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ORIGINAL PAPER

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# Determination of preferred conformations of brassinosteroids by means of NMR investigations and Boltzmann statistical analysis of simulated annealing calculations

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**Abstract** Brassinosteroids are a class of steroidal phytohormones with high growth-promoting properties. The preferred side-chain conformations of 10 brassinosteroids were determined by means of detailed NMR investigations and molecular-modeling studies. Vacuum conformations obtained by simulated annealing calculations and Boltzmann statistical analysis were compared with solution conformations derived from NOE experiments and molecular dynamic simulations, and with X-ray structures. In general, results from simulated annealing calculations and NMR-supported molecular dynamics simulations are in good agreement. For some of the compounds investigated the conformation was less well-defined at the end of the side-chain. It could be shown that the energetically most favorable and most probable conformations also include the conformations obtained by NMR supported molecular-dynamics calculations and by X-ray analysis. For the most bioactive compound brassinolide (**1**) the majority of conformations show a side-chain bent towards the β-face of the steroid skeleton, whereas for the less bioactive brassinosteroids, conformations with straight side-chains or side-chains bent towards the α-face are more frequent.

**Keywords** Simulated annealing · Brassinosteroids · Conformation · NMR

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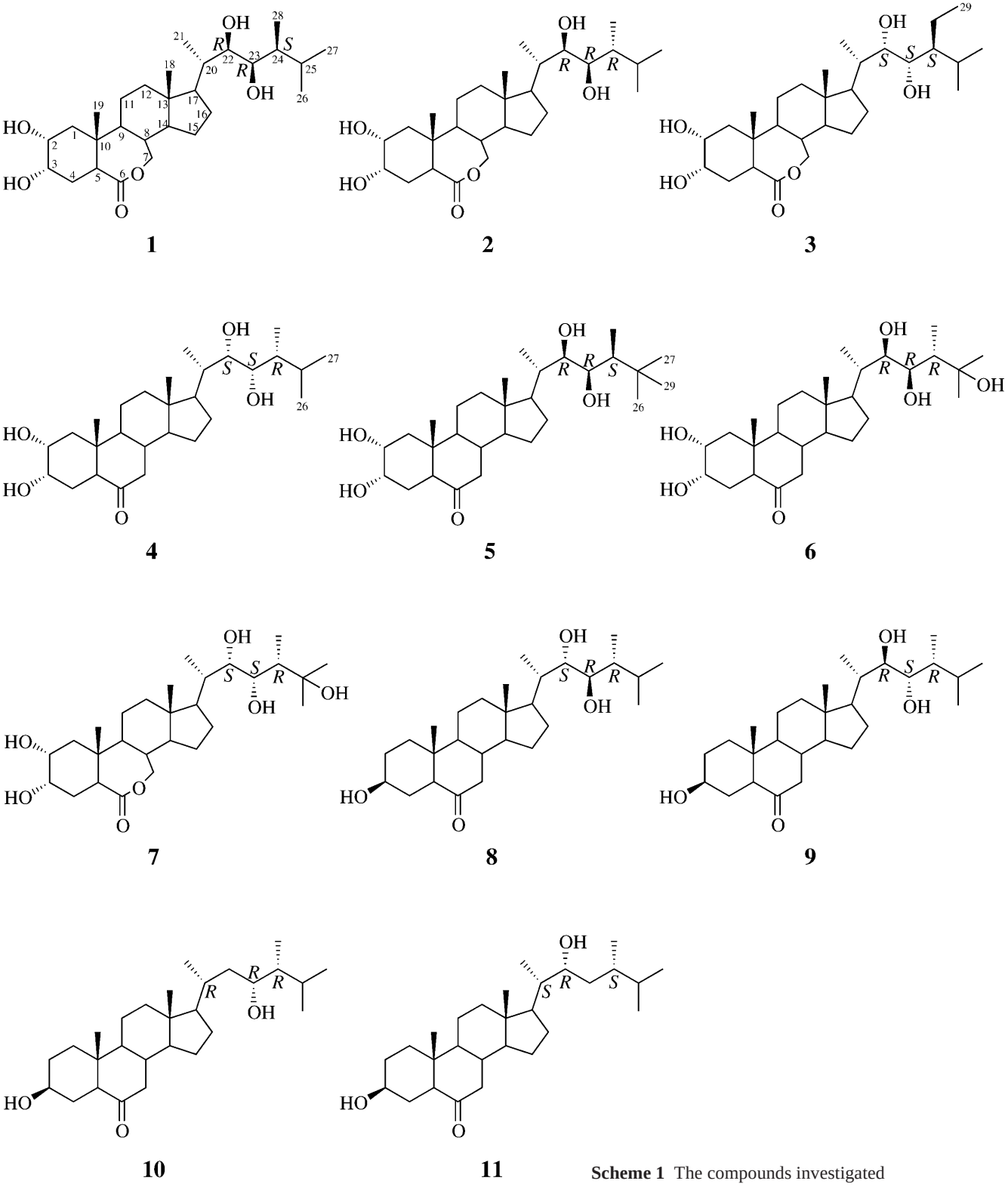
## Introduction

