Synthesis and Molecular Modeling: Related Approaches to Progress in Brassinosteroid Research

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ABSTRACT: In the field of brassinosteroids, which are potent plant growth regulators, we have developed a quantitative structure–activity relationship study to develop knowledge from a structural point of view and to find out new requirement definitions. This will help identify other suitable active brassinosteroid derivatives with a good activity/synthetic cost ratio for further application in agriculture. The methodology used to achieve this goal represents a multidisciplinary study involving synthesis, molecular modeling calculations, and bioactivity evaluation. The influence of different molecular properties in the bioactivity of a set of synthetic compounds (i.e., molecular electrostatic potential and the ability to form H bonds) is discussed. The molecular electrostatic potential is expressed in terms of the electrostatic Carbó similarity index (CI) between brassinolide (**1**) and other brassinosteroids. We have found that the electrostatic charges of the functional groups play an important role in the description of the activity, as evidenced by its good correlation with the CI in most cases. Deviation from this rule could be explained by the H bonding abilities of some of these compounds, which we believe may play an essential role in binding to the natural receptors.

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Brassinosteroids are potent naturally occurring plant growth regulators widely spread in the vegetal kingdom. They have an exciting potential use in agriculture owing to their capability of improving crop yield and quality as well as minimizing environmental stress and herbicidal injury and controlling pathogenic diseases (1,2).

At least 36 natural brassinosteroids have been identified from different parts of plants: pollen, seeds, leaves, shoots, etc., brassinolide (**1**) being the most potent natural brassinosteroid (3). From a structural point of view, brassinosteroids are polyhydroxylated steroids differing in their functionalities and the stereochemistry present in the A and B rings and the side chain.

Brassinosteroids have been tested as plant growth-promoting hormones in more than 20 bioassays typical for phytohormones such as auxins, gibberellins, or cytokinins (1). From all of them, the rice lamina inclination test is one of the most specific for brassinosteroids, being widely used for activity evaluation (4,5).

Because of the interest of such compounds and the high synthetic cost of the most active brassinosteroids, the goal of our study was to find other suitable active derivatives, with a good activity/synthetic cost ratio for agricultural application. For this purpose, minimal structural requirements for brassinosteroid activity should be known.

Different qualitative structure–activity relationships have already been established from the activity data obtained in special bioassay systems (6,7). Although these relationships are more or less strictly dependent upon the bioassay used, it is generally accepted that the structural requirements for a high brassinosteroid activity are: (i) 2α,3α-diol in A ring, (ii) 7-oxalactone better than 6-ketone in B ring, (iii) A/B *trans* fused ring junction, (iv) a *cis* C_{22},C_{23} diol preferably with 22*R*,23*R* configuration, and (v) a C_{24} methyl or ethyl substituent. Nevertheless, a closer look into these requirements and into how they were established (only a limited number of brassinosteroid analogs with few structural modifications have been assayed) reveals that they are far from being general from two points of view.

First, the brassinosteroid functionalities cannot be considered independently, as shown in these requirements, since we have found that they are closely related. Figure 1 shows activity data for nine brassinosteroids, measured using our modified rice lamina inclination test (Brosa, C., Soca, L., and Terricabras, E., manuscript in preparation) based on the procedure developed by Takeno and Pharis (4). In agreement with the general rule, it can be observed that compounds with lactone in B ring (**2**, **3**, **6**, and **7**) elicit higher activity than their corresponding 6-ketone analogs (**4**, **5**, **8,** and **9**). (see Scheme 1 for structures). In the same sense, compounds with 22*S*,23*S* configuration at the side-chain diol (**3**, **5**, **7,** and **9**) are less active than the corresponding 22*R*,23*R* ones (**2**, **4**, **6,** and **8**), but the values strongly depend not only on the configuration of the diol at C_{22},C_{23} but also on the type of alkyl substituent at C_{24}. Thus, for a similar functionality in the skeleton, compounds with stigmastane side chain (**2** to **5**) are more active than those with an ergostane side chain (**6** to **9**) except for **7** which is more active than **3**. Moreover, with respect to con-

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Abbreviations: CI, electrostatic Carbó similarity index; 3D, three-dimensional.

