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Unrevealed structural requirements for auxin-like molecules by theoretical and experimental evidences

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Abstract

An computational-biostatistical approach, supported by ab initio optimizations of auxin-like molecules, was used to find biologically meaningful relationships between quantum chemical variables and fresh bioassay's data. It is proven that the auxin-like recognition requires different molecular assembling states. We suggest that the carboxyl group is not the determining factor in explaining the biological auxin-like conduct. The biological effects depends essentially on the chemical condition of the ring system. The aim to find active molecules (quantum objects) via statistical grouping-analysis of molecular quantum similarity measures was verified by bioactivity assays. Next, this approach led to the discovery of a non-carboxylated active auxin-like molecule (2,6-dibromo-phenol). This is the first publication on structure activity relationship of auxin-like molecules, which relies on highly standardized bioassays of different auxins screened in parallel as well as analysed by multi-dimensional scaling.

Keywords: Auxin; Structure–activity relationship; Molecular quantum similarity measures; Plant growth regulation

1. Introduction

The analysis of chemical messengers is still one of the hot spots in plant physiology, biochemistry and molecular biology (Kulaeva and Prokoptseva, 2004), although phytohormones like auxin have been described decades ago (Went, 1935, 1945). Indole-3-acetic acid (IAA) and its analogs were found to be the typical auxin, mainly evaluated by cell elongation tests (Went and Thimann, 1937; Koepfli et al., 1938; Thimann and Schneider, 1938, 1939; Thimann, 1958; Porter and Thimann, 1965). However, the chemical space, which encompasses the term "auxin", is actually not easily achieved, since hundreds of substances were found to exhibit an auxin-like activity in several different bioassays. The analysis about structural requirements of auxins was hypothesized before by chemical intuition, but without convincing results (Went and Thimann, 1937; Koepfli et al., 1938; Jönsson, 1961; Porter and Thimann, 1965; Katekar, 1979). Different reasons can be found for the inadequacy:

- Most structure analysis was performed between 1930 and 1970, a technological scenery unable to provide substances sufficiently purified.
- All attempts to elucidate this relationship based on biological assays were performed in different labs, under different conditions, know how, and insufficient comparison of the different results obtained.

Abbreviations: PCA, principal component analysis; wt, wild type; MQSM, molecular quantum similarity measure; QSM, quantum similarity measure; DF, density function; DFT, density functional theory; HF, Hartree–Fock.

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- The existence of different auxin binding proteins, the wide diversity of the auxin molecules and its pleiotropic effects prevented the establishment of a convincing correlation between structure and biological activity up to now.

The term "auxin" is still available as a physiological definition and a well-defined molecular structure does not exist. The biochemical mechanisms of auxin-like molecules represent the result of an evolutionary process in plant kingdom (Dibbfuller and Morris, 1992; Cooke et al., 2002) and are thought to be in charge of particular specificities on their structure – activity relationships. IAA is still known to be an active molecule in all bioassays (Woodward and Bartel, 2005), and is reliant on its plasticity properties and metabolic interactions.

In a previous work, we were able to present a computational approach dealing with semi-empirical optimizations of the auxin molecules themselves. Our approach used molecular quantum similarity measures for the analysis of more than 240 auxin-like molecules (Ferro et al., 2006b). The finding of similarities in these molecules by focusing basically on intermolecular interaction descriptor, enabled us to cluster the auxins into different groups (Table 1). It was postulated that the auxin-like molecular recognition depends more on specific molecular assembling states than on a specific ring system or side chain.

Here, we present the significance of the similarity groups published before (Ferro et al., 2006b) in the context of their closer comparable biological activities. Furthermore, this is the first publication on structure activity relationship analysis of auxins, which relays on highly standardized bioassays, performed for all tested auxins in parallel. By this integral approach we were able to detect both nominal and continuous cause – effect relationships from quantitative analysis, which are objects to explain biological attributes such as:

- pleiotropic activity,
- the high number of active molecules,
- differentiated specificity exposed by auxin-like molecules.

Data obtained from bioassays and biostatistical analysis enabled us to further refine the structure–activity relationships of auxin-like molecules.

2. Results and discussion

2.1. Analysis and clustering of biological activities

Auxins are defined mainly by a set of physiological actions, but the structure–effect relationship is still based on chemical intuition. Recently, we presented a computational approach dealing with semi-empirical optimizations of the auxin molecules themselves (Ferro et al., 2006b) using Molecular Quantum Similarity Measures and additional quantum variables for the analysis of about 250 different auxin-like molecules. Additional statistical analysis identified relationships between eleven structural similarity groups. These groups could be assigned to five distinct groups according to their biological activity. However, the high variance especially of bioassay data prevented a clear discrimination. This is caused by limitations of the bioactivity data, which are available from literature. Due to different background, those data might not be comparable at all. Nevertheless, the clustering of auxins according their molecule structures revealed convincing congruences with known biological functions. For instance, the naturally occurring indole-3-acetic acid (IAA) and its synthetic analogs 1-naphthalene-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) belonged to a group sharing the same quantum spatial regions. Furthermore, neighbouring compounds within a group share similar biological activities as well (Ferro et al., 2006b).

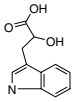
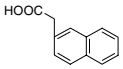
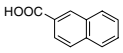
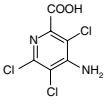
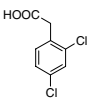
To obtain a better correlation of structural properties with their biological activities, we decided to choose representative substances out of each group for bioactivity analysis in defined and standardized bioassay systems. A list of the substances, which are the subject of this analysis is shown in Table 1.

An ideal bioassay offers information from the primary reaction (Veldstra, 1944) but due to their pleiotropic effects, many factors in the plant may influence the biological activity of auxin, e.g. the interactions with carriers influences membrane-permeability (Benkova et al., 2003; Paponov et al., 2005), the effect of auxin on endocytosis (Paciorek et al., 2005), or the rapid auxin conjugation (Cooke et al., 2003). It is difficult to distinguish between the primary reactions or even between primary effects.

The coleoptile test – widely used for measurements of elongation effects – is one of the less sensitive assay. Only certain kinds of active auxins can be detected while others are ineffective (Bandurski and Nonhebel, 1985). An additional disadvantage is the unspecific elongation response with other substances like gibberellin (Roesel and Haber, 1963), ethylene (Marinos, 1960) and brassinosteroids (He et al., 1991). Usually growth measurement in coleoptile tests are limited to growth of coleoptile's length only, misobey the increase in volume of the plant (Erickson, 1976). The individual cell length profiles within the overall length of the hypocotyl cannot readily be substituted by assumptions of uniform cell length or growth distribution within the hypocotyl (Barley, 2004). Therefore, differences in the responses of different cell types may influence each others. Biological activities must be tested in the intact organism, a test with isolated tissue limits the integration of pharmacokinetics/pharmacodynamic phenomena and provides an incomplete picture of the biological relevance (Abdel-Rahman and Kauffman, 2004).