Brassinosteroids are a class of steroidal phytohormones with high growth-promoting activity [1]. At present, more than 40 native brassinosteroids are known and many have been synthesized [2]. Structural variations lie in the substitution of rings A/B, different alkylation at C-24, various substitution at C-25 or different hydroxylation in the side-chain of the steroids (see Scheme 1). The stereochemistry at the asymmetric centers in the side-chain is also significant for the bioactivities of these plant hormones.

The conformations of the side-chains of 10 native and synthetic compounds were investigated by means of quantitative 2D NOE measurements and molecular-modeling studies. We have recently shown distinct differences in the solution side-chain conformation for the two most important native brassinosteroids brassinolide (**1**) and 24-epibrassinolide (**2**), and for two new synthetic analogues (22*S*,24*R*)-teasterone (**8**) and (23*S*,24*R*)-teasterone (**9**) [3, 4]. In continuation of these studies we now compare the side-chain conformations of 10 brassinosteroids obtained by means of simulated annealing calculations with the solution side-chain conformations and, if available, with X-ray structures.

Brosa et al. used an approach for structure–activity investigations of brassinosteroids considering electrostatic potentials and the ability to form hydrogen-bonds [5]. This approach is based on a postulated active conformation of brassinolide [6]. The authors established this active conformation by molecular dynamics simulations and a semi-systematic conformational search, as well as bioactivities of brassinosteroids in a modified rice-lamina inclination test [7]. Although it is usually assumed there is higher flexibility towards the end of a side-chain, Brosa et al. found a constant torsional angle (C23–C24–C25–H25) of 120° during the entire dynamic simulation [8]; this is hard to explain.

Because published results of bioassays of brassinosteroids are rather different and non-uniform [6, 9, 10] it has been difficult to analyze quantitative structure–activ-




**Scheme 1** The compounds investigated

ity relationships (QSAR). Despite these problems, we performed the present conformational studies as a first approach towards drawing a relationship between side-chain conformations and the bioactivities of brassinosteroids.

The conformational search was conducted by means of simulated annealing calculations and subsequent Boltzmann statistical analysis. We used these methods without distance restraints to compare these results to conformations obtained by NMR-supported molecular dynamics

simulations and to gain more information about compounds with less well-defined conformations at the end of the side-chain. The results of the two independent calculations were analyzed and compared with each other.

## Methods

*NMR experiments.* All $^1$H and 2D NMR spectra were recorded at 295 K on a Varian UNITY500 spectrometer operating at 499.84 MHz for $^1$H using a NALORAC 3 mm microsample inverse detection probe; $^{13}$C {$^1$H} and APT NMR spectra were recorded on a Varian GEMINI300 at 75.50 MHz or a Varian UNITY500 at 125.70 MHz. For 1D and 2D NMR experiments, solutions of the compounds in $CDCl_3$ were used; occasionally several drops of $CD_3OD$ were added for better solubility. Chemical shifts were referenced to internal TMS ($\delta$=0, $^1$H) and $CDCl_3$ ($\delta$=77.0, $^{13}$C). Samples for NOE measurements were carefully degassed by ultrasonic treatment or, alternatively, subjected to four freeze–thaw cycles and then flame-sealed under an argon atmosphere.

