecules will overcome such large energy barriers.

Table 1. Structures and activities of phytotropins. The torsion angles shown are defined in terms of the two carbon atoms defining the flexible bond and the carbon adjacent to each of these

Compound no.	Structure	Binding activity (pK_D)	Log *P* (Octanol–water)
			
1-1	R = H	5.41	4.51
1-2	R = Br	6.91	7.91
1-3	R = I	6.24	8.95
			
2-1	R = Phenyl	3.89	2.38
2-2	R = 4-Chlorophenyl	5.01	3.13
2-3	R = 1-Naphthyl	5.11	3.55
2-4	R = 2-Naphthyl	6.43	3.55
2-5	R = 1-Pyrenoyl	8.47	5.19
			
3-1	R = Phenyl	4.42	1.62
3-2	R = 1-Naphthyl	7.60	2.79
3-3	R = 2-Naphthyl	7.28	2.79
3-4	R = 2,6-Dichlorophenyl	Inactive	2.20
			
4-1		7.02	1.80
			
5-1		6.67	4.11



5-2		7.47	4.26



6-1	R = H	3.67	3.77
6-2	R = Cl	4.97	4.51

Table 2. The binding and conformational energies of phytotropins adopting the phytotropin recognition site model of ref. [12]

Phytotropin	Binding pK_D	Energy of conformation above global minimum (kJ/mol)
2-1	3.89	3
2-2	5.01	3
2-3	5.11	255
2-4	6.43	1
2-5	8.47	270
3-1	4.42	46
3-2	7.60	57
3-3	7.28	38
3-4	< 2	> 5000
5-1	6.67	9
5-2	7.47	9

Consequently, although this model is substantially accurate, it appears unlikely that the detailed topographical requirements proposed in ref. [12] are strictly correct.

A revised phytotropin binding model

It is likely that the essential details of the preceding model are correct but that the binding conformation (defining the relative orientation of the two aryl binding regions) differs from that proposed in the original model. In order to refine the model, conformational energy calculations were undertaken on a test set of phytotropins consisting of types 2, 3 and 5. Identification of the low energy conformations of these compounds, together with superimposition by computer graphics, should yield a binding conformation for each molecule which displays similar topography in each case, and is of low energy. Representative conformational energy surfaces for each of the three phytotropin types considered are given in Figs 1–3. Low energy conformations are listed in Table 3.

Since the pyrene derivative (PBA) of the type 2 phototropins (i.e. the arylbenzoic acids) is the most active, and is also quite conformationally restricted, it was used as a template in determining the most likely, active topography which the phytotropins must adopt at the receptor. Figure 1 shows the conformational energy surface arising from the rotation of the two torsion angles illustrated. The molecule is capable of adopting essentially only one conformation; the other conformation which appears on the energy surface is the mirror image of the first. The global energy minimum for this molecule is found at $\tau_1 = 110°$ and $\tau_2 = 110°$, this conformation is therefore close to, or coincident with, the binding conformation. The molecule is sufficiently hindered that moderate departures from this conformation entail significant energy penalties.

The other molecules in the set were then superimposed on this conformation of PBA. This was done by defining aryl receptor points perpendicular to each aromatic ring (above and below the plane) at a distance of 3.4 Å from the ring. The oxygen atoms in the benzoic acid moiety were also used as superimposition points. These receptor points are shown in Fig. 4. This method of receptor mapping is essentially the extended molecule approach of ref. [13].

The superimposition of the four receptor binding points on the benzoic acid moiety can be done unambiguously. Selections from the four possible pairs of aryl