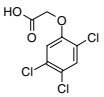
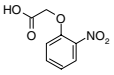
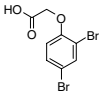
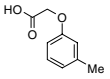
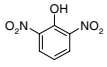
Here, we are interested in the comparison between various hormones in paralell using a standarized environment. The intention of our work is the relative activity of the different substances by a multidimentional analysis of

Table 1
Substances for the biological and chemical analysis

Name	Structure	CAS	pK_d ABP1	log$P$	Class[1]	Che. class. induction[2]	Outlier
Indole-3-acetic acid (IAA)		87-51-4	5.4	1.41	3[abc]	Root/call	x
Indole-3-butyric acid (IBA)		133-32-4	5	2.30	5[abc]	Root/call	x
DL-Indole-3-lactic acid (ILA)		832-97-3	-	1.22	1[bc]	Root/call	
Indole-3-acetamide (IAM)		879-37-8	2.1	0.53	3[abc]	Root/call	
3-Methyl-1H-indole (Skatole)		83-34-1	-	2.29	5[abc]	–	
1-Naphthalene acetic acid (NAA)		86-87-3	6.1	2.24	3[abc]	Root/call	x
2-Naphthaleneacetic acid (2-NAA)		581-96-4	5.9	2.81	7[ab]	Inactive	
1-Naphthoic acid		86-55-5	-	3.10	9[a]	Inactive	x
2-Naphthoic acid		93-09-4	-	3.28	2[abc]	–	
2,3,5-Triiodo benzoic acid (TIBA)		88-82-4	5,1	5.03	4[abc]	Callus	
Picloran		1918021	-	0.30	-	Callus	
Trysben		50317	-	2.71	9[a]	Callus	x
2-Fluorobenzoic acid (2-F-BA)		445-29-4	-	1.70	2[abc]	–	x
Dicamba		1918-00-9	-	2.21	-	Callus	
3-Fluor phenylacetic acid		331-25-9	-	1.65	11[c]	Callus	
2,4-Dichlorophenylacetic acid (2,4-Cl-PAA)		19719-28-9	-	1.75	2[abc]	Callus	

(*continued on next page*)

Table 1 (*continued*)

Name	Structure	CAS	pK_d ABP1	log*P*	Class[1]	Che. class. induction[2]	Outlier
2,6-Dichlorophenylacetic acid (2,6 Cl-PAA)		6575-24-2	-	2.47	4[abc]	Callus	
Phenoxy acetic acid (PHAA)		122-59-8	3.8	1.34	11[c]	Inactive	x
2,4,5-Trichlorphenoxyacetic acid (2,4,5-T)		93-76-5	-	3.31	7[ab]	Callus	
2,4-Dichlorphenoxyacetic acid (2,4-D)		94-75-7	-	2.81	4[abc]	-(Callus)	
2-Nitro phenoxyacetic acid ($2NO_2$-PHAA)		1878-87-1	-	1.13	4[abc]	Inactive	
2,4-Dibromophenoxyacetic acid (2,4 Br-PHAA)		10129-78-9	-	1.86	3[abc]	Callus	
3-Methyl phenoxy acetic acid (3Me-PHAA)		1643-15-8	-	1.78	4[abc]	Inactive	x
2,6-Dibromophenol (2,6-Br-Phe)		608-33-3	-	3.36	5[abc]	Root/call	
2-Chloro-6-nitrophenol ($2Cl\text{-}6NO_2$-Phe)		603-86-1	-	2.55	6[abc]	Root/call	
2,6-Dinitrophenol ($2,6\text{-}NO_2$-Phe)		573-56-8	-	1.37	7[ab]	Inactive	

Chemicals are from: Duchefa, Fluka and ABCR GmbH & Co KG, and Sigma-Aldrich. 4-Amino-3,5,6-tricloro picolinic acid (Picloram), 2,3,6-trichloro benzoic acid (Trysben); 3,6-dichloro-2-methoxybenzoic acid (Dicamba); (1) Classification due to Duncan multiple range test (Ferro et al., 2006b), in short: compounds have tendancy to maximal activity (a), be active (b), or be inactive (c); (2) Quantitative information, Table 2.

different tests, taking into account the response of each substance in the whole background of concentrations (Fig. 1). Dose response isotherma and its implications are not part of the scope of this work.

As expected, the biological activity depends even qualitatively on the structure differences and concentration of the substances, as it can be seen by the different responses of NAA and 2, 6-Br-Phe (Fig. 1). However, IAA did not induce callus significantly in any concentration, but roots only. The low activities of the compounds ILA and IAM are likely linked to the metabolic pathways of IAA (Carreno-Lopez et al., 2000) and not with their self activity. Some substances, like IAA and NAA applied to maize seedlings at lower concentrations showed significant inhibitory effects on root growth (Fig. 1).

Due to the multi-receptor and/or signal system of auxin, it is assumed more than one way of action (Niklas, 2003; Campanoni and Nick, 2005). Therefore various parameters were used for the evaluation of the biological activity. These parameters derived from the standardized experiments (Fig. 1 and Table 2) and are defined as follows (Fig. 2):

- Elongation of etiolated maize seedlings and tobacco explants were analysed in different concentration ranges (10^{-9}–10^{-7} M, 10^{-9}–10^{-4} M, and 10^{-7}–10^{-4} M) using Eq. (5) (Table 2). Root and mesocotyl-coleoptile lengths in maize as well as callus induction (C-ind) and root induction (R-ind) were analysed in tobacco. Yes/no-responses for C-ind and R-ind were recorded as well.
- The value ED50 represents the dose at which 50% of the sample units (explants) has a response, that represents a critical dose-effect. e.g. ED50 = corresponds to 5 out of 10 explants showed an response to the substance applied.

