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A theoretical approach to the solvation of brassinosteroids

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ABSTRACT

The interaction of three different brassinosteroids with water was studied by the Multiple Minima Hypersurface (MMH) procedure to model molecular interactions explicitly. The resulting thermodynamic data give useful information on properties of molecular association with water. This application can serve as a tool for future investigations and modelling concerning interactions of brassinosteroids with receptor proteins in plants. DFT/B3LYP calculations were also made in order to correlate and test the performance of the current AM1 Hamiltonian calculations of these complexes, which are inherent to MMH routine. Diol functionalities located in ring A and lateral chain appears as the sites that show the highest affinity to water. The oxalactone group does not appear to be a key structural requirement in the association with water. Parallel calculations with a "polarizable continuum method" (PCM) agreed with the reported experimental order of biological activities, where Brassinolide exhibited the best solubility features.

1. Introduction

Brassinosteroids (BRs) are plant growth promoting molecules found at low concentrations throughout the plant kingdom and are widely distributed in both reproductive and vegetative plant tissues [1,2]. Natural brassinosteroids identified so far, have a common 5α-cholestan skeleton, and their structural variations come from the kind and orientation of oxygenated functions in rings A and B. These modifications are produced by oxidation and reduction reactions during biosynthesis. These steroids can be classified as C_{27}, C_{28} or C_{29} brassinosteroids depending on the alkyl-substitution on the C_{24} in the side chain [3]. These side chain structures are all common in plants sterols.

Up to date, 61 naturally occurring BRs have been discovered [1]. Brassinolide (**Br**) [(22R,23R,24S)-2α,3α,22,23-tetrahydroxy-24-methyl-homo-7-oxa-5α-cholestan-6-one] is the most active brassinosteroid. It has a lactone function at C_6/C_7 in ring B, 2α,3α-vicinal hydroxyl at A-ring and in the lateral chain exhibits R configuration of the diol at C_{22}/C_{23} and 24S methyl substitution (Fig. 1).

As regards the B-ring oxidation, brassinosteroids are divided into 7-oxalactone, 6-ketone (6-oxo) and 6-deoxo (non-oxidized). In general, 7-oxalactone BRs have stronger biological activity than 6-ketone types (such as Castasterone (**Cs**)) and non-oxidized BRs reveal no activity in biological tests.

BRs with either a 3α-hydroxyl (e.g. Typhasterol (**Tp**)), 3β-hydroxyl or 3-ketone in the A-ring are precursors of BRs carrying 2α,3α-vicinal diol; those with 2α,3β-, 2β,3α-, or 2β,3β-vicinal diol can be metabolites of active BRs with a 2α,3α-vicinal diol. Decreasing order of activity (2α,3α > 2α,3β > 2β,3α > 2β,3β) shown by structure–activity relationship suggests that α-oriented hydroxyl group at C_2 is essential for a greater biological activity of BRs in plants [1,4]. Recent studies have shown that 3α,4α-diols are more active than 2α,3α. This fact is in strong contrast with the structure requirements mentioned above. Sisa et al. [5] suggest that the higher activity of unnatural 3α,4α-diols could be explained by twisting and distortion of the molecule due to the seven- or eight-membered B-ring and also by the position of a carbonyl group relative to the A-ring diol.

With the exception of some not fully characterized BRs with an oxo function at C_{23}, all bioactive BRs possess a vicinal 22R-23R-diol structural functionality, which appears essential for a high biological activity [1,6–8].

Although the existence and biological activity of these plant steroids have been described in a huge body of literature since 1979, significant progress has been produced in the last 10 years on our understanding of BRs biology, including their distribution in plants, the role of BRs in plant growth, BRs biosynthesis, perception, signal

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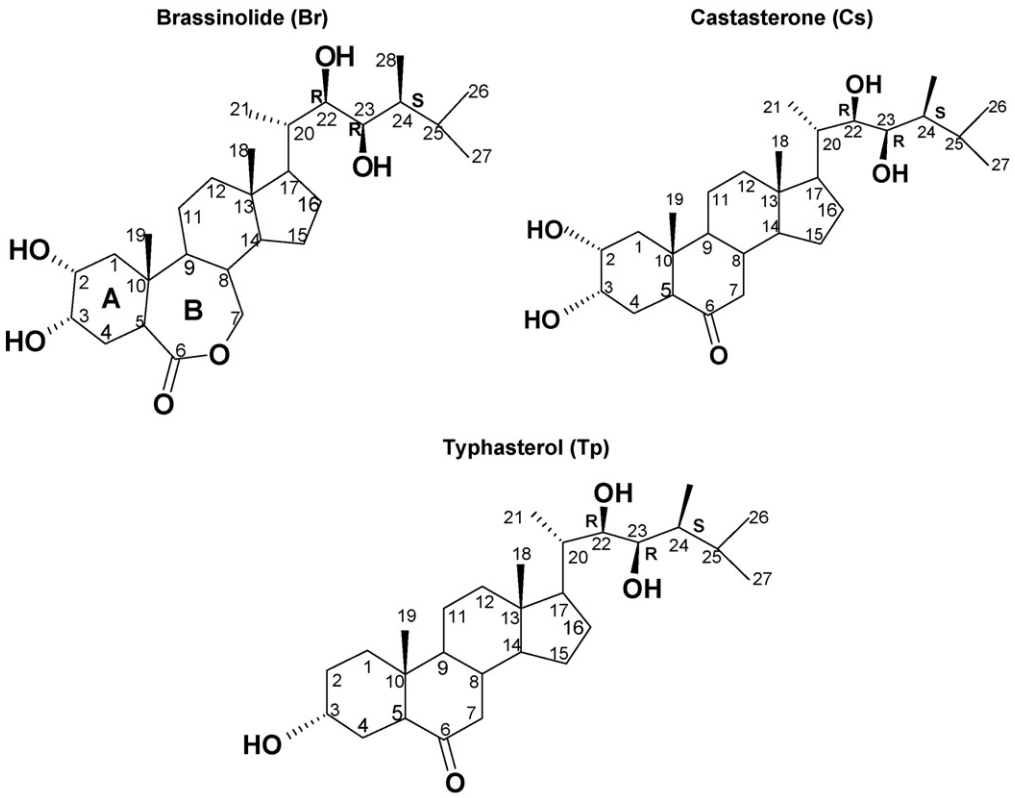

Fig. 1. Structures of brassinosteroids studied.

transduction and the interaction between BRs and other plants hormones [9]. Several previous experimental works suggest that transport inside cells play a determinant role in BRs activity [2].