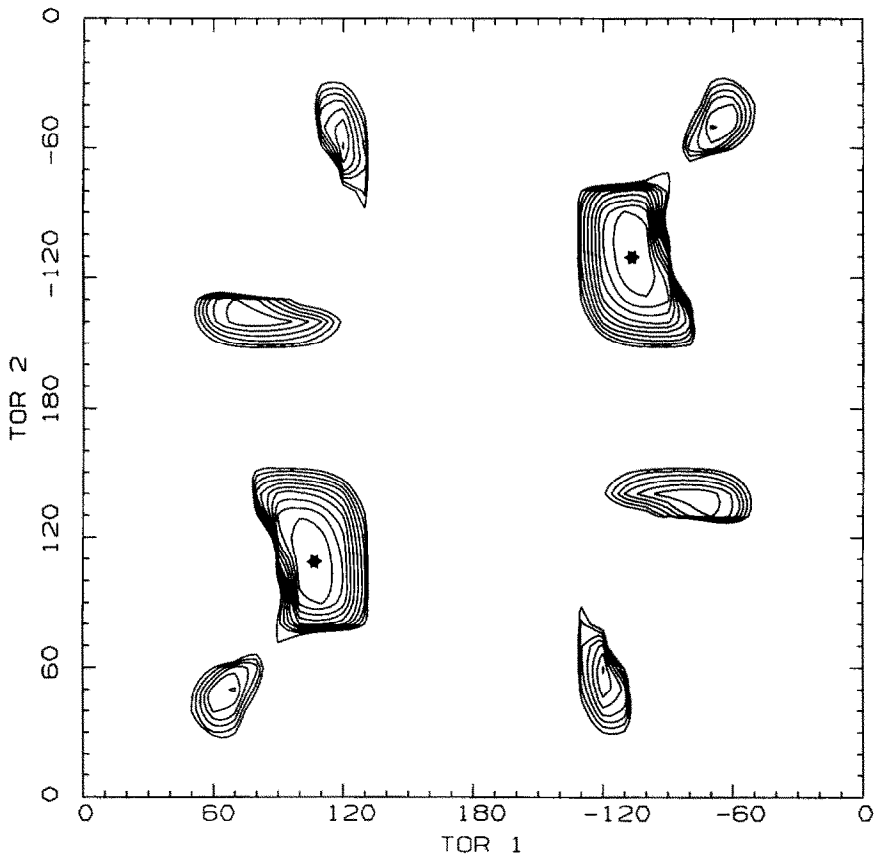


Fig. 1. Conformational energy surface for phytotropin 2-5 (PBA). The global minima are marked with stars. The contour interval is 10 kJ/mol.

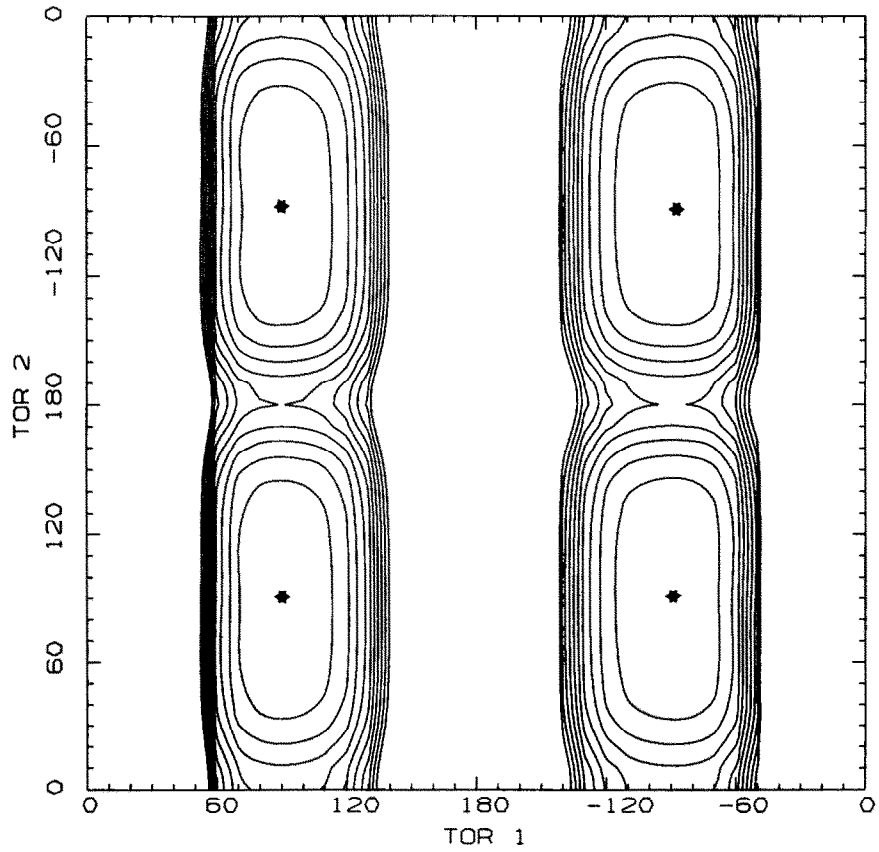


Fig. 2. Conformational energy surface for phytotropin 3-3. The global minima are marked with stars. The contour interval is 10 kJ/mol.