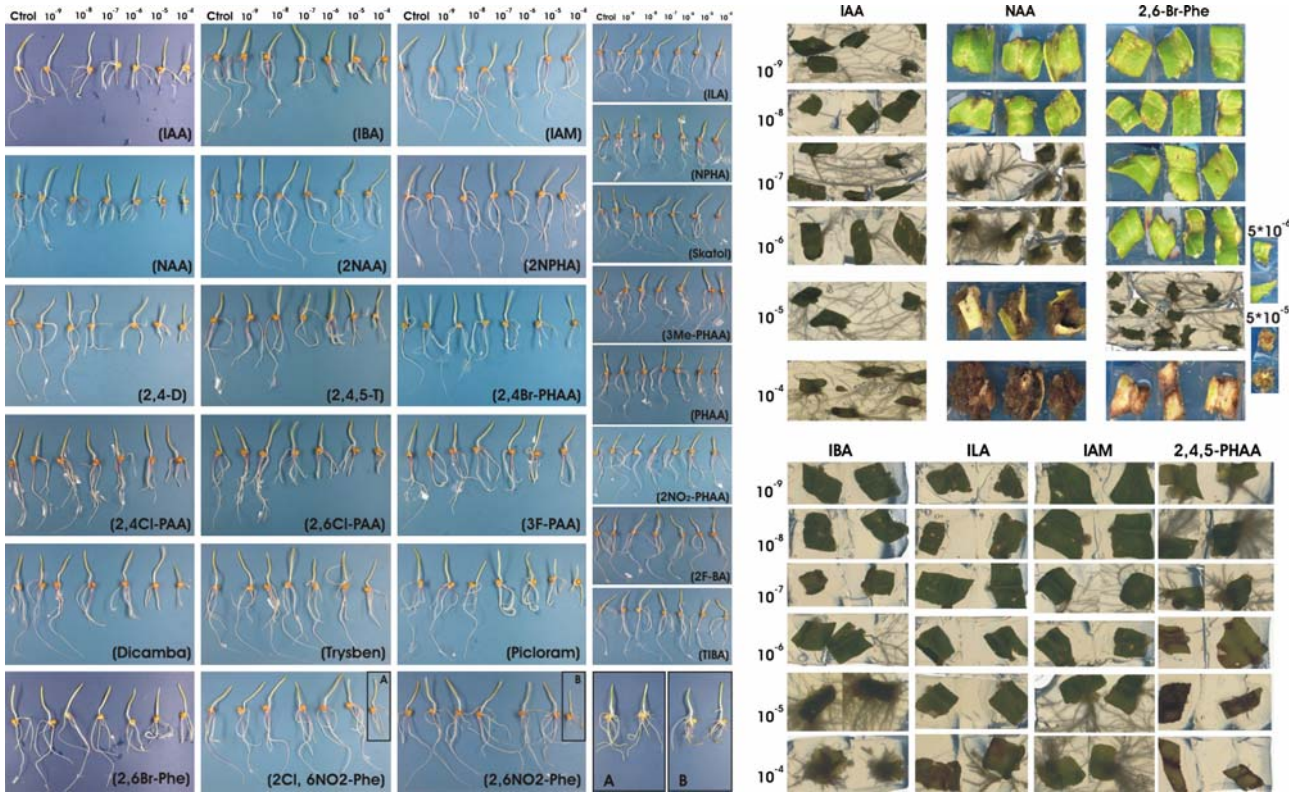


Fig. 1. Effects of auxin-like molecules on maize root inhibition (left panel). Concentrations of substances applied are indicated at the top of the panel. Control was done without any hormone added. One representative out of 10 seedlings is shown. Roots grown in 2Cl, 6NO_2-Phe (A), and 2,6NO_2-Phe (B) have been measured again two days later. These later analyses are shown at the right corner (A and B, respectively). Right panels show the influence of the different substances on tobacco leaves, either on root induction (upper right panel) or on callus induction (lower right panel). The concentrations applied are indicated at the left. Only those substance, which exhibited any effect, are shown but not those substances without any influence on the tobacco explants. The detailed values are summarized in Table 2. PRL (primary root length); MCL (mesocotyl–coleoptil length); R-ind (root induction), C-ind (callus induction).

- Furthermore, we used the log *P* (Table 2), analysed by Veldstra (1944), as a variable based on lipophilicity evaluated with the QSAR method. The lipophilicity log *P* correlates with membrane permeability and receptor binding of sample auxin molecules (Bertosa et al., 2003).
- Many putative auxin receptors have been described (overview: Napier et al., 2002), but best characterized one is the so-called auxin-binding-protein 1 (ABP1). Definitively being an auxin-binding protein, its physiological role is debated and it is not involved in all the different physiological auxin effects. Nevertheless, several auxin-like substance have been analysed for their binding behaviour to ABP1. Therefore, dissociation constants (Table 1) are valuable parameter, representing the fast auxin effects. We did not considered binding data on other, recently described ABPs (e.g. TIR1), since the amount of binding data obtained with different auxin analogs is not sufficient for our analysis.

In order to minimize biological variation and to focus on the analysis of the influence of the molecular structure, the values calculated by the different measurements are summarized in Table 2. All resulting variables were submitted to a cluster analysis (Fig. 2) as described in Sections 4 and 4.3 below. Interestingly, two separate clustered branches emerged from this analysis, representing the two prominent auxin responses: the effect of auxin on (elongation) growth in the upper branches and the effect of auxin on morphogenesis in the lower branches.

It is noteworthy that the response of auxin on the auxin binding protein ABP1 is located in the upper branch of Fig. 2, indicating a physiological role of ABP1 in elongation growth only, but not in morphogenesis. ABP1 binds a series of auxins with affinities that usually correlate with the efficiency of the compound to stimulate cell elongation (Jones, 1994; Jones et al., 1998; Chen et al., 2001). This behaviour could be predicted from the cluster analysis of Fig. 2.

2.2. Principle component analysis

The principle component analysis (PCA) allows the reduction of both the dimensionality of the problem and the redundancies of information in our dataset, keeping the "most important" aspects of the data. The PCA (Fig. 3) provides the chance to explore the individual contribution of the auxin like substances and distinguishing independent biological effects. Three principal components

Table 2
Quantitative bioassay results

Substance	Root length (cm)			Hypo length (cm)			Root induction		Callus induction	
	−9, −7	−9, −4	−7,−4	−9, −7	−9, −4	−7, −4	−9, −4	−7, −4	−9, −4	−7, −4
IAA	6.096	4.772	2.788	5.362	5.045	4.567	0.38	0.47	0.17	0.18
IBA	8.924	7.054	4.523	5.155	4.944	4.830	0.24	0.42	0.19	0.34
NAA	6.558	5.092	3.003	5.175	4.976	4.806	0.06	0.11	0.56	1.00
TIBA	13.161	12.533	12.227	5.529	5.325	5.209	0.04	0.06	0.00	0.00
Picloran	11.595	9.103	6.338	5.403	5.109	4.899	0.00	0.00	0.46	0.82
Dicamba	11.642	8.894	5.730	5.350	5.173	5.065	0.13	0.23	0.44	0.79
DL-ILA	11.974	11.764	11.861	5.280	5.175	5.083	0.08	0.15	0.12	0.13
245-T	9.290	7.394	5.233	4.394	4.511	4.467	0.12	0.03	0.75	0.64
2-NAA	12.923	12.503	12.716	5.714	5.714	5.820	0.00	0.00	0.03	0.05
2,6 Cl-PAA	9.600	7.407	4.367	4.999	5.011	4.985	0.03	0.00	0.25	0.39
Trysben	13.927	12.697	11.769	5.534	5.509	5.493	0.00	0.00	0.16	0.29
$2NO_2$-PHAA	10.348	9.622	9.107	5.332	5.159	4.965	0.00	0.00	0.00	0.00
3Me-PHAA	10.945	10.250	9.746	5.045	5.091	5.092	0.00	0.00	0.00	0.00
3-F-PAA	7.317	6.961	6.349	4.779	4.922	5.151	0.00	0.00	0.36	0.64
PHAA	8.904	8.896	8.792	5.015	4.932	4.720	0.00	0.00	0.00	0.00
Naphthoic acid	10.515	9.367	7.731	5.012	4.938	4.920	0.00	0.00	0.00	0.00
2,4 Br-PHAA	8.835	6.955	4.375	4.903	4.792	4.635	0.01	0.02	0.30	0.54
2,6-Br-Phe	9.127	7.820	3.906	5.152	5.185	3.572	0.12	0.21	0.19	0.34
2Cl-6NO_2-Phe	12.905	12.258	12.297	5.573	5.419	5.308	0.00	0.00	0.13	0.23
2.6-NO_2-Phe	12.698	11.575	10.751	5.703	5.648	5.549	0.00	0.00	0.03	0.05
I-3-Acetamide	12.132	11.662	11.470	5.379	5.282	5.083	0.19	0.34	0.04	0.00
2.4-Cl-PAA	9.230	7.911	6.441	4.918	4.901	4.794	0.00	0.00	0.09	0.16
Skatol	11.954	11.481	11.014	5.650	5.554	5.344	–	–	–	–
2.4-D	10.839	8.035	3.946	5.517	5.127	4.650	–	–	–	–
2-F-BA	12.226	12.710	13.799	5.094	5.155	5.228	–	–	–	–
2-Naphthoic acid	14.107	12.232	10.848	5.902	5.638	5.497	–	–	–	–

Each number was calculated by the equation: $Y_i = \frac{(\sum y_i)}{\sum c_j}$

Root and hypocotyl length were clustered in cm at different concentration ranges: 10^{-9}–10^{-7} M (=−9, −7). 10^{-9}–10^{-4} M (=−9, −4). and 10^{-7}–10^{-4} M (=−7, −4). Data for root and callus induction bases on a yes-no answer, calculated by the equation above.