In a previous study by Brosa et al. [10], a model based on brassinosteroid–receptor interaction was proposed. According their results the activity exhibited by brassinosteroids can be explained by considering the putative H-bonding interactions in the brassinosteroid–receptor complex, and most importantly this H-bonding seems to be essential for activity when it is located in a specific zone. The zone that seems to be most important for increasing biological activity is the electronegative part of the side chain, and it appeared to be even more important than the diol system of the A-ring. Another requirement was that these molecules must be in a so-called "active conformation", which is defined as the one that is able to bind to the receptor. Brosa et al. also tested with a common theoretical "docking" procedure the interaction between BRs side chain analogs and different probes that simulated different kind of interactions. Water was chosen among the probes tested in that work owing to the well-known capacity for acting both as acceptor and donor of hydrogen bonds. The diol group of Brassinolide shows a strong interaction with all the probes used.

In order to interpret molecular interactions of brassinosteroids with the biological media we show here the results of modelling preferred clusters of these phytohormones with water according the MMH routine. It allows us to select those preferred cluster structures that represent the highest expected populations in their respective molecular ensembles. Water solvations probably have an important role in BR transport in plants and in BR binding to their receptor protein BRI1.

Three natural brassinosteroids with different structural characteristics and biological activities have been selected for study in their association with water molecules (Fig. 1). The first one is Brassinolide, which possesses the highest biological activity of all natural brassinosteroids. The second one is Castasterone, the best known 6-oxo BR, which is a direct biosynthetic precursor of Brassinolide [11] and is believed to have an independent biological activity in some plants. The last one is Typhasterol, which also has a 6-ketone group in ring B, but does not possess the α-hydroxyl group in C_2.

The order of bioactivity (relative activities) shown by these BRs in the rice lamina inclination test [12] is the following:

$$\mathbf{Br}\ (100\%) > \mathbf{Cs}\ (20\%) > \mathbf{Tp}\ (2\%)$$

2. Methodology

In order to explore the conformational space in the interaction of each brassinosteroid with water, Multiple Minima Hypersurfaces (MMH) [13–18] procedures were applied. Then a molecular alignment approach with the most relevant local minima obtained by the MMH method in the case of association with one water molecule was performed. This molecular alignment procedure called Topo-Geometrical Superposition Approach (TGSA) [19] allows us to obtain the set of relevant semiempirical local minima, which is later refined using a density functional theory method (DFT), B3LYP with the 6-31G and the 6-31+G* basis set [20]. The PCM method [21,22] has been used to calculate bulk solvent effects.

MMH is a very useful and reliable approach for localizing the minima of hydrogen-bonded and weakly interacting systems and therefore has been successfully employed in several studies [15,23–27]. This procedure combines quantum mechanical methods for the calculations of energy with statistical mechanics to obtain thermodynamic quantities related to the molecular association process. The main procedure of this approach will be outlined briefly.

An appropriate construction of several random molecular geometries initially generates a set of n solute-solvent cluster configurations, starting from the independently optimized structures of BRs (solute) and water (solvent). Randomness has been carefully tested [13]. It produces a set of typical clusters of local minima in the solute-solvent interaction configuration space after an appropriate molecular optimization procedure of the previously obtained random structures, normally following an energy gradient pathway. The energy, ε_i, of every ith cluster of the ensemble is thus obtained. As our attention is focused on finding a reduced set of configurations, which can represent the most significant contributions to the ith state for the whole system we select only those that can represent a significant population after a Bolztmann distribution treatment of such ε_i. In some cases different arrangements converge to the same minimum and they must be eliminated after deciding if they are redundant or not. Non-redundant are, for example, those minima that correspond to multiplicities originated in molecular symmetry. For this purpose a Tanimoto similarity index was used for discriminating such statistically redundant information.

Obviously, the partition function of molecular association must be calculated by assuming the appropriate energy scale with respect to a reference value that is chosen to be the same set of non-interacting molecules. This means that we make the approximation of considering the translational, rotational and vibrational states of clustered molecules as identical to those in the reference states, which means that the association process is taken as isothermal.

Thus, the association process between a solute and n solvent molecules can be described by

$$X + n(\text{solvent}) \rightarrow X(\text{solvent})_n$$

Thermodynamic properties such as association energy, entropy, and Helmholtz free energy are then calculated by this procedure [13,14].

It must be noticed that this approach can provide, at the same time, important model structures of solute-solvent cluster interactions with true statistical significance together with the thermodynamic variables.

Previous studies report that the AM1 semiempirical method reproduces quite well the geometry of natural brassinosteroids and some analogues [28,29]. The most important fact to decide exploration of hypersurfaces with parametric, i.e. semiempirical Hamiltonians, is that they allow a huge number of calculations with a computational cost that is lower than that with ab initio methods and the computations are much faster for large supermolecules [30], as in our case. Semiempirical calculations should be a good choice to explore the energy hypersurfaces of the complexes, although they must be correlated with other independent calculations for testing reliability.

Then, to obtain energy minima structures of isolated molecules, the AM1 semiempirical Hamiltonian was applied to three brassinosteroids i.e. **Br**, **Cs**, **Tp** and also to the water molecule, using the MOPAC v. 6.0 program [31]. In the case of **Br** we used as input the X-ray structure [32].

In all calculations the eigenvector-following routine "EF" for searching minima was used, and all convergence thresholds were refined 100 times with respect to the defaults.

A study of the association energy evolution per mol of solvent, corresponding to solvated shells with an increasing number of solvent molecules was performed. It has been built by taking into account all 100 optimized sets formed by solute (**Br**, **Cs**, **Tp**) and n solvent molecules (water), with n = 1 to 5. The procedure explained in detail and all programs for processing are available on a web page [16]. Hundreds of thousands of SCF cycles with their respective supermolecular geometries were calculated during the process for each set. The program used for processing the output data is called Q3 [16].

In some cases the Tanimoto index does not completely eliminate the similarity degeneracy produced with clusters that possess the same energy and molecular geometry. Then we used a molecular alignment procedure with the most relevant local minima obtained by the MMH method in the case of association with one water molecule (n = 1). This procedure, called Topo-Geometrical Superposition Approach (TGSA) [19], is only based on comparisons of atom types and interatomic distances. Hence, the procedure can handle large molecular sets within affordable computational costs. This algorithm performs the molecular superposition within a molecular set in two ways: (1) calculating all pairwise alignments; and (2) choosing a molecule as a template [19]. Then we calculate all pairwise alignments between the most important configurations of each brassinosteroids with one molecule of water, to be absolutely sure that no redundant degenerate clusters were obtained for further analysis.

As mentioned before, semiempirical results provide a preliminary overview of the interactions in these complexes. For this reason, the set of relevant semiempirical local minima obtained after TGSA analysis were reoptimized using the B3LYP/6-31G scheme. All calculations were performed with Gaussian 03 package [33].

Finally the PCM method [21,22] has been used to calculate bulk solvent effects. In this method the solvent, i.e. water in this case, is represented by an infinite dielectric medium characterized by the dielectric constant of the bulk and the so-called UAHF predefined radii are used for building the effective cavity occupied [34]. The solvent effects are considered by PCM single point energy calculations at the B3LYP/6-31G and B3LYP/6-31+G* levels applied to the BSSE uncorrected structures of the most important previously optimized clusters [35].