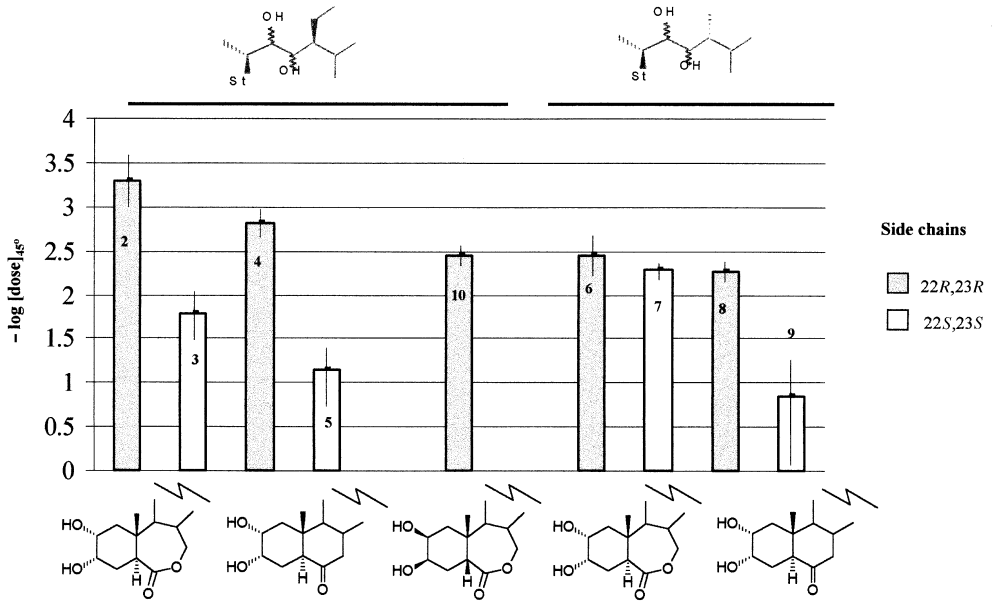


FIG. 1. Brassinosteroid activity in the rice lamina inclination test. Numbers on bars correspond to compounds in Scheme 1. Bars represent the activity value of each compound.

figuration of the diol, lactone **6** is less active than the 22*R*,23*R* ketone analog **4**, thereby not meeting the requirements. But when the configuration changes from 22*R*,23*R* to 22*S*,23*S* the activity also changes, and now becomes higher for **7** than for **5**. Therefore, these results clearly indicate the existence of a relationship between functionalities and thus a strong interdependence among requirements (ii), (iv), and (v). Furthermore, similar relationships are observed for the rest of the requirements.

The second point for which we believe that these requirements are far from being general is related to the brassinosteroids involved. The postulated requirements limit the scope of applicability to the functionalities involved in the tested set of brassinosteroids. For instance, they were developed without taking into account analogs having a 2β,3β diol and/or A/B *cis* junction. No brassinosteroids with these functionalities were examined. So, why were they not considered for the requirements if there was no proof of their activity? After synthesizing such compounds and evaluating their activity, we obtained a very high activity for **10**, with 2β,3β diol and A/B *cis* junction (Fig. 1) (8,9).