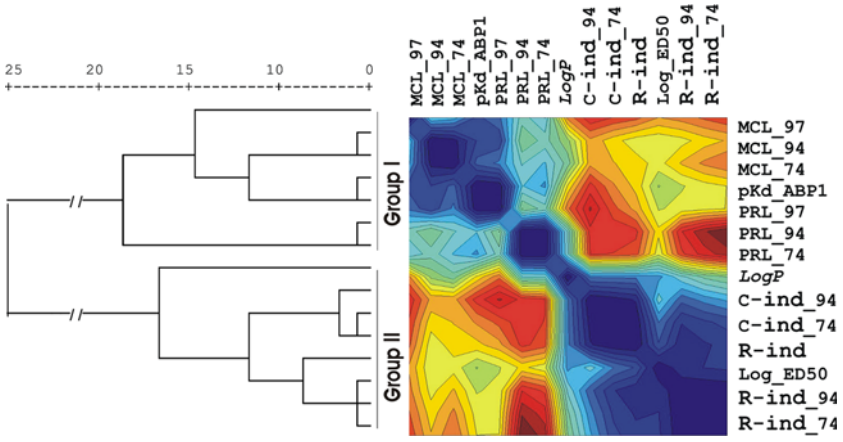


Fig. 2. The matrix (right panel) represents the relationship among the different variables. The variables are plotted in rows and columns as indicated. Colors scheme proceeds from red → yellow → blue with increasing relationship of the variables. The corresponding dendrogram at the right is a multiscaling representation of the analyzed variables in all molecules tested by the different bioassays. It clearly can be seen that the dendrogram falls in two main branches. In the upper group auxin effects, related to elongation growth can be found. The lower group consists of morphogenetic effects. The two separated groups are also represented by the blue areas in the matrix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were found to be informative with a percentage of variance of 49.88% (growth factor), 24.94% (root induction factor), and 13.99% (callus induction factor). An assessment of the relative biological activity (Fig. 3) shows a relation between chemical structure and biological activity by means of a multidimensional approach of the auxin effects.

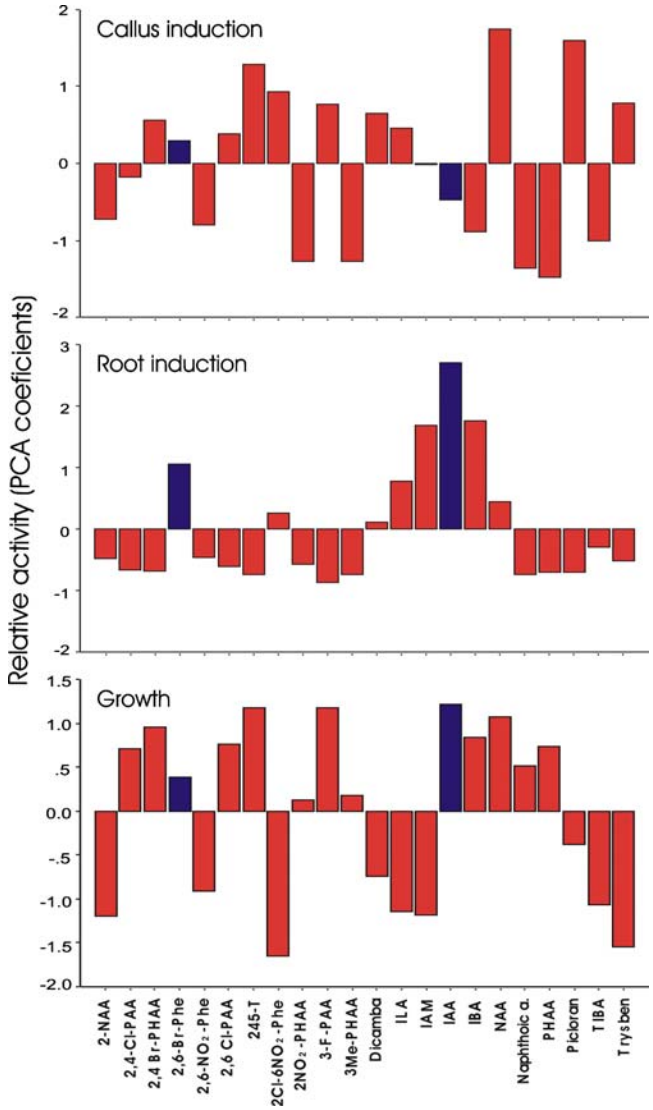


Fig. 3. Graphical representation of the PCA coefficients for the informative PCAs callus induction, root induction, and elongation growth. The coordinate system lists the coefficients against the various substances tested. This graph correlates the relative biological activity of each substance tested for with the physiological event, respectively.

2.3. Structure–function relationships

On basis of these results, we were able to consider:

- the relativity of the physiological effects;
- the formation of two groups of clustered variables (Fig. 2); and
- the existence of independent principal components able to characterize the structural influence of the molecules on basis of the biological activity (Fig. 3).

Auxin induction of root and callus are qualitative events, which depend on structure and concentration. Root induction is hardly linked to callus induction and it depends more on the properties of the molecule than on its concentration (Fig. 2).

The molecular discrimination based on molecular variables (analysis of discriminants, Fig. 4) according to the clustered morphogenetics effects (previous analysis of effects) revealed the structural causes for their different biological behaviour:

- one group is able to produce root and callus (•),
- a second group produces callus only (▲), and
- a third group is inactive (▽).