3. Results and discussion

The activity of BRs could be related with the formation of hydrogen bonds in the receptor binding sites [10,36]. Moreover the exploration of the sites of the BRs molecules that more likely will interact with water by hydrogen-bond interactions is important to understand how may occur the hydrophilic interactions of these phytohormones with the receptor.

The first step of this study was to explore hypersurfaces of the brassinosteroids–water complexes. The solvation shell refers to the amount of solvent molecules to be considered in a single solute-solvent cell to achieve the most significant effects [37]. A study of the evolution of the association energy (E^{assoc}, mol) of solvent corresponding to solvation shells with one up to five solvent molecules was performed. It was built by taking into account all 100 optimized sets. It must be stated that in order to obtain a full representation of solvent effects on the solute, a larger number of solvent molecules is needed. Perhaps the best cluster is produced when the association energy per solvent molecule converges to a certain value [13,37]. However, since our purpose is to take into account the main effects of the environmental molecules on the physical and chemical solute properties, we have decided that a relatively small solvation shell of five water molecules should represent the main perturbations to these properties with respect to the isolated solute.

Thermodynamic results are shown in Table 1 and some conclusions can be obtained after processing 100 configurations of each brassinosteroid with one up to five molecules of water. The thermodynamic properties of **Br** with n = 4 and 5 do not change significantly with respect to the cluster containing three solvent

Table 1
Association energies (E^{assoc}, kJ/mol), Helmholtz free energies (A^{assoc}, kJ/mol) and entropies (S, J/kmol) of selected BRs as obtained at the AM1 level.

No. molecules H_2O	Brassinolide (**Br**)			Castasterone (**Cs**)			Typhasterol (**Tp**)		
	E^{assoc}	A^{assoc}	S	E^{assoc}	A^{assoc}	S	E^{assoc}	A^{assoc}	S
1	−29.8	−37.1	24.4	−31.1	−37.7	22.1	−25.5	−30.5	16.7
2	−30.4	−38.9	28.4	−31.1	−38.4	24.5	−26.8	−31.4	15.5
3	−30.6	−37.7	23.8	−31.3	−40.0	29.1	−30.6	−35.3	15.5
4	−30.9	−40.1	30.9	−29.3	−38.9	32.2	−27.4	−34.6	24.2
5	−31.0	−40.5	31.8	−31.2	−39.9	29.3	−29.8	−37.1	24.4

molecules. Then in the case of Brassinolide three molecules of water, as microstates in a canonical statistical ensemble, can represent the main perturbation to these properties with respect to the isolated solute molecule. The thermodynamic properties of the three kinds of BRs molecular clusters studied are very similar, but as can be seen from Fig. 2a, the association energies of **Br** and **Cs** behave in a different way to the same property of **Tp**. In Fig. 2b the Helmholtz free energies of association (A^{assoc}, mol) versus the number of water molecules are presented. The presence of two groups can be clearly distinguished. **Br** and **Cs** possess greater absolute A^{assoc} than **Tp**. This is expected and is related to a self-evident molecular similarity principle: the more similar two molecules are, the more similar will be their properties.

The only feature that distinguishes **Br** and **Cs** is the structure of the B-ring (Fig. 1). The fact that the thermodynamic behaviour of both molecules is very similar also means that the oxalactone group is not so important in the association of these molecules with water. Table 1 also shows that the absolute values of the energies and Helmholtz free energies of association of **Tp** are lower than those of all the other molecules in the study. Typhasterol shows the lowest association for water and forms the least stable complex with it. This is also expected, since this molecule does not contain the diol system in ring A. It is the less hydrophilic, and also possesses the lowest biological activity [1–4,12]. The fact that this molecule shows the lowest association for water could be a strong indication that the diol group in ring A is very important in the association and stabilities of these complexes.

The entropy can be understood as depending on the number of possible minima structures that contributes to the partition function. In this sense, optimized clusters having the same or