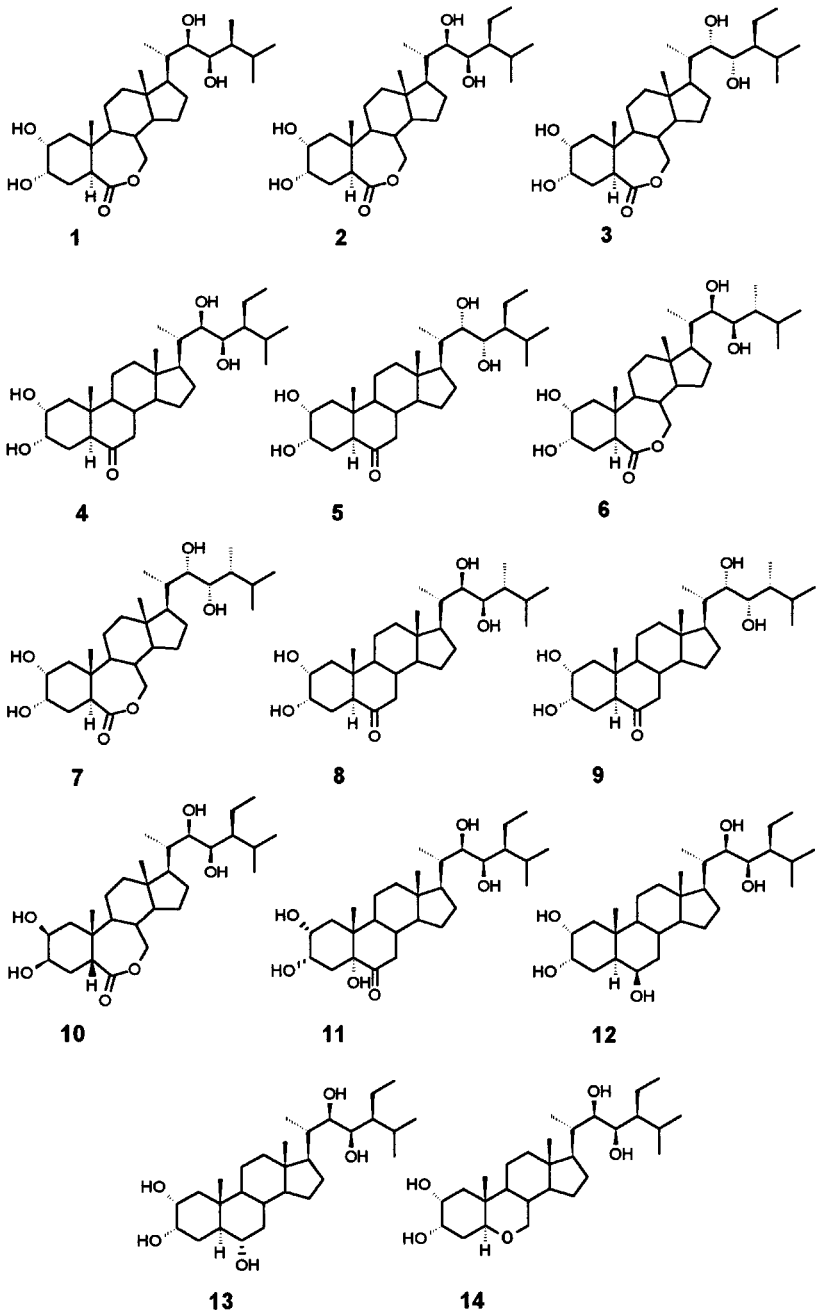
In short, the close relationship between the brassinosteroid functionalities and the high activity elicited by this new brassinosteroid **10** indicates the weakness of the requirements postulated in the literature for a high brassinosteroid activity. Thus, a more accurate way to define the structural requirements should be found.

In assuming that brassinosteroids may act at the molecular level through a mechanism similar to that of animal steroid hormones, a receptor/ligand complex which binds to nuclear or cytoplasmatic sites to regulate the expression of specific genes should be involved. Therefore, the active brassinosteroids should have a single defined three-dimensional (3D) "active conformation" which best fits the receptor or receptors. On this active conformation, the atoms directly involved in binding with the brassinosteroid receptor ought to have the same spatial situation in all active molecules. Thus, the more complementary is the active conformation of a defined brassinosteroid to the 3D structure of the receptor, the more active it should be. Since brassinolide (**1**) is the most active natural brassinosteroid found, we can assume that its active conformation will be the one that best fits to the receptor and that can be taken as reference. In this sense, a new way to define the structural requirements for a high brassinosteroid activity has been considered.

The strategy to achieve this goal involves molecular modeling techniques which also enable the establishment of a quantitative structure–activity relationship (QSAR). Apart from providing information about brassinosteroid receptor binding, the results will eventually be of help in the design of the most suitable brassinosteroids for agricultural applications. An analysis of the results obtained in the application of this approach is presented here.

MATERIALS AND METHODS

To establish a QSAR, a broad set of brassinosteroids having sufficient structural modifications, together with their corresponding strictly homogeneous activity data with statistical parameters, has been used. Different parameters have been calculated and their correlations with activity have been investigated. In Scheme 1 are presented the brassinosteroids mentioned in the present paper. Except for brassinolide (**1**), we have synthesized all of them (8–15). The first difficulty in this strategy is the lack of homogeneity and statistical parameters on the activity data obtained from the literature, so a bioassay was developed in order to derive strictly homogeneous activity data and their corresponding statistical para-



SCHEME 1

meters (Brosa, C., Soca, L., and Terricabras, E., manuscript in preparation).