These groups could be discriminated in two ways by the use of chemical descriptors in a first approach (morphogenesis):

1. The following variables were able to discriminate different biological activities: molecular volume, HOMO energy, hardness ($\eta = 1/2(\mathrm{HOMO} - \mathrm{LUMO})$) calculated by density functional theory (DFT), ($\eta_{(\mathrm{DFT})}$) and one factor of overlap self - similarity matrix not related to IAA (Fig. 4A). The overlap-self similarity matrix was processed by factorial analysis and shows similarity factors not related to the IAA molecule.

$$D_1 = 36.10 + 66.49\eta_{(\mathrm{DFT})} - 0.01\mathrm{Vol} + 65.34\varepsilon_{\mathrm{HOMO}} + 0.27F3 - \mathrm{Sim\ OVER} \quad (1a)$$

$$D_2 = -0.92 + 67.19\eta_{(\mathrm{DFT})} + 0.01\mathrm{Vol} - 1.24\varepsilon_{\mathrm{HOMO}} - 1.49F3 - \mathrm{Sim\ OVER} \quad (1b)$$

2. The influence of the indol-N-atom on the HOMO orbital, positions 10 and 11 on HOMO and HOMO − 1 and the hardness calculated by Hartree–Fock ($\eta_{(\mathrm{HF})}$) were considered (Fig. 4B)

$$D_1 = 10.32 + 56.75\eta_{(\mathrm{HF})} - 2.25N8_{\mathrm{HOMO}} + 1.86C10_{\mathrm{HOMO}} + 2.36P11 \quad (2a)$$

$$D_2 = 1.23 - 6.27\eta_{(\mathrm{HF})} - 3.17N8_{\mathrm{HOMO}} + 1.66C10_{\mathrm{HOMO}} - 1.30P11 \quad (2b)$$

The 72.7% and 77.3% grouped cases, respectively, were correctly classified by cross-validation. The use of both, descriptors of electronic molecular structures and intermolecular interaction descriptors facilitated the explanation of the biological behaviour (Ferro et al., 2006a). The important role of the N-indole region for auxin action was documented long before. Correlation between biological activity and the existence of the N-indole was found by Porter and Thimann (1965), independent of the charge separation theory as explanation. Porter and Thimann (1965) as well as Kaethner (1977) created an special region for this N-indole in their binding site proposals. We proved statistically the importance of this region due to its significance to the outer molecular orbitals (HOMO and HOMO − 1). The indole-N-atom is the most negative atom in the molecule, and this variable has been used as a molecular descriptor before (Vaes et al., 1996). The indole compounds analysed

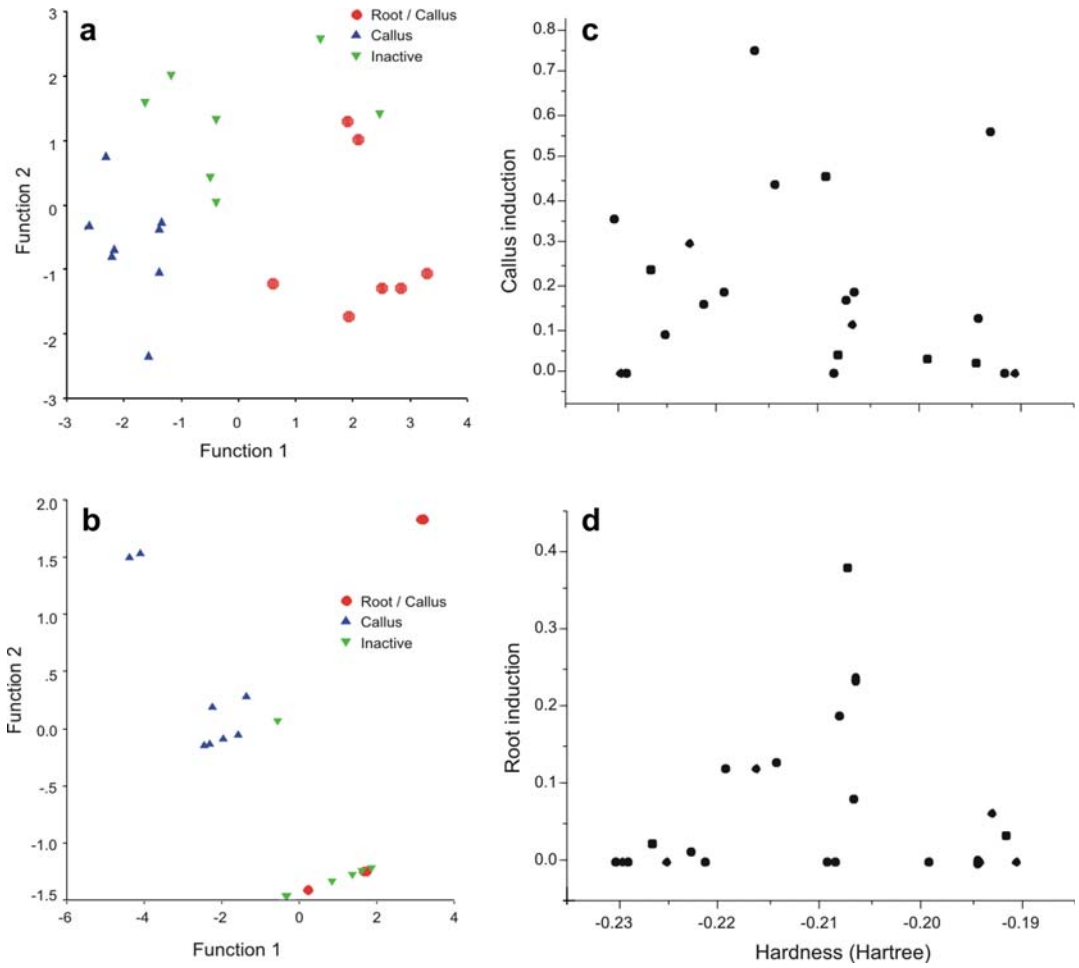


Fig. 4. Overview of the structural classification of auxin-like molecules based on *in vitro* tests. A representation of the discriminant functions ((a) "Eqs. (1a) and (1b)" and (b) "Eqs. (2a) and (2b)") show the significance of the structural parameters to discriminate between biological inactive molecules and molecules causing callus and root induction. Different shapes represent the different molecular characterizations. The scatter plots (c,d) represent graphically the influence of Hardness (η). Hardness was found to be the most important variable for the quantitative relationship between bioassay results and molecule structure.

were very specific in root induction, with few or no callus induction. Even in molecules without self activity (IAA metabolites) the indole ring system is an attractive molecular prerequisite for root induction.

The second approach (growth) focuses on the first group of the cluster (Fig. 2). The relative activity of the substances is characterized basically by the length of the primary root. In this case the hardness (η) results in a very significant variable. The linear regression equations without outliers are

$$\text{Biol. Activity} = -45.3483\eta_{(\text{HF})} \quad R^2 = 0.929 \tag{3}$$

$$\text{Biol. Activity} = -97.2737\eta_{(\text{DFT})} \quad R^2 = 0.906 \tag{4}$$

Hardness (η) results in a variable, which is responsible for the biological activity. This is the third time we found this variable to be significant. The linear dependency between hardness and the biological activity of the selected molecules is strong by both HF and DFT calculations (Fig. 5). In both calculations, the level of predictability is higher than 80%, independently of the intercept.

However, the outliers indicate that some points (molecules) do not line up with the rest of the analysed molecules. Among the outliers very familiar compounds can be found, like active auxins (IAA, IBA and NAA) and inactive ones (Trysben, 3-Me-PHAA and PHAA). In order to know the cause of these behaviours, we performed a discriminant analysis between the molecules adjusted linearly and outliers. Despite of the small statistical sample, discriminant analysis considers:

- the influence of the significance of position 8 to HOMO,
- the significance of C15 to HOMO (Fig. 7), and
- the influence of a second component factor of the overlap matrix.