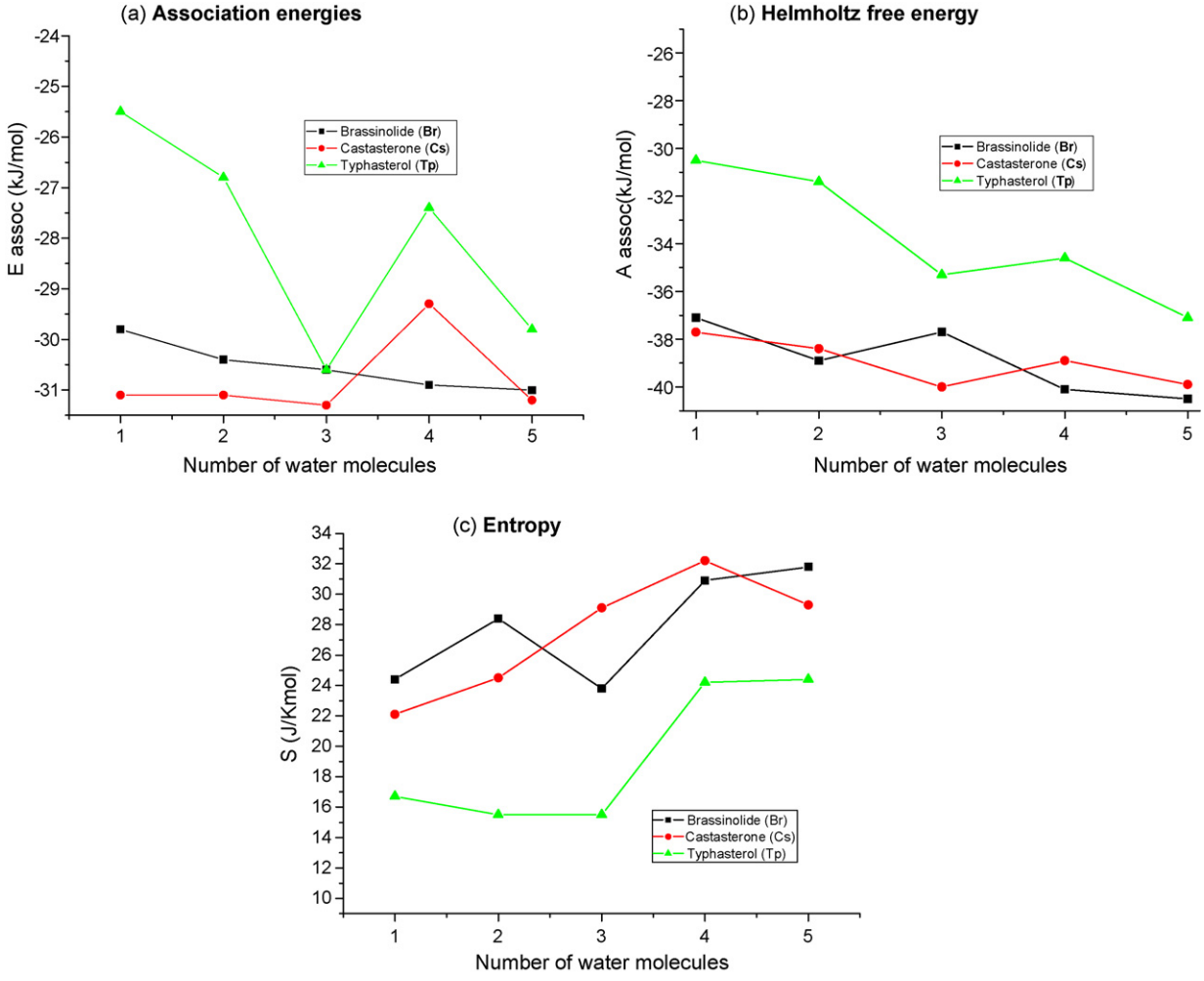

Fig. 2. Values of association energies of Brs versus the number of solvent molecules.

nearly the same energy and different molecular arrangements are relevant to the ensemble and contribute in a significant way to the entropy [38] and therefore to the probability of such structures to determine the biological activity.

In Fig. 2c the entropies of these systems (S, mol) versus the number of water molecules are presented. As expected the tendency of the entropies is to increase with the number of water molecules, because as the degrees of freedom of the system increase several conformations become possible. In some cases the entropy is higher than in others. For example, in **Br** with $n = 1$ and 2 the entropy is greater than when the solute is surrounded by three water molecules, and the increase in the entropy is derived from the fact that the system shows several different configurations with small differences of energy if compared to the global minimum. The same two groups can also be distinguished here. Fig. 2c shows that **Br** and **Cs** present higher entropies than **Tp**. For the systems **Br** and **Cs**, the water molecules can interact with the diol groups in ring A and lateral chain, the oxalactone ring of **Br** and the carbonyl group of **Cs**. Nevertheless, in the case of **Tp** the entropy is reduced because the diol system of ring A is absent.

Thermodynamic data confirm us that the most similar BRs of those studied here are **Br** and **Cs**. The diol system in ring A appears essential to provide a high association for water while the oxalactone group is not a key functionality in the association of these molecules with water.

The energetic data computed with AM1 must be used with care. Nevertheless one of the values of MMH methodology is the calculation of thermodynamic association properties. The statistics obtained by MMH usually produce reasonable results, probably due to systematic error cancellations [14].

3.1. Topo-geometrical superposition analysis

One of the abilities of the MMH procedures is the selection of the configurations of major stability. Therefore, we can check the preferential positions of interactions between the solvent molecules and the solute, after an accurate exploration of the potential energy surface by a stochastic method [38].

TGSA [19] is an algorithm that only requires the atomic numbers and coordinates of the molecules to be superposed, it considers the molecules as rigid bodies [39]. In these cases the quality of the superposition, evaluated by the Carbó index [40] is quite high. This is in disagreement with our requirements since we need clusters with different configuration and with an important contribution to the partition function. The problem is that as we are applying TGSA [19] to the most important configurations given as a result of the MMH procedure, the solute is the same. Therefore, it is the part of the system that superposes almost entirely and then the only feature that differentiates each configuration from another is the position of the solvent. This explains why Carbó index values are so high. Then it was necessary to choose a similarity Carbó index threshold, above which the clusters are either the same or almost the same. This value was fixed to 0.9990. Then, if two clusters possess a Carbó index lower than this fixed value, we can consider these two configurations as different.

The relative populations, energies of each configuration and the Carbó similarity index after pairwise superpositions with TGSA99 program [19] of each cluster are summarized in Table 2. In each case the configurations that represent the 50% of all cluster population were selected. This program was only carried out to all systems with one molecule of water because when $n \geq 2$ we could have the possibility of two clusters having at least a solvent molecule in the same place. In this case the similarity would be very high and therefore it would be reasonable that these clusters are almost the same, eliminating by this way useful information for the system in study.

For the dimers of **Br**, 4 configurations were found to contribute 46% to the system (see Table 2). The cluster Br3 was eliminated because the similarity Carbó index of the pair Br2-Br3 is 1. We then keep the cluster Br2 since it possesses a lower energy than the cluster Br3. Although the cluster Br4 possesses the highest energy of the four configurations we cannot eliminate it, because this cluster shows a different zone of interaction, i.e. the oxalactone group.

Then Br1, Br2 and Br4 represent the hydrophilic interactions of water with the molecule of Brassinolide and can be seen in Fig. 3a–c.

For **Cs** with one molecule of water, 4 configurations were found to show similar energies, which represent almost 54% of the whole system population (Table 2). The clusters Cs2 and Cs3 are eliminated because the similarity Carbó index is 1. Cs1 is the minimum-energy cluster and the water molecule interacts with the diol system in ring A. Then, in order to keep non-degenerate and non-redundant information we eliminate the Cs2 and Cs3 clusters since both possess similarity Carbó indexes greater than 0.9990, i.e. 0.9997. From the 100 configurations generated by the MMH procedure only one, representing 0.3% of all cluster population showed the water molecule interacting with the carbonyl group of ring B. Cs1 and Cs4 structures are shown in Fig. 3d and e.

Tp shows two configurations with a total contribution of 52% and with a Carbó index of 0.9980. In this case only two configurations have shown the H_2O molecule interacting with the carbonyl group and with a very low contribution (3%). Tp1 and Tp2 structures are shown in Fig. 3f and g

As a result of the TGSA procedure [19] a total of 7 non-degenerate structures were finally obtained in the case of $n = 1$ for all brassinosteroids studied i.e. 3 for Brassinolide (Br1, Br2, Br4), 2 for Castasterone (Cs1, Cs4) and 2 for Typhasterol (Tp1, Tp2). The similarity degeneracy was completely eliminated from our results.

Table 2
Relative populations of principal minima clusters, energies of the minima and Carbó indexes (C_{AB}) of the BRs studied.