In following this methodology, a modified active analog approach has been used to obtain the active conformer for each brassinosteroid. This study involves a conformational analysis, a 3D structure comparison, and a conformer alignment (16). The conformational analysis has been carried out by a systematic tree analysis and corroborated by molecular dynamics. For the 3D structure comparison, a program has been developed to calculate three different similarity indexes among the conformers of each compound: the square residue (SQR), the root mean square (RMS), and the molecular overlay volume (MOV). In the last step, the structure alignment was performed by optimization of the electrostatic Carbó similarity index (CI) (17) calculated using the ASP program (18).

The ability to form H bonds has been calculated with the GRID program (19). The preliminary results are given using water as a probe.

RESULTS AND DISCUSSION

The CI was selected to align the molecules since it is a measure of the similarity of electrostatic potential between two molecules. Moreover, these kinds of electrostatic interactions

TABLE 1
Brassinosteroid Electrostatic Carbó Similarity Index (CI) and Rice Lamina Inclination Test Activity

Compound[a]	CI[b]	–log (dose)$_{45°}$
1	1	5.93
2	0.82	3.29
4	0.76	2.80
6	0.97	2.45
10	0.76	2.45
7	0.72	2.27
8	0.81	2.27
3	0.75	1.78
11	0.76	1.53
12	0.58	1.36
5	0.74	1.14
13	0.48	1.13
9	0.52	0.84
14	0.67	0.47

[a]Compound numbers correspond to structures presented in Scheme 1.
[b]The value of the CI depends on the alignment. The confidence limit is ±0.05.

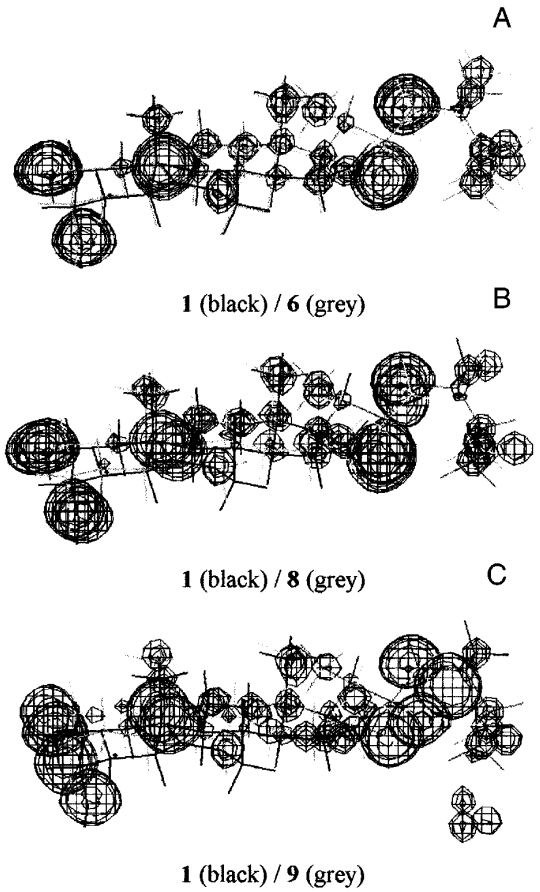


FIG. 2. Overlaid molecular electrostatic potential maps at –30 KT level between brassinolide (**1**) (in black) and three brassinosteroids (**6**, **8**, **9**) (in grey). The electrostatic Carbó similarity index/activities for these compounds are: (A) 24-epibrassinolide (**6**), 0.97/2.45; (B) 24-epicastasterone (**8**), 0.81/2.27; and (22*S*, 23*S*) 24-epicastasterone (**9**), 0.54/0.84.

are very important for compound binding to a receptor. Thus, the more similar a compound is to brassinolide (1), the higher the CI.

Table 1 shows the CI and the activity data for some of the brassinosteroids studied. In general, activity correlates with the CI and is higher when the CI is over 0.75, although there are some exceptions.