Both position C15 and C8 are important auxin regions: C15 is the position C4 for indole and C8 for the naphtaleneacetic acid ring system. Substitutions in this regions

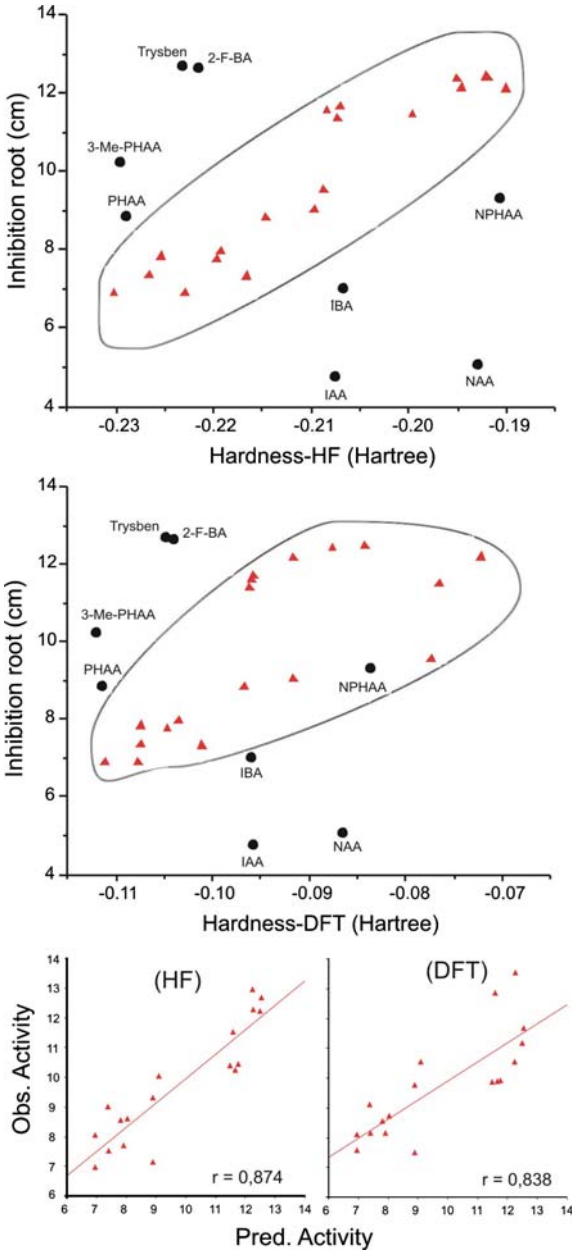


Fig. 5. The scatterplots represent the relationship between root inhibition and hardness η. Hardness was calculated with both methods HF (Hartree–Fock) and DFT (density functional theory). Bioassay data are also shown in Table 2. Each dot represents a molecule tested. A linear dependence (triangles) was found. Some outliers above and below are most interesting from the biological point of view (see text). The predictability of both lineal model is shown as well.

cause dramatical changes in the activities, being able to see in compounds like 4-Cl-IAA or 8-Cl-NAA. The region 8 corresponds to the position of the N–H of the indole system. In case of auxin molecules the discriminant factors (principal component) of the overlap matrices represent less than 10% of the information of the whole overlap self-similarity matrix. A further analysis of the molecules significantly included in the component revealed that molecules like NAA, 2,6-Br-Phe, Naphthoic acid and Picloram are not sharing the same charge localization of indole derivatives, TIBA, Dicamba and 2,4-D. This could explain some differences in activity between IAA and NAA.

2.4. 2,6-Dibromo-phenol

Attractive and fully surprising results of the present work are represented by the positive biological activity of a non-carboxylated compound (2,6-dibromo-phenol). This was expected by a previous classification (Ferro et al., 2006b) (Figs. 1 and 4). It is the first time that, in practical terms, a new active compound is found via quantum similarity measures (Carbó-Dorca, pers. com.).

The auxin-like activity of this substance support our results, that statistically the distances between the COOH group and the ring system are not significant. Biological activity and metabolism of phenol derivates have already been identified to be active disubstituted phenols at positions 2 and 6. But the issue was focused on the mimic of conformational geometries and charge separation of the COOH and NO_2 groups in respect to the ring (Harper and Wain, 1969, 1971 ; Farrimond et al., 1980). NO_2 is a withdrawing substituent, while for unpolarized π-systems the dominant interaction is π-repulsion (Hunter et al., 2001). Electron availability (Katekar, 1979) and the softness as measure of the chemical polarisability influences the degree of auxin-like activity. The ring system and its substitutions generate the decisive factors.

Of the nearly 3200 known naturally occurring organohalogen compounds, more than 1600 contain bromine (Gribble, 1999). 2,6-Br-Phenol is a versatile molecule known as pheromone and in marine algae (Whitfield et al., 1999; Leonovich, 2004). As a biological remark we want to mention recent experiments on gene silencing activities of siRNAs with a ribo-difluorotoluyl nucleotide. These experiments demonstrated the importance of stacking interactions rather than hydrogen bonding in the fidelity of DNA replication (Xia et al., 2006).

2.5. Common analysis of both approach

The chemical space, which encompasses the auxin definition, suggests a multi-dimensional molecular space characterized by the plasticity of its biological interactions. Being the hardness (η) gap between anti-bonding and bonding molecular orbitals, a reflection of the molecular stability (Gilman, 1997), was commonly implied in every statistical result of this paper. The ionization potential (I) and electron affinity (A) of a molecule should be achieved easily by a calculation of the ground state-geometry of the neutral molecule and similar calculations for its positive and negative ions. This approach is rarely performed, in its essence, the correlation energy errors depend on the number of electron pairs in the molecule, in our practice, a descriptor for next works with hundreds of molecules is being needed. But, the Koopmans' theorem assume $-\varepsilon\mathrm{HOMO} = I$ and $-\varepsilon\mathrm{LUMO} = A$ without the necessity to take into account relaxations in the ionized sates (Pearson, 1986).

The necessity of electron transfer or rearrangement for the reaction in auxin-like molecules has been statistically suggested by the analysis of self-similarity Coulomb matrix of almost 250 molecules (Ferro et al., 2006b). Now it is confirmed that soft auxin-like molecules are more active than the hard ones (Pearson, 1986) by mean of calculus of hardness using HF and DFT algorithms. Density functional theory (DFT) has been found to provide a rigorous theoretical background for hardness and related concepts (Chattadaj et al., 2004). However the DFT method can be seen as a generalization of the HF method (Zevallos and Toro-Labbé, 2003). Both can be used to analyse hardness by means of the Koopman's theorem. In our study both methods produced the same outliers and only molecules with NO_2 groups and both variants of naphthoxyacetic acid had different hardness, but without significance statistical differences in the predictability.

The existence of a large number of auxin-like molecules and their pleiotropic effect implies the principle of "a separate key to a back door" to the enzyme-substrate correlations in auxins (Veldstra, 1944). The physiological activity is a result of the interaction of its effector chemical fragments with a receptor to form the given character (quality) (Gafurov and Zefirov, 2004).

Different abilities of the substituents to bind to the accessory binding areas (Katekar and Geissler, 1983) can be the reasons for the statistical outliers in the structure–activity. Another possibility for the outliers in Fig. 5 may – at least in part – be explained by other physiological influences. NAA and IAA are transported by the auxin efflux carrier, while other substances like 2,4-D are not. At least IAA is relatively fast metabolised compared to many synthetic auxins. Since some other outliers do not fit into these schemes, it can be proposed that there is another variable needed to fully explain the structure–activity relationship for these molecules. Nevertheless, data presented in Fig. 5 show that hardness is the most important variable for the majority of the analyzed molecules.