BRs	Cluster	n/N %	ε_i (kJ/mol)	C_{AB}
Brassinolide (**Br**)	Br1	14	−32.2	$C_{Br1-Br2} = 0.9973$, $C_{Br1-Br3} = 0.9973$, $C_{Br1-Br4} = 0.9988$
	Br2	14	−32.1	$C_{Br2-Br3} = 1$, $C_{Br2-Br4} = 0.9979$
	Br3	12	−31.8	$C_{Br3-Br4} = 0.9977$
	Br4	6	−30.3	
Castasterone (**Cs**)	Cs1	21	−33.8	$C_{Cs1-Cs2} = 0.9997$, $C_{Cs1-Cs3} = 0.9997$, $C_{Cs1-Cs4} = 0.9971$
	Cs2	13	−32.6	$C_{Cs2-Cs3} = 1$, $C_{Cs2-Cs4} = 0.9979$
	Cs3	11	−32.2	$C_{Cs3-Cs4} = 0.9979$
	Cs4	9	−31.8	
Typhasterol (**Tp**)	Tp1	32	−27.7	$C_{Tp1-Tp2} = 0.9980$
	Tp2	20	−26.5	

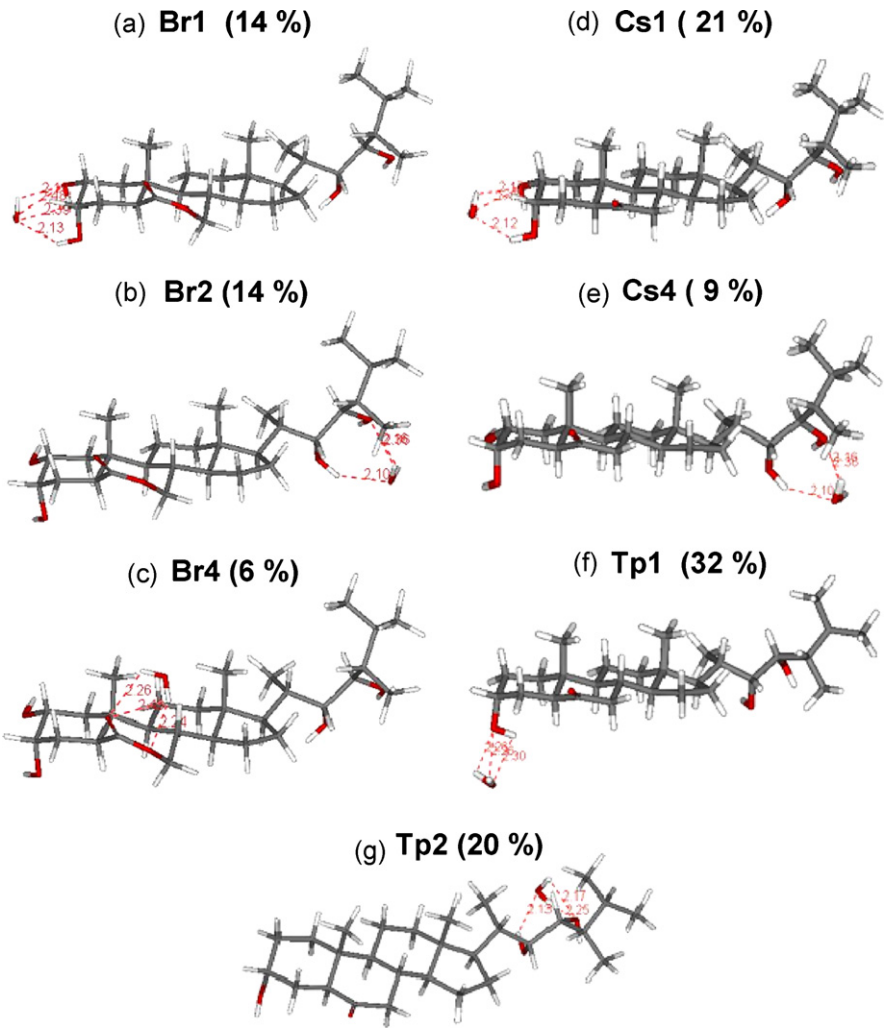

Fig. 3. Most stable clusters obtained by MMH procedure applied to Brassinolide (**Br**), Castasterone (**Cs**) and Typhasterol (**Tp**) and relative population in parenthesis.

Superposition of the most important clusters of Brassinolide, Castasterone and Typhasterol are shown in Supplementary material.

3.2. Interaction between brassinosteroids and one water molecule

In the association of water with the diol group in positions 2 and 3 of ring A (structures Br1, Cs1) the OH in position 2 is reoriented, favouring then the interaction of the hydrogen of the hydroxyl group with the oxygen of the water, OH···OH_2 (Fig. 3a). The most common interaction of the OH in C_2 produced in these cases is of the type HO···H_2O. This interaction is observed in both structures (Fig. 3a and d) and represents the interaction of the oxygen of the hydroxyl group with the two hydrogen atoms of the water molecule .The interaction of the OH group in position 3 is of the type OH···OH_2.

The cases of Tp1 and Tp2 are different; the OH in 2 is absent (Fig. 3f and g). The OH in 3 has a different orientation that makes possible not only the interaction OH···OH_2 presented in the structures Br1 and Cs1, Fig. 3a and d, but also the interaction HO···H_2O that can be seen for Tp1 in Fig. 3f. The different orientation of the OH in position 3 in structures Tp1 and Tp2 with respect to Br1, Cs1 and Br2, Cs4 could be due to the lack of the OH in position 2. The presence of intramolecular hydrogen bonds between the OH in the diol group of ring A force the OH in 3 to be oriented towards the OH in 2. Therefore when the OH in position 2 is not present the OH in 3 can rotate freely and adopt other conformations.

In the association of water with the diol group of the side chain (structures Br2, Cs4 and Tp2) the OH in position 22 seems to be more important in the formation of hydrogen bonds. As can be seen from Fig. 3b, e and g the hydrogen of OH in position 22 is always oriented towards the oxygen atom of the water, contrary to the OH in position 23, where the hydrogen is oriented towards the other face of the molecule. The conformation of the diol system in ring A of structures Br2 and Cs4 remains unaltered since the interaction of the water molecules is with the lateral chain of the molecule (Fig. 3b and e). The hydroxyl group of C_{22} experienced always the interaction OH···OH_2, and the OH in C_{23} the type HO···H_2O.

Br4 is the only structure that possesses a water molecule interacting with the polar groups of ring B (Fig. 3c).

3.3. Refinement of geometries

The geometries obtained by the AM1 semiempirical Hamiltonian were further optimized with density functional B3LYP/6-31G calculations. Therefore, the most important configurations obtained for compounds studied in the case of $n = 1$ were optimized at this expectedly higher level because B3LYP is one of the most widely used hybrid density functionals in biological

Table 3
B3LYP calculations. Association energies on the CP-uncorrected and corrected PES with 6-31G and 6-31+G* basis set.