For example, Figure 2 shows how the electrostatic potential contour map at –30 KT for brassinolide (**1**) overlays with that of **6** (Fig. 2A), **8** (Fig. 2B), and **9** (Fig. 2C). In agreement with its high activity, all the electrostatic potential sites for 24-epibrassinolide (**6**) (in grey) overlay perfectly with brassinolide (**1**) (in black), with a very high CI (0.97). In the case of 24-epicastasterone (**8**), which shows lower activity than **6**, only the B-ring region does not overlay well with the lactone of brassinolide (**1**), decreasing its CI (0.81). Finally, in the case of (22*S*,23*S*) 24-epicastasterone (**9**), which displayed very low activity, neither the region of the A ring and side chain nor the region of the B ring overlays well with brassinolide (**1**), as is indicated by its very low CI (0.52). These compounds are a good example of how the activity decreases along with CI.

Furthermore, a similar relationship is observed for compounds **2**, **4**, **12**, and **13**, differing only on the B ring (Fig. 6A).

Note that 28-homobrassinolide (**2**), 28-homocastasterone (**4**), 24-epibrassinolide (**6**), and 24-epicastasterone (**8**) fulfill the requirements postulated in the literature, although they show different activities, ranging from 3.29 to 2.27 in a logarithmic scale. These activity differences are better explained through their CI. Thus, it seems that this similarity index (CI) is a more precise way to establish the requirements. Nevertheless, in some cases the activity cannot be explained by the CI. Thus, in the case of the lactone **10**, the activity is higher than expected for a CI of 0.76. Conversely, the activity of the 6-oxa analog **14** is lower than expected for a CI of 0.67.

Moreover, the three compounds represented in Figure 3 (**4**, **10**, and **11**) have a very similar CI but they elicit different activities. Looking at the overlaid maps (Fig. 3), one can observe that, for **10** (Fig. 3A), although having a 2β,3β-diol and A/B *cis* junction, all the electrostatic potential sites of this compound overlay very well with the ones of brassinolide (**1**) except for the region near the hydroxy group at C_2. This could indicate the minor importance of this hydroxy group to the activity. Concerning the hydroxyketone **11** (Fig. 3B), the regions corresponding to the diols of the side chain and A ring are larger than those of brassinolide (**1**). Therefore, although this similarity index proved to be better than the previous requirements, it is not enough to set up a brassinosteroid CI-related activity index. Other factors may be involved.

In considering the initial hypothesis, if brassinosteroids acted through a mechanism similar to that of animal steroid hormones, the brassinosteroid receptor complex could involve H bonds between the protein residues and the steroid. Thus, if the region where the probability to form H bonds and the interaction energy for these compounds was found, it should be possible to discriminate between them and, moreover, to gain more information about the mechanisms of brassinosteroid receptor interaction.

The GRID methodology (19) has been used to calculate

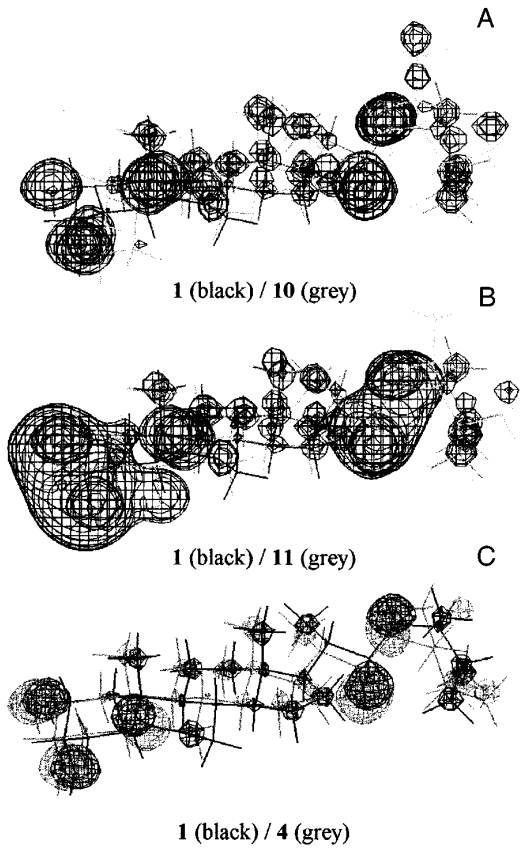


FIG. 3. Overlaid molecular electrostatic potential maps at –30 KT level between brassinolide (**1**) (in black) and three brassinosteroids (**4**, **10**, **11**) (in grey). The electrostatic Carbó similarity index/activities for these compounds are: (A) **10** 0.76/2.45; (B) **11** 0.76/1.53; (C) 28-homocastasterone (**4**), 0.76/2.80.

the energy interaction between the brassinosteroid and a probe that simulates an interaction by the H bond. On this preliminary calculation, water has been chosen as probe owing to its capability to act both as acceptor and donor of H bonds.