Hardness is a global reactivity descriptor and appears to be inadequate to explain site selectivity (Chattadaj et al., 2004), of a phytoregulator as well. Other variables reported for the discriminant analysis could be helpful: like the principal components of the overlap matrix and punctual atomic significances on the outer molecular orbitals. The physical meaning of the overlap operator has a large set of possible applications: the volume of the molecules is determinant in the analysed system (Carbó-Dorca and Gironés, 2004) or as an estimation of charge localization (electronic charge) (Sola et al., 1996).

A statistical overview of the occupied outer molecular orbital elucidates the energetic closeness of HOMO and HOMO − 1 orbitals, to which COOH, does not contribute significantly (Fig. 6). These two orbitals are energetically far away from the rest of the occupied molecular orbitals. In contrast to bigger molecules such as ecdysteroids (Ferro et al., 2006a), in auxin-like molecules the most probable orbitals to produce a reaction are HOMO and HOMO − 1. The contributions of atoms at positions 10 and 11 to molecular orbitals (HOMO, HOMO − 1) infer a statistical resemblance between different ring systems depending on their substitutions. TIBA orbital localizations are quite different from the other molecules due to the influence of the two iodine atoms in *ortho*-position (Fig. 6).

Other variables not commented before, because of the low percentages of the cross-validation of the multivariable analysis, were found statistically significant in specific situations. First, the number of Br and F is critical as well. Halogen substitutions in organic molecules will affect their metabolic degradations but also their intrinsic activity and they are very decisive for auxin activity (Katekar, 1979; Sexton, 1963). Second, the existence of position 4 (the methylene carbon sp^2 hybrids) was determinant for molecules with activity at lower concentrations (between 10^{-9} and 10^{-7}). It was claimed (Katekar, 1979; Katekar

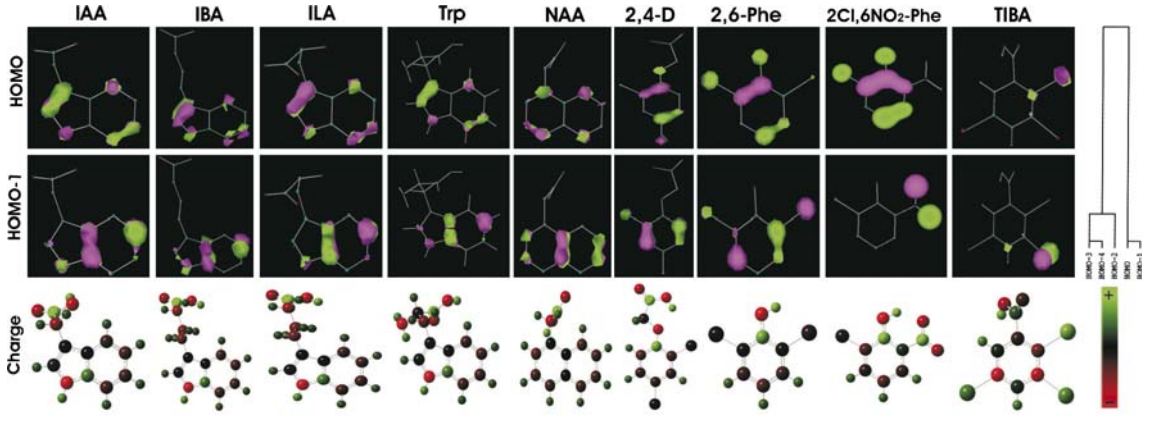


Fig. 6. Graphical view of spatial representation of the outer molecular orbitals, HOMO and HOMO − 1 in auxin-like molecules. Dendrogram (right) shows the energetic isolation of the HOMO and HOMO − 1 orbitals from the rest of the outer molecular orbitals.

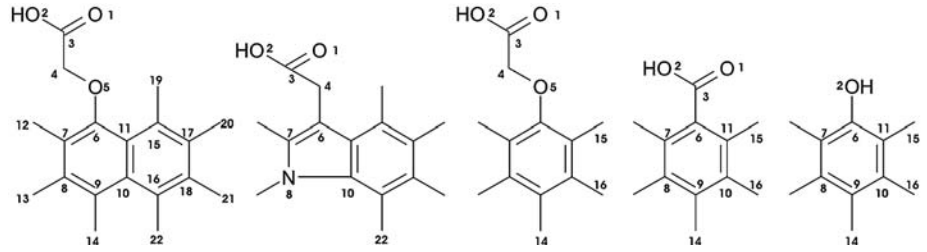


Fig. 7. Representation of the atom distributions to homogenize the analysis of auxin-like molecules by fix positions.

and Geissler, 1982, 1983) that C4 (Fig. 7) acts as a buffer area between the COOH group and the ring system.

The carboxyl group has been considered as the vital molecular site in auxins. Paradoxically, it is chemically and physically identical in all compounds, of which acidity is separated from the ring electronic effects by the buffering effect of the intervening methylene group (Katekar and Geissler, 1982, 1983). In contrast, tryptophan (indole) is the only heterocyclic aminoacid ring system, whose electronic structure has been preserved throughout all auxin analogs (Fig. 6). The COOH group does not influence any outer molecular orbital related to the activity (Fig. 6). However, fluoride substitutions in the first carbon of the side-chain changes the electronic structure of the indole ring and modify the biological activity totally (Fig. 6) (Zhang and Hasenstein, 2000). We suggest that differences in the activity are due to differences in the ability to bind to the electron acceptor.

The structure–activity findings are consistent with both unspecific reactions like callus induction and very specific reactions like root induction. An analysis of the packing behaviour depending on size and chemical nature of the aromatic rings in the Protein Data Bank showed that the tryptophan (indole) prefers edge-to-face interactions (Samanta et al., 1999). Additional molecular interactions analysis confirms an obvious way to effect binding to a tryptophan by hydrogen bond to the indole NH proton. One way of edge-to-face interactions of the indole ring is the formation of a NH ... π bond (Taylor, 2002). Tryptophan could be the only heterocyclic amino acid, which may confer to the specificity of indolic auxins in root induction and particularly is responsible for the high activity of indole-3-butyric acid (IBA), whose distance between ring system and COOH is uncommon for active auxins (Jönsson, 1955).

Indolic compounds can induce roots at different concentrations without any influence on other tissues. Other molecules like NAA or 2,6-Br-Phe provoked root inductions or a mass of undifferentiated plant cells (callus) at highest concentrations, which can be regarded to stress response. Others are able to produce solely callus. This suggests unspecific non-bonding interactions of non-indolic rings. Successful biological auxin-like activity requires both the preferred geometries of non-bonded contact and the likelihood of their occurrence.

3. Conclusions

An assessment of structure–property relationship of auxin-like molecules was performed. Our strategy bases on a multi-dimensional scale of the biological activity and a dynamic view of the structural requirements. It was demonstrated that the mixture of both electronic structure and intermolecular interaction descriptors was able to discriminate this multi-dimensional biological view. Our findings can open the spectrum of new structural relationships emerging from new molecules. For the first time, QMSM method has been useful to detect a new active molecule with unexpected characteristics showing empirical and experimental evidence. A compound without COOH (2,6-Br-phenol) in the side chain was able to induce root, callus and inhibit root elongation.