BRs	Cluster	ΔE (kJ/mol)[a]	BSSE (kJ/mol)[b]	ΔE^{CP} (kJ/mol)[c]	ΔE^{CP} (kJ/mol)[d]	ΔE^{CP} (kJ/mol)[e]
Brassinolide (**Br**)	Br1	−69.65	19.66	−49.99	−51.46	−33.01
	Br2	−67.55	23.56	−44.00	−45.80	−33.99
	Br4	−46.38	19.29	−27.09	−29.98	−26.54
Castasterone (**Cs**)	Cs4	−67.22	23.51	−43.71	−45.40	−33.88
	Cs1	−64.06	20.86	−43.23	−44.30	−33.36
Typhasterol (**Tp**)	Tp2	−64.56	21.15	−43.40	−44.20	−29.85
	Tp1	−50.90	20.24	−30.66	−33.19	−22.77

[a] Energy of stabilization as $\Delta E = E_{(BS-H_2O)} - E_{(BS)} - E_{(H_2O)}$ at the B3LYP/6-31G level without BSSE correction.
[b] BSSE is the total defect of energy originated due to the basis set superposition error at the uncorrected geometry using the B3LYP/6-31G basis set.
[c] ΔE^{CP} is the CP-corrected interaction energy at the uncorrected geometry using the B3LYP/6-31G basis set.
[d] ΔE^{CP} is the CP-corrected interaction energy at the CP-corrected geometry using the B3LYP/6-31G basis set.
[e] ΔE^{CP} is the CP-corrected interaction energy at the B3LYP/6-31+G*//B3LYP/6-31G.

systems [41–43]. The stabilization energies obtained at the DFT level were corrected using the counterpoise procedure (CP) of Boys and Bernardi [44] although there is a tendency to believe that DFT calculations are much less influenced by the Basis Set Superposition Error's (BSSE) worst consequences than correlated ab initio methods [45]. The first step was the complete optimization of the clusters on the CP-uncorrected potential energy surface (PES) with a single point evaluation of BSSE. Then the uncorrected minima obtained were optimized in the CP-corrected PES. Finally a single point evaluation of the energies of the 7 CP-corrected geometries with the 6-31+G* basis set have been performed.

Calculations of interaction energies at the B3LYP/6-31G level have been performed and the results are presented in Table 3. Clearly the CP-optimized geometry (Table 3d) possesses a larger stabilization than the normally optimized geometry plus the single point CP correction (Table 3c) [46,47].

In some cases the effect of BSSE in intermolecular distances is well known to be critical, mostly when there is a very weak complex [48,49].

The magnitude of BSSE correction at energy minima configuration is about 20–40% of total uncorrected energy of interaction. When calculating interaction energies of molecules that differ quantitatively in size, the BSSE could be very high, as is our case, since the small molecule possess a high BSSE and the larger molecule makes its estimation difficult [50]. Br1 possesses the lowest magnitudes of BSSE. Although the BSSE of these complexes are high and the interaction energies should be corrected for it, the differences between the uncorrected and corrected minima

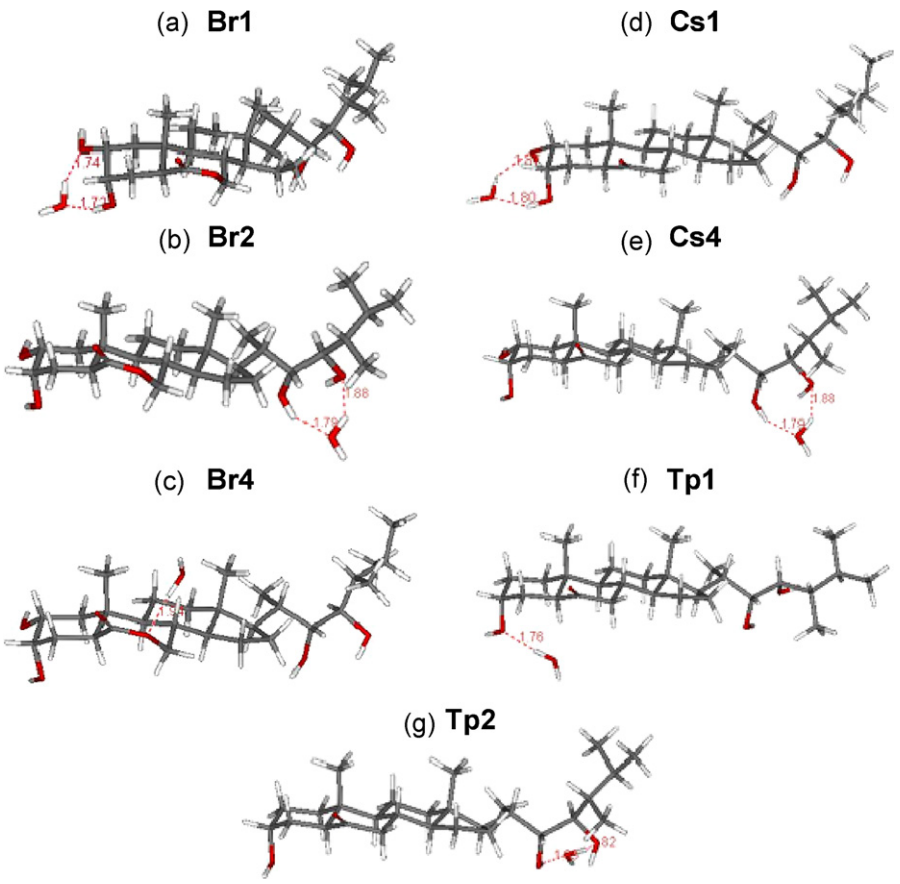

Fig. 4. Brassinolide (**Br**), Castasterone (**Cs**) and Typhasterol (**Tp**) CP-corrected structures.

geometries are very small. For the sake of simplicity uncorrected geometries are not shown in this work.

In order to analyze the effect of BSSE correction on geometries of clusters BRs···H_2O and its differences with semiempirical calculations, the CP-corrected structures are presented in Fig. 4a–g. Moreover these structures were superposed with the 7 clusters obtained by the MMH procedure (Fig. 3a–g) and presented in Supplementary material. As expected in all cases the water molecule is closer to the polar sites of the Brs studied, because an optimization at a higher level was performed to the clusters obtained by MMH. The interaction HO···H_2O has changed in all cases by the type HO···HOH as can be seen in Fig. 4a–g. Then the clusters Br1, Cs1 and Tp1, which earlier had shown the first type of interaction, now possess the oxygen of the hydroxyl group interacting with a hydrogen atom of the water molecule (Fig. 4a, d and f).

In Br4 the water molecule is not interacting with the entire oxalactone group (Fig. 3c) but only with the ether function C–O···HOH (Fig. 4c).

The most stable complexes were obtained when the water molecule is interacting with both diol groups (ring A and lateral chain) see Table 3d. These clusters are presented in decreasing order of energy: Br1 and Br2 (Fig. 4a and b), Cs4 and Cs1 (Fig. 4e and d), and Tp2 (Fig. 4g). The diol group in ring A is very important because the interaction energy decreases when is not present in the molecule, that is the case of Tp1 (Fig. 4f). In Tp2 as the water molecule interacts with the diol system of the lateral chain; the interaction energies are very similar to Br2 and Cs4. When the interaction is produced with the oxalactone group in ring B the energy also decreases, that is the case of Br4 (Fig. 4c). This indicates that the interaction of water with the oxalactone group is not favoured.