Figure 4 shows the GRID map at a –4 kcal/mol for brassinolide (**1**); the areas in white represent the highest probability to form H bonds.

Figure 5 shows the GRID map junctions only for the A and B rings between brassinolide (**1**) and **4**, **10**, and **11**, the same three compounds whose electrostatic potential maps are shown in Figure 3. The junction (in white) represents the common area between brassinolide (**1**) and each compound with a higher probability to form H bonds. As it has been indicated, all these compounds have the same CI but elicit very different activities. From these pictures, we see that, whereas the area corresponding to the hydroxy groups on the A ring for **4** and **11** is similar to that of brassinolide (**1**), there is only a small area on this region in **10**. This is in agreement with what was observed on the electrostatic potential map of **10** (Fig. 3), where the hydroxy group at C_2 was far more distant. If one takes into account the junction on the B ring, one observes that **4** presents a hole in the center and that its activity falls from 5.93 for brassinolide (**1**) to 2.80. This area becomes smaller for the hydroxyketone **11** and the activity falls again to 1.53. Moreover, in this case, another factor could decrease the ability of H bonding with the receptor owing to its capability of forming an intramolecular H bond between the two hydroxy groups at C_3,C_5. This phenomenon is observed in the active conformation found for **11** (16). Therefore, the size and shape of the area where the probability to form H bonds is higher seem to be activity-related. For the lactone **10**, two fea-

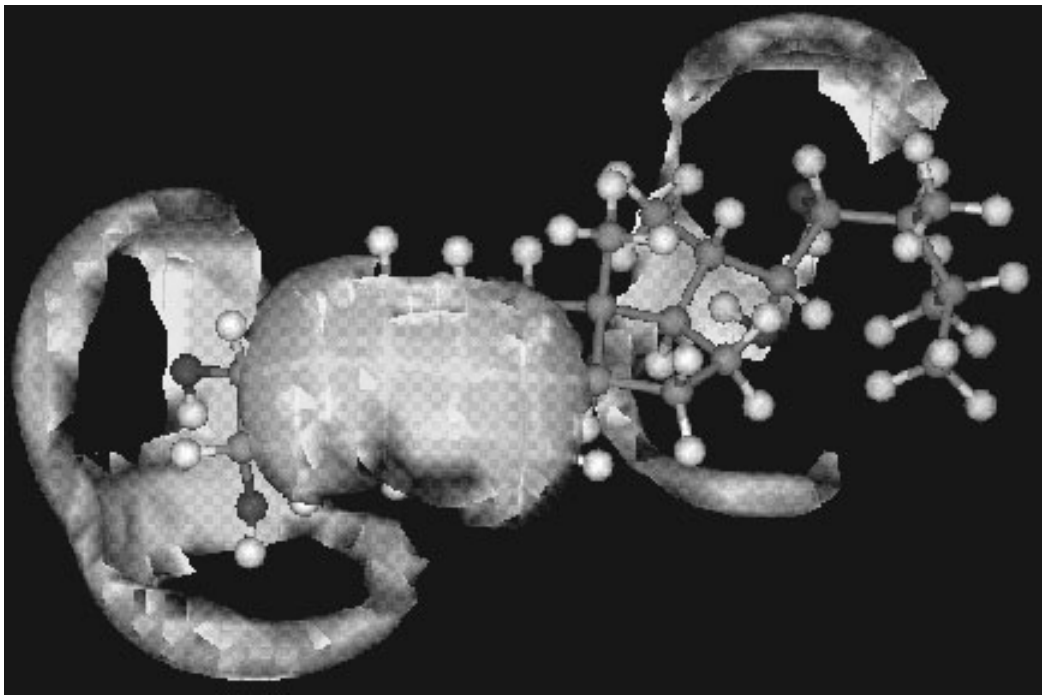


FIG. 4. GRID map for brassinolide (**1**) using a water probe at –4 kcal/mol.