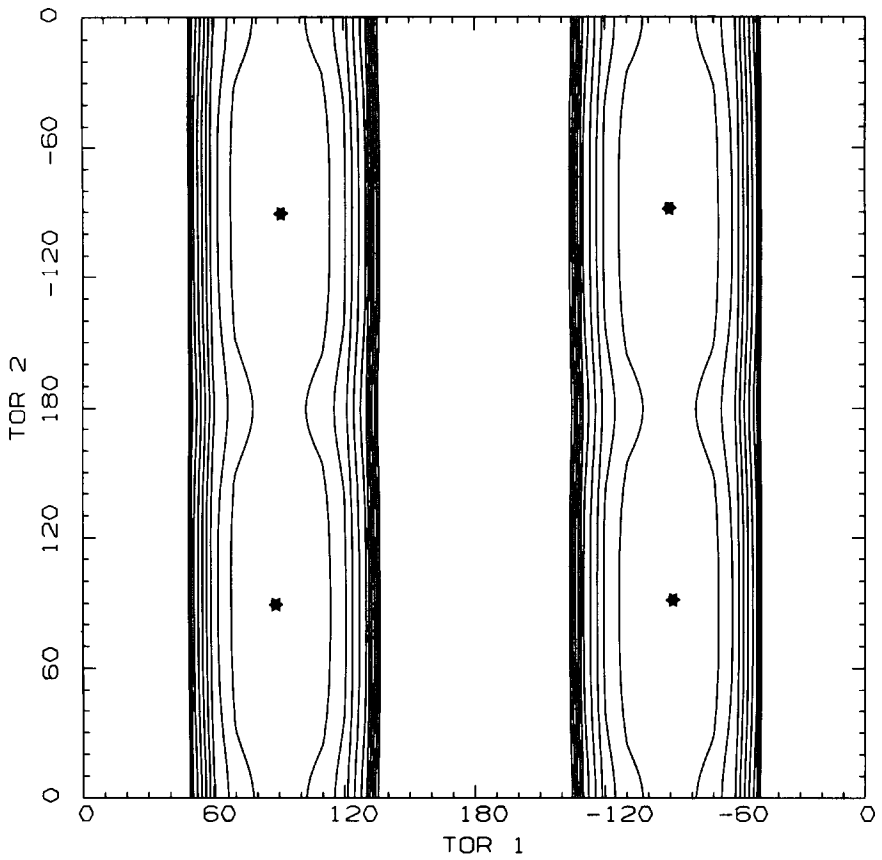


Fig. 3. Conformational energy surface for phytotropin 5-1. The global minima are marked with stars. The contour interval is 10 kJ/mol.

Table 3. Low energy conformations of phytotropins (the angle ranges represent the regions in which the energies are less than 40 kJ/mol above global minimum)

Phytotropin	τ_1(°)	τ_2(°)
2-1	± 90±20	0±60
		180±60
2-2	±90±20	0±60
		180±60
2-3	100±10	120±20
2-4	−100±10	−120±20
		180±60
2-5	110±10	110±20
	−110±10	−110±20
3-1	±90±30	20−350
3-2	100±30	−80±30
	−100±30	80±30
	− 90±30	±80±50
3-3	− 90±30	±90±60
3-4	90±40	±90±40
	−100±40	80±20
		−100±20
5-1	±90±30	0−360
5-2	± 90±30	0−360

binding points in the pyrene moiety were chosen to give the best, low energy superimposition between the pyrene compound and the others in the set. The energy of the conformation corresponding to the best fit for each

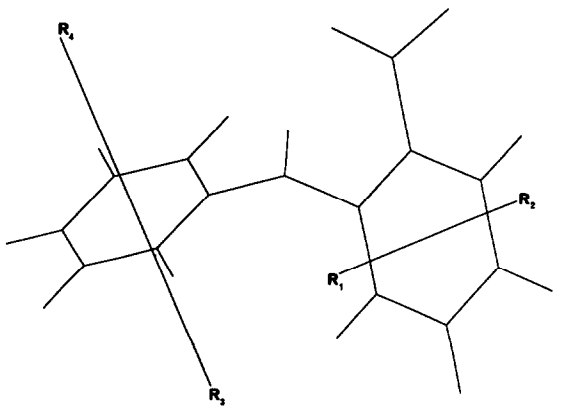


Fig. 4. Illustration of the aromatic ring receptor binding points used in the superimposition of phytotropins. The binding points R_1–R_4 are 3.4 Å from the ring and perpendicular to it. The oxygen atoms of the carboxyl group were also used as binding points.