All experiments were performed by use of standard pulse sequences given by the manufacturers. DQFCOSY, NOESY, and gradient-supported HSQC spectra were recorded and processed in the phase-sensitive mode with quadrature detection in both dimensions; gradient-supported HMBC spectra were processed in absolute value mode.

To relate NOESY cross-peak intensities to inter-nuclear distances of the dipolar coupled proton pairs, NOESY spectra with a 500-ms mixing time were processed for compounds **1**–**4** and **8**–**9** with the NMR TRIAD program (SYBYL 6.5 software package, TRIPOS, St Louis, MO, USA). The complete relaxation matrix analysis program, MARDIGRAS [11, 12, 13] as implemented in SYBYL 6.5 was used. For more details see Ref. [3].

For compounds **5**, **7**, **10**, and **11**, classification of the NOESY cross peak intensities as strong (distance range 1.8–3.0 Å), medium (1.8–4.0 Å), and weak (1.8–5.0 Å) were used to obtain interproton distances.

## Molecular modeling

### Determination of solution conformations

All molecular-mechanics calculations were performed on Silicon Graphics workstations using the TRIPOS force field [14] of the molecular software package SYBYL 6.5.

High-temperature restrained molecular dynamics simulations were utilized for conformational searches. The first derived set of NMR upper and lower distances were added to the force field as pseudo-quadratic potentials with a force constant of 25 kcal mol$^{-1}$ Å$^{-2}$. The simulation temperature and length were 1000 K and 100 ps, respectively; an NTV ensemble, an integration time step of 0.5 fs, and a Boltzmann distribution of starting velocities were used. A non-bonded cut-off of 8 Å for van der Waals interactions was applied and non-bonded lists were updated every 5 fs. No electrostatic interactions were considered at this point. For all compounds, 100 conformations were extracted from the trajectory and energy minimized by use of the Powell method [15] until a gradient of $10^{-3}$ kcal mol$^{-1}$ Å$^{-1}$ was achieved.

For further refinement, the brassinosteroids were surrounded with ~670 chloroform solvent molecules (precomputed solvent box, 44.5 Å length in each dimension) and restrained-energy minimization with the full set of distance–range constraints (force constant 75 kcal mol$^{-1}$ Å$^{-2}$) was performed for 5000 Powell steps. Partial charge contributions were calculated by use of the method of to Gasteiger and coworkers [16, 17] and electrostatic interactions were taken into account by using a constant dielectric function with $\varepsilon$ = 1. Periodic boundary conditions were applied. Subsequently, the molecular ensembles were subjected to restraint molecular-dynamics simulations at 300 K for 100 ps. The force field set-up and molecular dynamics parameters were identical with those of the energy minimization and the high-temperature simulations, respectively. From the trajectories 5, relating to different values of side-chain dihedral angles, conformations were selected manually. The whole molecular ensembles, including both the steroid and all solvent molecules, were energy minimized to a gradient of less than $10^{-3}$ kcal mol$^{-1}$ Å$^{-1}$.

To check whether the conformations obtained are stable without NMR-derived distance constraints imposed by the TRIPOS force field, additional molecular dynamic simulations and energy minimization of the conformations without distance restraints were performed.

### Simulated annealing calculations

The simulated annealing calculations were conduced under vacuum, without restraints, over 500 cycles with heating to 1000 K over 1500 fs and subsequent annealing to 0 K, also over 1500 fs, with an exponential function. The 500 conformations obtained at 0 K were minimized. The minimization set-up was identical with that of the energy minimization after the molecular dynamics simulation. The steroid skeleton was defined as aggregate.