It is generally believed that diffuse functions are very important for DFT calculations, particularly when studying weak interactions. Then we went over the calculations of the 7 CP-corrected structures with a higher level of theory, the B3LYP/6-31+G*, which possesses diffuse functions that are so important to describe relative energies. We found that the calculated relative energy among the selected Br4 cluster were very similar by either of the two methods: a full optimization at the B3LYP/6-31+G* level or a single point calculation using this level of theory with the B3LYP/6-31G CP-corrected geometry. Hence it appeared that an alternative was desirable. These results also show that the geometries of Br4 cluster are not much different by either method. Therefore, the single point energy calculation at the B3LYP/6-31+G* level using the counterpoise-corrected B3LYP/6-31G geometries seemed to be an expedient choice (B3LYP/6-31+G*//B3LYP/6-31G). As can be seen in Table 3e, the order of stabilities is almost the same: Br4 and Tp1 possess the lowest stabilities meanwhile the clusters where the water molecule interaction is produced with the diol groups of BRs shows the greatest stabilities (Br2, Cs4, Cs1 and Br1).

Table 4
NBO natural atomic charges (a.u.) of the main groups (diol groups of ring A, lateral chain, and oxalactone group in ring B).

	Brassinolide (**Br**)	Castasterone (**Cs**)	Typhasterol (**Tp**)
Ring A			
OH-2	O −0.78, H 0.48	O −0.78, H 0.48	–
OH-3	O −0.77, H 0.49	O −0.78, H 0.49	O −0.77, H 0.47
Ring B			
O-6	−0.56	−0.53	−0.53
-O-	−0.54	–	–
Lateral chain			
O-22	O −0.78, H 0.48	O −0.78, H 0.48	O −0.78, H 0.48
O-23	O −0.78, H 0.49	O −0.78, H 0.49	O −0.78, H 0.49

Since **Br** present more polar sites than the other BRs it is expected that this compound exhibit a higher association for water. Previous results have shown that almost no water molecules interact with the ring B, unless the oxalactone ring is present (**Br**). To explain this fact a Natural Bond Orbital (NBO) [51,52] analysis of population has been performed with the B3LYP method and the natural atomic charges are presented in Table 4. This analysis allows us to differentiate the regions of the molecule that possess an accumulation of charges from the zones with low charge density in a quantitative way. As can be seen in Table 4 the zones with higher charge density are the oxygen atoms corresponding to the hydroxyl groups that form the diol groups of ring A and the lateral chain, and therefore the hydrogen atoms of these hydroxyl groups possess higher positive charges. The oxygen atoms that correspond to the lactone group possess a smaller charge. This could be the reason why the water molecule will tend preferentially to associate with the diol groups of ring A and the lateral chain. In the cases when the oxalactone functionality is not present (**Cs** and **Tp**) the probability to found association by the carbonyl group is even lower, since the charge density of this zone has decreased.

3.4. Polarizable continuum model

In Table 5 the total Gibbs free energies of solvation of the solutes (Brassinolide, Castasterone, Typhasterol) and complexes (7 cluster uncorrected structures) at the 6-31G and 6-31+G* levels are shown. Both theoretical levels agree that **Br**, followed by **Cs**, appears the best solvation. We have tried to calculate the effect of solvation on the interaction energies. However, it is known that PCM has its obvious limitations associated with the need to constrain an arbitrarily shaped solute to an artificial cavity, and

Table 5
Computed solvation free energies of the solute and complexes.

Solute	ΔG_{solute} (kJ/mol)[a]	ΔG_{solute} (kJ/mol)[b]	Complexes	$\Delta G_{complex}$ (kJ/mol)[c]	$\Delta G_{complex}$ (kJ/mol)[d]
Br	−18.49	−39.66	Br1	−24.94	−23.14
			Br4	−13.68	−14.27
			Br2	−12.47	−12.80
Cs	−6.86	−28.17	Cs1	−5.15	−7.11
			Cs4	−1.42	−4.30
Tp	9.96	−9.89	Tp1	3.31	−3.49
			Tp2	16.61	2.44

[a] Total Gibbs free energies of solvation of the solutes at the B3LYP/6-31G level.
[b] Total Gibbs free energies of solvation of the solutes at the B3LYP/6-31+G* level.
[c] Total Gibbs free energies of solvation of the uncorrected clusters at the B3LYP/6-31G level.
[d] Total Gibbs free energies of solvation of the uncorrected clusters at the B3LYP/6-31+G* level.

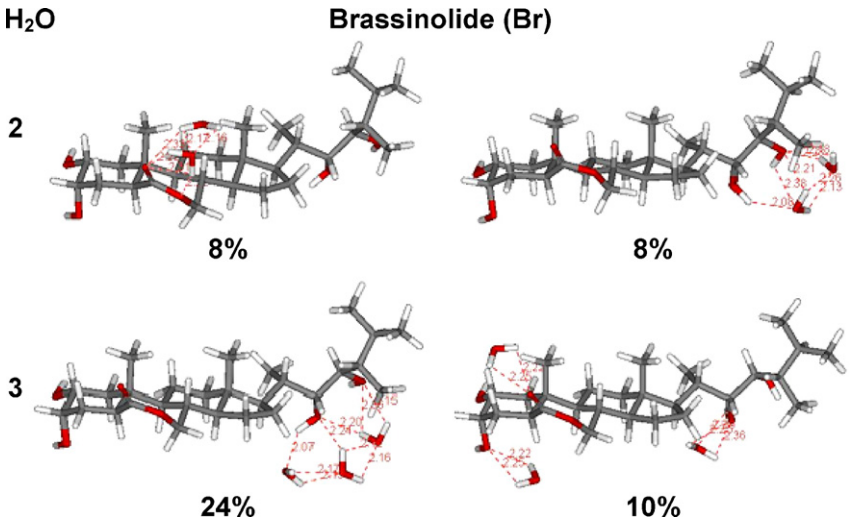

Fig. 5. Preferential positions of interactions between Brassinolide (**Br**) and 2 or 3 water molecules and relative populations.

these molecules possess a large area to be acceptably covered by the spheres described in the PCM method. Nevertheless, the two levels of calculation correctly described the order of association for water.

3.5. Interaction of brassinosteroids with n = 2 to 5 water molecules

The significant minima obtained for Brassinolide, Castasterone and Typhasterol surrounded by 2–5 water molecules are shown in Figs. 5–7 and in Supplementary material. The global minimum obtained in these cases corresponds to configurations where the water is forming solvent clusters.

When the MMH procedure is applied to **Br** with $n = 3$, the cluster which contributes 10% to the partition function of this system confirms the main three hydrophilic sites of association of this molecule with water (see Fig. 5), found earlier with $n = 1$ and presented in Fig. 3a–c. When $n = 4$ and 5 the minimum-energy configuration possesses the n molecules interacting with the oxalactone functionality and the diol group of the lateral chain respectively. The other two configurations contribute 5% and possess the n water molecules interacting with the different polar sites of the molecule. In these cases ($n = 4$ and 5) the entropy is very high since the possibilities of different arrangements of water molecules around the solute increases, see Table 1. Even in the case where $n = 5$ all the water molecules interact with the solute as well as among them (see Supplementary material).