molecule was then calculated and compared with the global energy minimum for that molecule. The energies of the best fit conformations are given in Table 4. The fit of the other members in the set to the assumed binding conformation of PBA was also attempted using the two pairs of aryl binding points only (neglecting the binding of the carboxyl oxygens). In this case the fit was slightly improved but several of the molecules adopted conformations with unfavourably high energies.

The results of the superimposition of the aryl and carboxyl receptor binding points for all molecules in the test series are given in Fig. 5. There is a good overlap of the benzoic acid moiety in each case with differences in the carboxyl oxygen positions being sufficiently small that hydrogen bonding interactions with a receptor would still be possible.

The overlap of the aromatic moiety at the Ar_2 binding position is also good. The arylphthalamic acids all produce good overlap with the amide linkage locked *trans* although the Ar_2 binding occurs in a slightly different plane to that of the arylbenzoic acids and type 5 phytotropins. All molecules in the test series except 3-4 have low energies when adopting the conformations shown in Fig. 5, as Table 4 shows. It is quite clear, however, that the 2,6-dichlorophenyl phthalamic acid (3–4) cannot adopt the required binding conformation due to steric hindrance from the two *ortho* chlorine atoms. This is consistent with its negligible activity. The relationship between the Ar_1 and Ar_2 binding regions in space, as proposed by the revised phytotropin binding model, is presented in Fig. 6.

Table 4. Relative energies of phytotropins in binding conformation

Phytotropin	Energy relative to global minimum (kJ/mol)
2-1	3
2-2	3
2-3	0
2-4	15
2-5	0
3-1	22
3-2	16
3-3	13
3-4	500
5-1	21
5-2	26

Binding of the Ar_2 region

It is apparent from inspection of the proposed receptor model given in Fig. 6 and the energies of the molecules in their binding conformations that the binding activities of

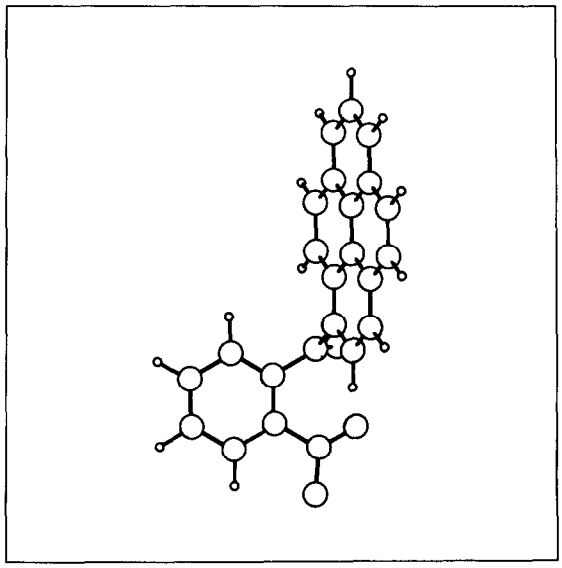


Fig. 6. View of the phytotropin 2-5 in its proposed binding conformation. The benzoic acid moiety (Ar_1) is in the plane of the page and the second aryl binding region (Ar_2) is above it. The relative positions of Ar_1 and Ar_2 define the binding model.