This procedure was repeated with different initial conformations, on the one hand with the energetically most favorable conformation from run1 (run2) and on the other hand with the energetically most unfavorable conformation from run1 (run3). For all 1500 conformations, the torsional angles along the carbon skeleton of the side-chain were calculated (H17–C17–C20–C22, C17–C20–C22–C23, C20–C22–C23–C24, C22–C23–C24–C25, C23–C24–C25–H25). The conformations were clustered in families in respect of these five torsional angles allowing a range of 30°. For 24-epibrassinolide (**2**) and 25-methylcastasterone (**5**), a range of 10° had to be used to obtain consistent families. To analyze the families not only with regard to the frequency of occurrence (how many structures are included in one family) but also with regard to the energy of the conformations, a Boltzmann distribution was applied.

The following equation was used for the calculation of the probability (over a Boltzmann distribution):

$$N_f = \frac{\sum_{i}^{n} e^{-\frac{E_i}{kT}}}{\sum_{i}^{1500} e^{-\frac{E_i}{kT}}} \quad (1)$$

where i is the number of conformations, n is the number of conformations in one family, f is the family, and $E_i$ is the energy of conformation i from simulated annealing calculations.

For comparison of the different conformations and calculation of the root mean square (rms) the carbon and the oxygen atoms of the side-chains (C-17, C-20 to C-28/C-29 and O-22, O-23, O-25) were used.

## Results and discussion

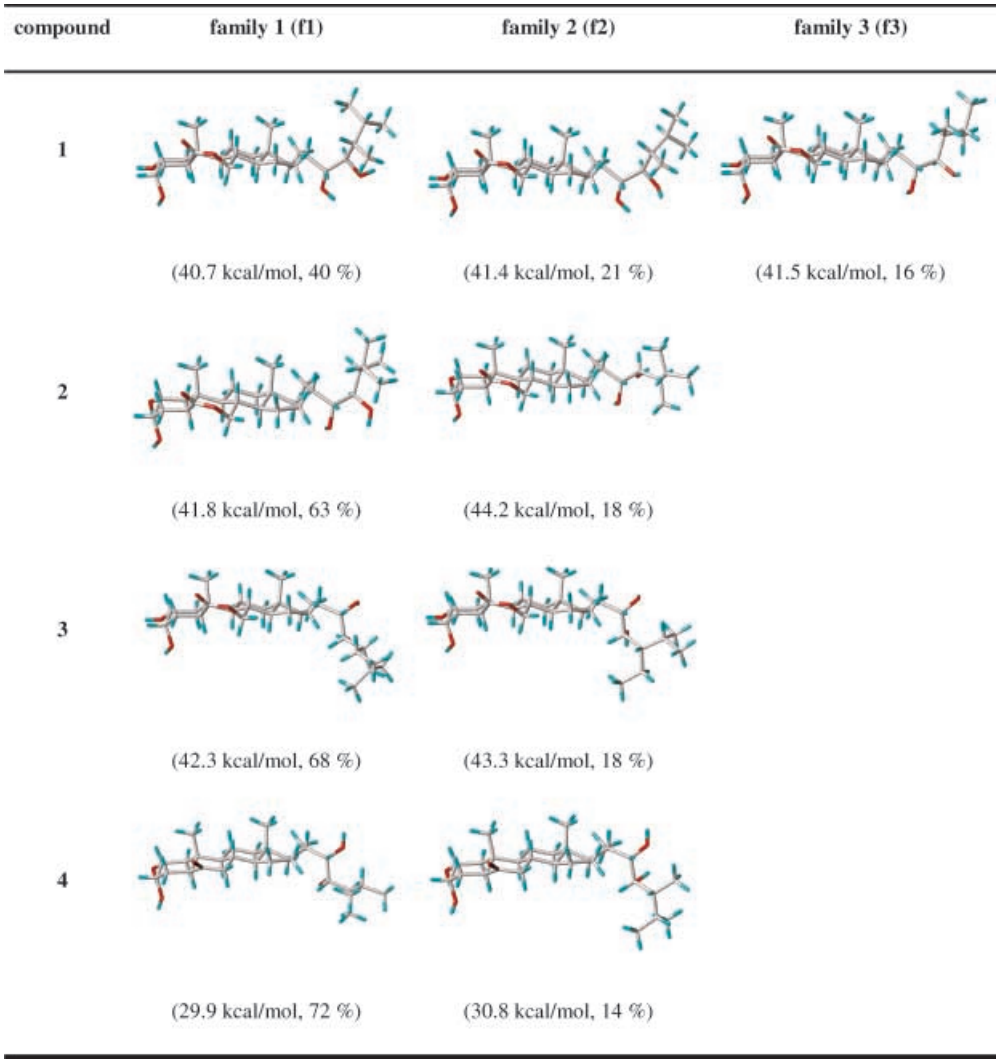
The $^{1}H$ and $^{13}C$ chemical shifts of **1**–**3** and **7**–**9** have already been published [3, 4, 18, 19]. For **4**, **5**, **10**, and **11** unambiguous assignment of NMR signals was achieved by combined use of one- and two-dimensional NMR experiments including acquisition of the gradient-supported COSY, HSQC, and HMBC spectra. NOE measurements were performed as 2D NOESY and as NOE difference experiments. 24-Epi-25-methylcastasterone (**6**) was, unfortunately, not available; for comparison with 25-methylcastasterone (**5**), however, simulated annealing calculations were also performed for **6**.