The interaction of **Cs** with water shows a similar behaviour to that of **Br**. This is an expected result since these molecules are very similar. In this case the water molecules interact preferentially with both diol systems (ring A, and lateral chain) as oxalactone function has changed by a carbonyl group (see Fig. 6). In spite of the differences found when $n = 1$ the clusters with $n = 3$, 4 and 5 exhibit the interaction C═O···H_2O with an important contribution to the partition function. When the system possesses more than two water molecules the diol groups are not available because they are already interacting with water. Then when $n = 3$, 4 and 5 the solvent has two possibilities, either interacts first with the oxalactone group (available site) or associates directly with the water already present. Once again it is demonstrated that the preferable sites of association are the diol groups presented in these structures. The fact that diol groups are very important could also be related with the different conformations that may adopt the lateral chain and the availability that confers to this particular group (diol).

In **Tp** the lack of OH in C_2 facilitates the association of the OH in C_3 and carbonyl group (ring B) through hydrogen bonds formed by

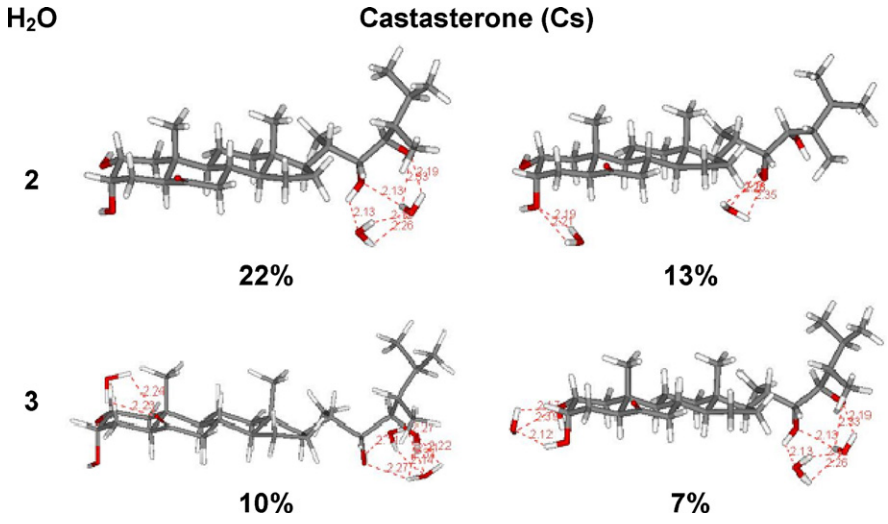

Fig. 6. Preferential positions of interactions between Castasterone (**Cs**) and 2 or 3 water molecules and relative populations.

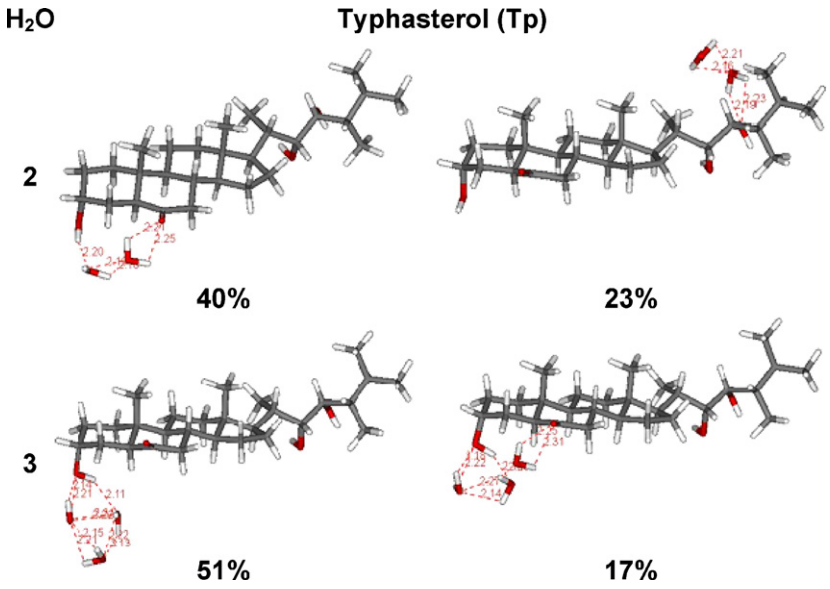

Fig. 7. Preferential positions of interactions between Typhasterol (**Tp**) and 2 or 3 water molecules and relative populations.

the molecules of water. This trend is only observed in the case of **Tp**. This behaviour is found for $n = 2, 3$ (see Fig. 7) and in the case of $n = 5$.

When $n = 2$ and 4 the water molecules do not interact with the entire diol group of the lateral chain, but specifically with the –OH in C_{23}. These clusters where the interaction is through –OH in C_{23} possess the greatest contribution to the partition function. Nevertheless, the MMH method has well explored all the possible configurations since it has also generated clusters with water molecules interacting with –OH in C_{22} but with a low contribution to the partition function.

3.6. Supporting material

- Superposition of the most important clusters of Brassinolide, Castasterone and Typhasterol are shown in Figs. 1S, 2S and 3S.
- Superposition of the CP-corrected structures of Brasinolide, Castasterone and Typhasterol with the clusters optimized by the AM1 semiempirical method can be found in Figs. 4S, 5S and 6S.
- Clusters with $n = 4$ and 5 of BRs studied can be found in Figs. 7S, 8S and 9S.

4. Conclusions

A theoretical model for understanding the interaction between brassinosteroids and water molecules has been built. These are the main conclusions of this study:

- The MMH method allows us to explore the hydrophilic interaction sites of these molecules using the AM1 semiempirical Hamiltonian.
- All analyzed theoretical complexes of association between water and BRs are thermodynamically stable, and thermodynamic properties of Brassinolide and Castasterone are very similar, due to the structure similarity.
- The positive values of association entropies are related to several stable complexes obtained and also to the presence of different sites of association of these compounds for water.
- Calculations performed show that the association of brassinosteroids with water preferentially occurs by the hydroxyl groups of these molecules. These sites are (diol ring A and lateral chain) the most active positions of brassinosteroids for hydrophilic interactions.
- The interaction energies of the complexes brassinosteroids-water calculated at the DFT/B3LYP/6-31G and 6-31+G* levels follow the same behaviour as the biological activities of the three molecules studied.
- The magnitude of BSSE of the complexes is large but it does not change significantly the geometries.
- PCM calculations at the B3LYP/6-31G and 6-31+G* levels correctly describe the order of association for water of the brassinosteroids studied.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2008.09.013.

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