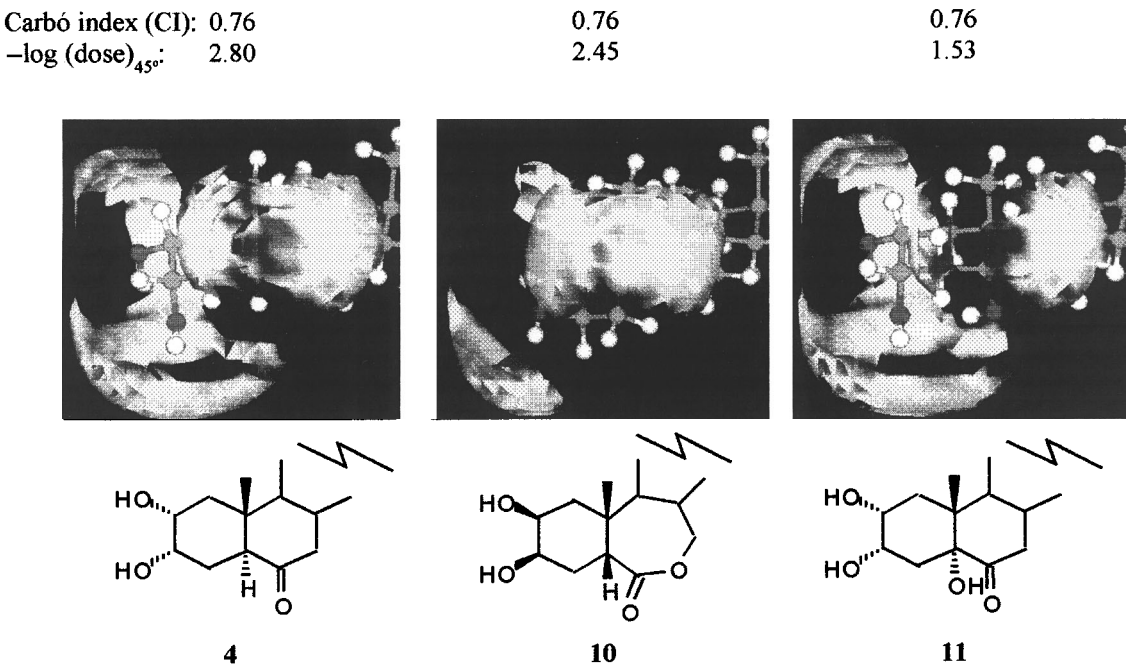


FIG. 5. GRID map surface junction for water probe between brassinolide (**1**) and three brassinosteroids (**4**, **10**, **11**). See Scheme 1 for structures **4**, **10**, and **11**.

tures of the molecule seem to compensate each other. On one hand, the area of the B ring is highly similar to brassinolide (**1**), so the activity should be higher than elicited; on the other hand, there is only a small common area for the hydroxy group at C_3, which forces its activity to decrease. Therefore, qualitatively one can assume that the ability to form H bonds