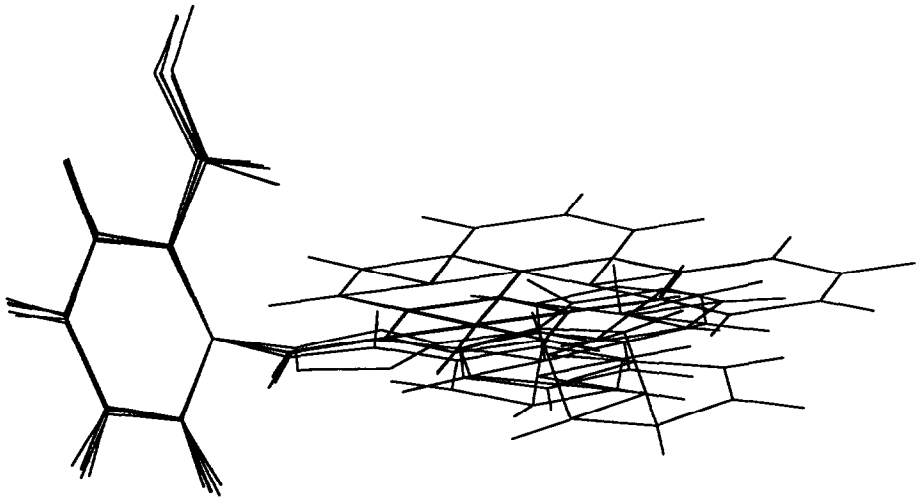


Fig. 5. Superimposition of phytotropins of types 2,3 and 5 in their proposed binding conformations.

the phytotropins in the test set are influenced by the nature of the aromatic moiety in the Ar_2 region. Exceptions to this occur where other steric effects such as ortho substituents are significant, as in compound 3-4. There is no obvious relationship between the goodness of fit of a particular molecule to the model and its gravitropic activity or between energy of its binding conformation and activity.

In the phytotropin recognition site model [12], it was proposed that the nucleus binding at the Ar_2 receptor point would do so more tightly in certain parts of the receptor binding region than others. The main evidence for this is in the higher gravitropic activity observed for compounds which had a three atom unit between the Ar_1 aryl group and the first aromatic ring in the Ar_2 area, and the higher gravitropic response of several isomeric dichlorophenylphthalamic acids (except for the 2,6-derivative where steric effects are important). In order to understand the binding to the Ar_2 region more fully, electrostatic potentials appropriate to each group binding to the Ar_2 region were generated.

Figure 7 shows the potential (the field experienced by a proton approaching the aromatic moiety in the specified plane) around several of the benzoylbenzoic acids. The potential is calculated in a plane parallel to the aromatic ring plane and 2Å below it. The potential wells generated by the electronegative carbonyl and carboxyl groups are clearly apparent. There were no obvious correlations between the depths of these potential wells and the binding affinities of the relevant phytotropins. The binding affinities of these phytotropins did bear some relation to the shapes of the aromatic moieties in the Ar_2 region, as suggested by Katekar *et al.* [12]. In the naphthyl derivatives, 2-3 and 2-4, the potential in the plane perpendicular to the naphthyl moiety was more similar to the equivalent potential around PBA (2-5) for the β analogues than for the α analogues. The electrostatic potential in the Ar_1 (benzoic acid) binding region is given in Fig. 8.

The potential wells resulting from the influence of the carboxyl and carbonyl groups are also clearly evident, providing an indication of the spacial requirements for electrostatic complementarity of a receptor at this point. This property is clearly quite specific as substitution in Ar_1 decreases gravitropic activity in type 4 compounds [14] and also in the fluoresceins [15]. The tetrachloro analogue of NPA also has a reduced ability to bind to the receptor [16].

The calculated lipophilicities of the phytotropins of types 2, 3 and 5 showed no obvious correlation with activity within the test set (see Table 1). The binding affinities of the phytotropins in the test set appeared to be at least partly influenced by the lipophilicity of the Ar_2 moiety (except where steric influences on the binding conformation were dominant).

Assessment of the revised model

The activities of the phytotropin types 1,4 and 6 not previously considered can now be assessed in terms of their binding to the receptor model. The substituted fluoresceins (type 1) are particularly suitable for assessing the revised phytotropin binding model as they have

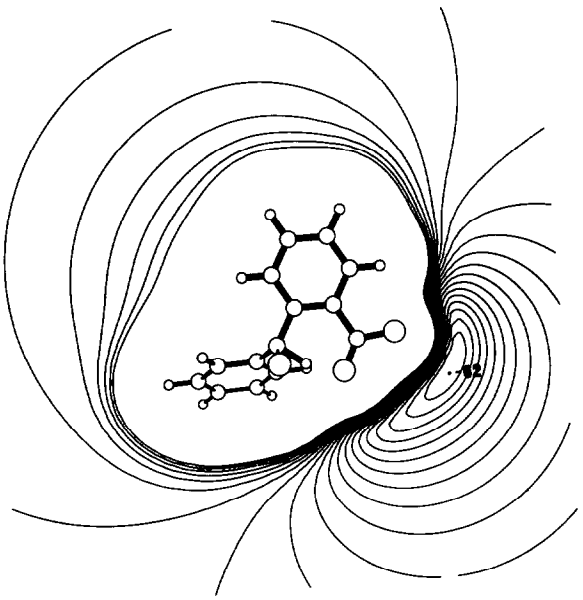


Fig. 8. Molecular electrostatic potential around the benzoic acid moiety (Ar_1). The contour interval is 4.2 kJ/mol. Numbers indicate depth of potential wells in kJ/mol. The potential is calculated in the plane of the benzoic acid moiety.