Solution conformations were obtained by molecular-modeling calculation with distance restraints derived from NOE measurements as described in detail for compounds **1**, **2**, **8** and **9** in previous publications [3, 4].

Significantly different NOE contacts were observed for the two geminal methyl groups at the ends of the side-chains of **1**–**4** and **8**–**9**; this enabled unequivocal assignment of Me-26 (pro-*R*) and Me-27 (pro-*S*). For **7**, **10**, and **11** this was not possible, because of scarce and/or similar NOE contacts of the diastereotopic methyl groups. Thus, for these brassinosteroids the solution side-chain conformation could not be well-defined, especially at the end of the side-chain.

Simulated annealing calculations were performed to obtain more information about the side-chain conformation of brassinosteroids, which is essential for their bioactivity. For all compounds three simulated annealing

**Scheme 2** The three most probable conformation families with their energies and probabilities, in %, for **1**–**4**



runs were done with different start conformations. Because these three runs yielded similar results, in each case the following discussion is confined to the results of one run. All discussed energies are calculated without any restraints and aggregates.

The conformations obtained were combined into families with pronounced similarity in terms of their torsional angles. The calculation of the Boltzmann distribution of the conformational families is described in detail in the Methods section. The conformational family with the greatest number of included conformations is not necessarily the most probable family, because the energy of the conformations must also be considered.

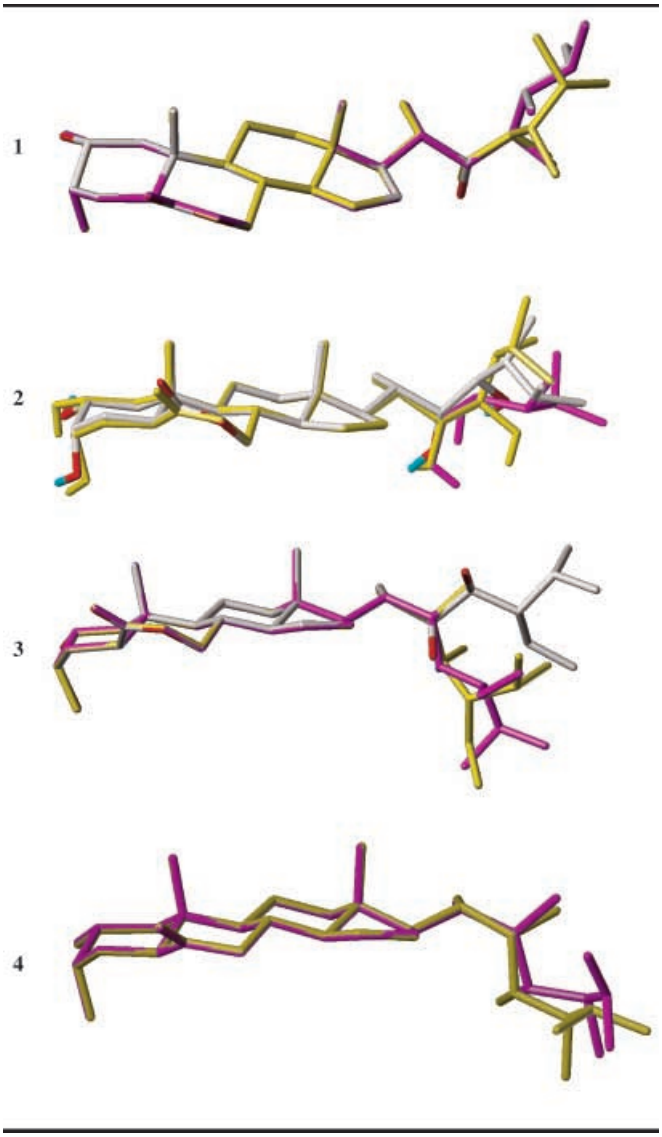
In Scheme 2 the conformations of these families with a probability of more than 10% are displayed for compounds **1**–**4**.