Hardness ($\eta = 1/2(\mathrm{HOMO} - \mathrm{LUMO})$) represents a variable, statistically related to auxin activity. The molecular regions 8, 9 and 15 are statistically detected as significant depending on their influence on the outer Molecular Orbitals.

4. Experimental

4.1. Bioassays

Maize seeds from KWS SAAT AG (Einbeck, Germany) and tobacco leaf explants (*Nicotiana tabacum* cv. *samsun*) were used to perform assays of growth and morphogenesis.

The maize seeds were soaked in sterile water to stimulate the germination. After 8 hours they were placed on soaked cotton wool for 14 h in the dark. After a negative selection of non-germinated and extreme seedling size, the maize seeds were rolled (10 seeds per roll) in filter paper. Each of them were placed vertically in a plastic flask, which contained 50 ml of the particular auxin-like substance (Table 1). The whole procedure was done under dark conditions at 20 °C and the evaluations were accomplished four days later.

The sterile tobacco leaf explants were placed in a MS medium (Murashige and Skoog, 1962) supplemented with vitamins. The whole experiment was performed under a 12 h photoperiod at 20 °C. The different substances were always tested in parallel to achieve full comparability.

The evaluation of data was performed after six weeks of growing.

4.2. Outputs and design of biological variables

The main goal of this work is the comparison of the different behaviour of compounds tested. Different output variables, referring to the diverse physiological effects were measured. For the maize experiment the variables were: primary root length (PRL), mesocotyl–coleoptil length (MCL) and number of secondary roots. For the tobacco experiments the variables were: root induction (R-ind), callus induction (C-ind) and lethality.

Dealing with the output variables we developed other variables able to perform a structure–activity analysis: ED_{50}, minimal active concentration, maximal active concentration, lethal concentration, variances. Log$P$ was obtained from SRC Physical Properties Database (http://www.syrres.com/esc/datalog.htm). Additionally, we resume an average value in respect to all the concentrations analysed for each output variable, in an attempt to focus on the molecular influence:

$$Y_i = \frac{\left(\sum \frac{y_i}{c_j}\right)}{\sum c_j} \tag{5}$$

where Y_i the resume value, y_i the mean per treatment and c_j is the concentration Three sets of concentrations were considered for each variable: 10^{-9} M – 10^{-4} M, 10^{-9} M–10^{-7} M, and 10^{-7} M–10^{-4} M. Therefore we obtained three sets of concentrations for the different effects: PRL-94, PRL-97, PRL-74. The same type of classification was used for the other variables: mesocotyl-coleoptile length (MCL-94, MCL-97 and MCL-74); root induction (R-ind-94, R-ind-97 and R-ind-74), callus induction (C-ind-94, C-ind-97 and C-ind-74). Finally, the relative biological effects of the molecules were statistically analysed by principal component analysis to remove collinearity and repetitive information among the predictor variables and to focus on causal effects.

4.3. Statistical assessment

First of all, a molecular representation, based on naphtoxyacetic acid, was carried out. This represents schematically the atom positions of any kind of auxin - like molecule, and it was able to be treated statistically (Fig. 7).

A statistical featuring of biological variables was done using a classification of the range standardized (−1 to 1) by different methods of cluster analysis. This yielded in a consistent dendrogram with two groups of related variables. Based on that, two different classifications of the molecules for each case were achieved.

The repetitive information of the similarity matrixes was eliminated by principal component analysis (Ferro et al., 2006b). These components can be considered to be a discreet distribution of the quantum objects within a three-dimensional similarity space. Therefore, all molecules are not exactly related from the quantum point of view and the components are used to find relations with effects.

Next, discriminant analysis were performed to find relationships between the biological classifications and molecular properties by means of both descriptors of intermolecular interaction and quantum-chemical descriptors related to intramolecular electronic properties (Raevsky, 1999; Ferro et al., 2006a). Lineal regression analysis was carried out in particular cases, which were found to be consistent with the phenomenological facts.

4.4. Molecular modelling and quantum molecular similarity measures

The first molecular conformations were optimized using the MM+ force field (in the Hyperchem program), no cutoffs for non-bonded interactions and electrostatic interaction bond dipoles (Allinger, 1977) and additionally semiempirical PM3 calculus (MOPAC v. 6, March 1997, Stewart, 1991). Subsequently, the final geometry was performed with quantum chemical optimizations at the ab initio level, with Hartree–Fock (HF) algorithm or density functional theory (DFT), using Gaussian 03W, Version 6. The basis set defined for most molecules was 6-31G* (Petersson and Al-Laham, 1991). In case of the remaining molecules, which includes iodine atoms the base CEP-31G was used (Stevens et al., 1984).

Next, an analysis of molecular quantum similarity measure (MQSM) was applied to the molecules applied in the biological tests. The quantum similarity methods used in the present paper are essentially the same than those exposed in the previous work on auxins by the same authors (Ferro et al., 2006b). A molecular quantum similarity measure (MQSM) (Carbó et al., 1980) can be defined as the scalar product between the first-order molecular density functions (DF) of two compared molecules, weighted by a non-differential positive definite operator (Ω):

$$Z_{AB}(\Omega) = \int\int \rho_A(\mathbf{r}_1)\Omega(\mathbf{r}_1,\mathbf{r}_2)\rho_B(\mathbf{r}_2)\,d\mathbf{r}_1\,d\mathbf{r}_2 \tag{6}$$

where A and B are the two molecules being compared, $\mathbf{r}_1$ and $\mathbf{r}_2$ are the electron coordinates, and ρ_A and ρ_B the corresponding first-order density functions.

According to the form of the weighting operator, different types of MQSM can be defined. As described previously (Ferro et al., 2006b), two kinds of MQSM have been used in the present study: the so-called Overlap QSM (Carbó et al., 1980), and the Coulomb QSM (Carbó and Domingo, 1987). The molecular DF has been adjusted using the Promolecular Atomic Shell Approximation (ASA) (Gironés et al., 1998; Amat and Carbó-Dorca, 2000). This electron density fitting algorithm adjusts the first-order molecular electronic density functions to linear combinations of spherically symmetric functions. In the present study, the presence of bromine and iodine atoms forced the selection of the Huzinaga basis set, which pro-

vides fitted functions from H to Rn (Amat and Carbó-Dorca, 1999). The number of terms in the expansion of the atomic basis set for each atom can be found at http://iqc.udg.es/cat/similarity/ASA/table432.html, whilst the ASA exponents and coefficients for each atom can be downloaded from the web site (http://iqc.udg.es/cat/similarity/ASA/Huzinaga432/).

Similarity measures also depend on the relative orientation of the molecules being compared. In this study the field–based maximum similarity superposition algorithm (Constans et al., 1997) was used to superimpose molecular structures. Once calculated, the whole set of pairwise MQSM are stored in the similarity matrix (SM): $\mathbf{Z} = \{Z_{AB}\}$, where Z is a squared matrix of dimension N, i.e. the number of molecules.

Acknowledgements

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