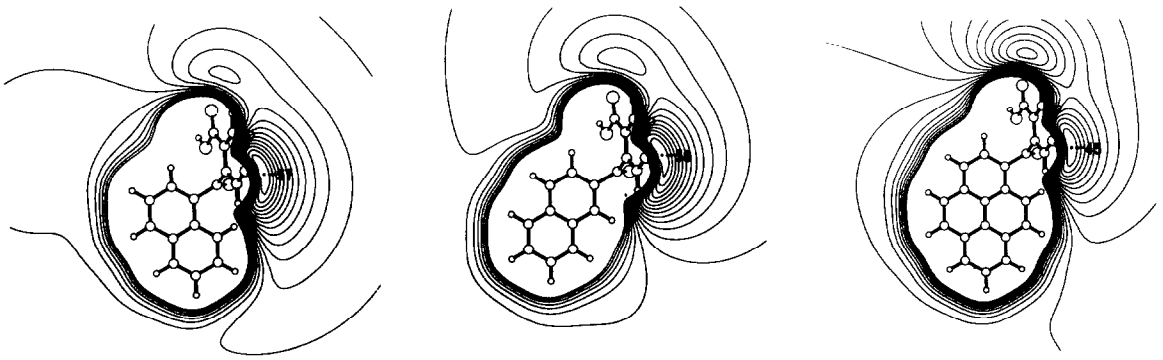


Fig. 7. Molecular electrostatic potentials around three phytotropins:- (a) compound 2-3, (b) compound 2-4, (c) compound 2-5, The contour interval is 4.2 kJ/mol. Numbers indicate the depth of the potential wells in kJ/mol. The potential is calculated in a plane 2 Å below the ring plane.

essentially only one conformational degree of freedom and a crystal structure is known [17]. The relatively low activity of the parent species 1-1 indicates that the fluoresceins may not be binding as efficiently to the NPA site as their tricyclic structure (in the Ar_2 region) would suggest. An unweighted superimposition of the substituted fluoresceins onto the very active pyrenoylbenzoic acid (2-5) template indicated that only a moderately good fit was possible between the Ar_2 polycyclic moieties. The fluoresceins bound at the NPA receptor in a slightly different plane than the PBA and the aromatic regions could not overlap completely. Addition of electron-rich substituents around the tricyclic rings, as in 1–2 and 1-3, increases the area of the region of high electron density accessible to the Ar_2 binding region. This, together with the increase in the lipophilicity of the Ar_2 binding group, may be responsible for the higher activities of these compounds. The very high lipophilicity of 1-3 (a log *P* octanol–water of approximately 9) may impair its transport to the site of action and thus reduce its *in vivo* activity. The preferred binding conformation was one having a fluorescein torsion angle value of $\tau_1 = 125°$ (or the topographically equivalent value of 305°).

The type 4 phytotropins adopt essentially the same topography as the type 5 compounds and can be accommodated easily into the binding model. Being β-diketones they have a planar arrangement of the diketone moiety due to the contribution of several resonance forms and hydrogen bonding between the carbonyl oxygens.

The arylbenzoic acids 6-1 and 6-2 have relatively low binding affinities (and poor biological activity). This is consistent with the receptor binding model as the aryl group is too close to the benzoic acid moiety to effectively bind to the Ar_2 region. Addition of a *p*-chloro group would increase activity as the region of high electron density available to the binding region Ar_2 is larger in this case.

The revised topographical model appears better able to account for the *in vitro* activities of the phytotropins considered here and provides a more precise description of the relative orientations of the two aryl binding regions on the receptor. It will be used in the design of new compounds with potentially higher phytotropic activity.