For brassinolide (**1**), three families were found with a probability of more than 10% (Scheme 2). The most probable family f1 (40%) is not the family with the highest number of conformations, but has the lowest energy and an rms value of 0.9 Å when compared with the NMR-supported solution conformation and the X-ray conformation [20]. Although the side-chain is always turned to the steroidal β-face (the angular methyl groups Me-18 and Me-19 in the steroidal skeleton are defined as β-oriented), greater conformational flexibility was found towards the end of the side-chain. The conformation of the family f3 has the best correlation with the solution conformation (rms=0.06 Å) and also to the X-ray structure (rms=0.06 Å). The solution conformation and the X-ray structure are almost identical (Scheme 3).

To combine similar conformations of 24-epibrassinolide (**2**) in conformational families a deviation of the torsional angles of 10° was used as a classification parameter to obtain consistent families. Two families were found with a probability of more than 10% (Scheme 2). The side-chain conformations of these families differ in the terminal regions. They vary from a stretched conformation to a β-oriented conformation. Similar behaviour was found for the solution conformation [3]. A conformation of the most probable family f1 (63%) has an rms value of 0.9 Å when compared with the solution conformation and of 1.6 Å when compared with the X-ray structure [20] (Scheme 3). The family with the best correlation to the solution conformation (rms=0.09 Å) has a probability of only 2.5%. Relatively few conformations are included in this family but it has a low energy of 43.8 kcal $mol^{-1}$. The family with the best correlation to the X-ray structure (rms=0.2 Å) has a very low probability of 0.0001%. The conformational family f1 (highest probability) has a side-chain conformation similar to brassinolide (**1**), whereas the other highly probable families have a more stretched side-chain.

Two conformational families with a probability of more than 10% were also found for 22,23-diepi-28-homobrassinolide (**3**) (Scheme 2). The conformation of the most probable family f1 (68%) has a rms value of 1.6 Å when compared both with the solution conformation, and with the X-ray structure [21] (Scheme 3). The family with the best correlation to the solution conformation (rms=0.2 Å), with a probability of 8%, differs from family f1 only in the position of the ethyl and the isopropyl group of the side-chain. The family with the best correlation to the X-ray structure (rms=0.5 Å) has a low probability of only 0.07%. The side-chain of the X-ray structure is stretched in comparison with the side-chains of the solution conformations and with the most probable simulated annealing conformation, which are more α-oriented.

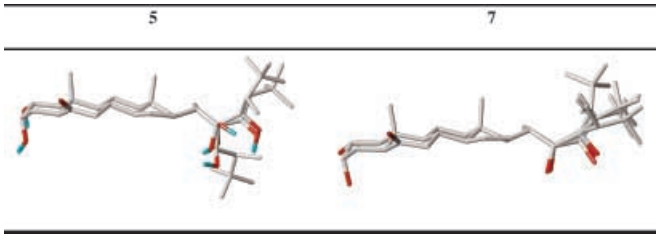
For 22,23,24-trisepicastasterone (**4**) no well-defined defined solution conformation was found. None of the conformations obtained met all the observed NOE contacts. Thus, **4** must exist in at least two side-chain conformations, which have to be in rapid exchange on the