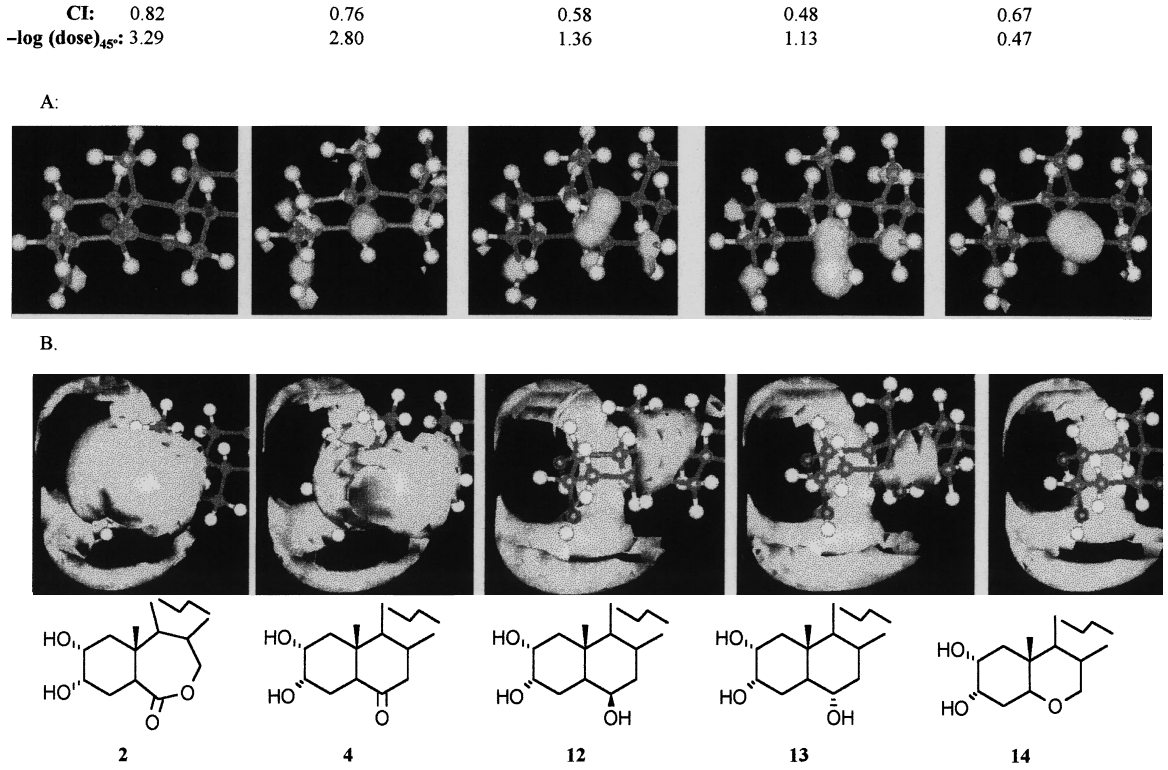


FIG. 6. (A) Surface difference in molecular electrostatic potential between brassinolide (**1**) and five brassinosteroids (**2**, **4**, **12**, **13**, **14**). (B) GRID map surface junction for water probe between brassinolide (**1**) and five brassinosteroids (**2**, **4**, **12**, **13**, **14**). See Scheme 1 for structures. See Figure 5 for abbreviation.

to the receptor could be suitable to describe the activity.

The 6-oxabrassinosteroid **14** is another example on how the ability to form H bonds is activity-related. Thus, on Figure 6A, the surface difference of molecular electrostatic potentials (in white) for A and B rings, between brassinolide (**1**) and 5 analogs differing only in B ring (**2**, **4**, **12**, **13**, and **14**) is shown. Qualitatively, one can observe that the white area, which is the difference between the electrostatic potential map for brassinolide (**1**) and each compound, increases and the activity decreases progressively in all cases as well as with the CI, except for the 6-oxabrassinosteroid **14**, for which its CI is higher than expected. An explanation for this exception can be found, again bearing in mind the ability of this group to form H bonds.

Looking at the surface junction of GRID maps between brassinolide (**1**) and the same five analogs already mentioned (**2**, **4**, **12**, **13**, and **14**) (Fig. 6B), one can see that the area on B ring, where the probability to form H bond is higher, gradually decreases in the series from **2**, up to disappearance for **14**. Qualitatively there is a very close relationship between this area and the activity. So, the impossibility of forming H bond on the B ring of the 6-oxa analog **14** is in agreement with its very low activity even with a CI higher than expected.

These results are the first reported evidence of the limit of scope of applicability and the weakness of the requirements so far postulated for a high brassinosteroid activity. In this sense, our new brassinosteroids having 2β,3β-diol and A/B *cis* ring junction (**10**), hydroxyketone (**11**), 6-hydroxy (**12** and **13**) or 6-oxa (**14**) functionalities, which had not been considered in these requirements, have shown activity as plant growth regulators and in some cases have even shown a high activity.

Moreover, in our aim to find new requirements definition, we found that the electrostatic charges of the functional groups play an important role in the description of the activity as evidenced by its good correlation with the CI in most of the cases studied. The differences observed in some of them could be explained by their ability to form H bonds. This could be one of the ways through which brassinosteroids interact with the receptor or receptors.

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