EXPERIMENTAL

Computational methods. Conformational energy calculations were performed on a CYBER 845 and 205 computers using the CONES program [18]. Comparison of molecular structures by computer graphics, and additional energy calculations, were conducted on a PDP11/34 computer using the CRYSX program [18]. Both programs perform classical conformational calculations by pairwise summation of the Van der Waals interaction between non-bonded atoms, together with electrostatic potentials. The force field parameterizations used were those of refs [19, 20] which have been used to study a number of related systems [21, 22]. The results obtained are usually consistent with those of moleculear orbital calculations [23].

The molecular geometries used in the calculations were obtained from crystal structures of 2-[(naphthalen-2-yl) carbanoyl)]benzoic acid [11], 2-(5-phenyl-1,3,4-oxadiazol-2-yl)benzoic acid [11], 2-(phenyl carbanoyl)benzoic acid [11] and 2-(2,6-dichlorophenylcarbanoyl)benzoic acid [10]. Other molecular geometries were based on crystal structures of related compounds and bondlengths and angles from standard compilations [24]. The torsion angles varied in the conformational analysis, and the atoms defining those torsion angles are given in Table 1. The torsion angles were defined according to the convention of ref. [25]. Conformational energy contour maps were prepared using a modification of the contouring program KONTOR [26] and molecular structural diagrams were produced by the PLUTO program (Motherwell, unpublished). Electrostatic potential calculations were performed with a semi-empirical program ELCPOT [18] using charges obtained from the modified CNDO/2 program INCOUL [27]. Calculations were done with the molecules in their proposed binding conformations and potentials are generated in the specified planes. Lipophilicities of phytotropins and fragments were obtained from calculated log *P* (octanol–water) values from the CLOGP3 program [28].

Bioassay

Plant Material. For compatibility with previous results, [12] seeds of the same variety of maize were used (*Zea mays* L.cv.PX-82). The seeds were surface sterilized with a 4% solution of NaClO for 45 sec, followed by rinsing in H_2O for at least 12 hr to facilitate imbibition. Germination took place in darkness at 25° in plastic trays containing a (1:1) mixture of perlite and vermiculite. After 5–6 days, coleoptiles were harvested as required and kept chilled on ice. These and subsequent procedures were performed in daylight.

Preparation of binding fractions. In general, the method previously described was followed [12]. As is usual with this type of procedure, the preparation deteriorates rapidly at room temperature. For consistent results, the preparation and assay were carried out within the day, keeping the tmperature at 0–4°. The tissue (usually 7–12 g/assay) was cut into small pieces with a cold razor blade and homogenized, by pestle and mortar, in an equal volume of grinding buffer (0.25 M sucrose, 50 mM Tris; 1 mM disodium EDTA; 0.1 mM $MgCl_2$, adjusted with acetic acid to pH 8.0). The homogenate was squeezed through 20 μm nylon cloth and the residue reground and extracted twice more in equal vols of the buffer. The amount of buffer used was calculated to give 0.33 g fr. w of tissue per assay for all tissues. The combined extracts were centrifuged at 4000 *g* for 20 min and the pellets discarded. The supernatant was recentrifuged for 30 min at 38 000 *g*.

Binding assay. The membrane pellet was resuspended in binding medium (0.25 M) sucrose, 10 mM sodium citrate, 5 mM $MgSO_4$ adjusted with acetic acid to pH 5.5) using a Teflon–glass homogenizer. To each ml of binding fraction 20 μl of ^{3}H NPA was added to give a final concn of 10^{-8} M). The radiolabelled binding fraction was divided into 3 ml aliquots to which 30 μl of an ethanolic solution of the test compounds were added at various concentrations. The blank control samples were obtained by the addition of 30 μl ethanol. Samples of CPD at 10^{-7} and 10^{-4} M were also included as standards in each experiment. Triplicate 0.9 ml samples were taken from each aliquot and placed in 1 ml polycarbonate tubes. All manipulations were carried out at 0–4°. After 30 min the tubes were centrifuged for 45 min at 38 000 *g* (Beckman fixed angle rotor type-25). The supernatants were decanted and the surfaces of the pellets gently washed with 1000 μl H_2O. The membrane pellets were allowed to dissolve overnight in 200 μl 10 mM Tris. The pellets were recovered using 3 × 200 μl aliquots of 10 mM Tris, transferred to scintillation vials and counted in 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole/l of toluene, mixed 2:1 v/v with Triton X-100). Total radioactivity was measured, using a Beckman LS 6800 liquid scintillation counter.

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