**Scheme 3** Comparison of the most probable simulated annealing conformation (*yellow*) with the solution conformation (*magenta*) and the X-ray structure (*varicoloured*) of **1**–**4** (for clarity protons are not shown; the X-ray structure of **4** was not available)

chemical shift time scale because no separate $^{1}H$ and $^{13}C$ signals were observed. Nevertheless, the solution conformation that was in agreement with the most NOE contacts was used for comparison with simulated annealing results. The conformation of the most probable conformational family f1 from simulated annealing calculations (72%) has a rms value of 0.7 Å when compared to the solution conformation (Scheme 3). The simulated annealing family (0.6%) sharing best correlation with this solution conformation has a rms value of 0.5 Å. All conformations from simulated annealing calculations have a side-chain bent towards the steroidal α-face (Scheme 3). They differ only in the torsional angle at the end of the side-chain. An X-ray structure of **4** was not available.

No X-ray quality crystals could be obtained for 25-methylcastasterone (**5**), and thus no X-ray structure was available. However, no well-defined solution conformation was found for this compound because of insufficient useful NOE contacts. The NMR signals of the three methyl groups attached to C-25 are isochronous, the NOE's of these groups cannot be assigned and hence cannot be used as restraints. For compound **5** a high conformational flexibility in the molecular dynamic simulation even at 300 K was found (Scheme 4).



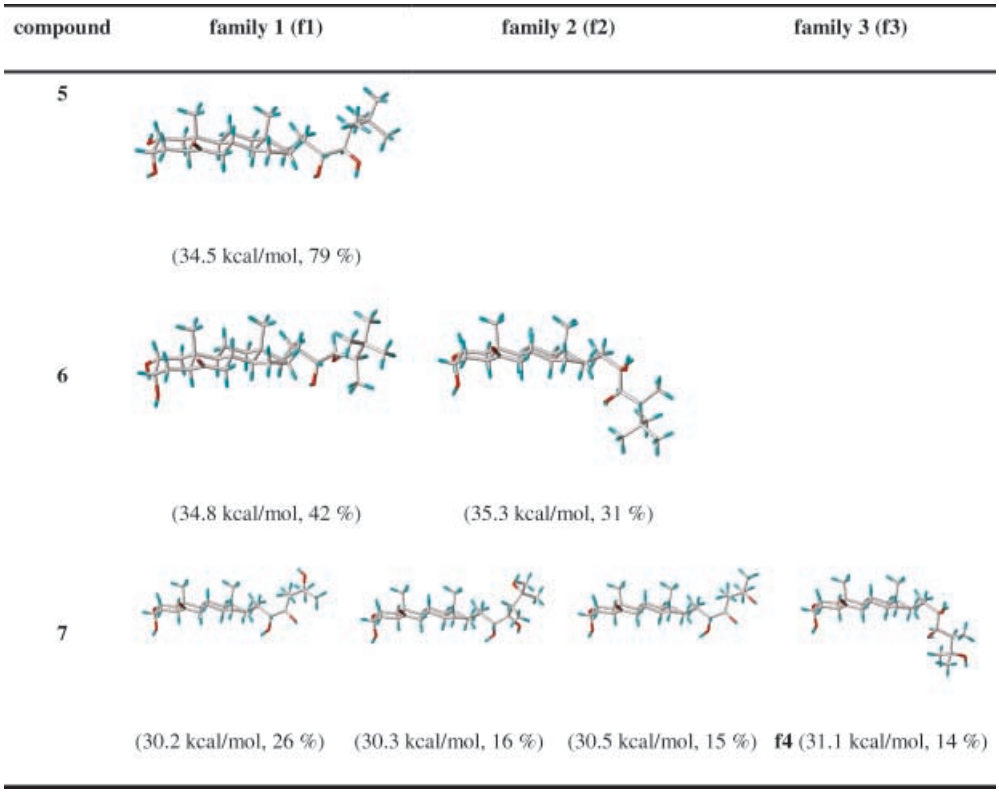

**Scheme 4** Possible solution conformations of compounds **5** and **7** (for clarity protons are not shown)

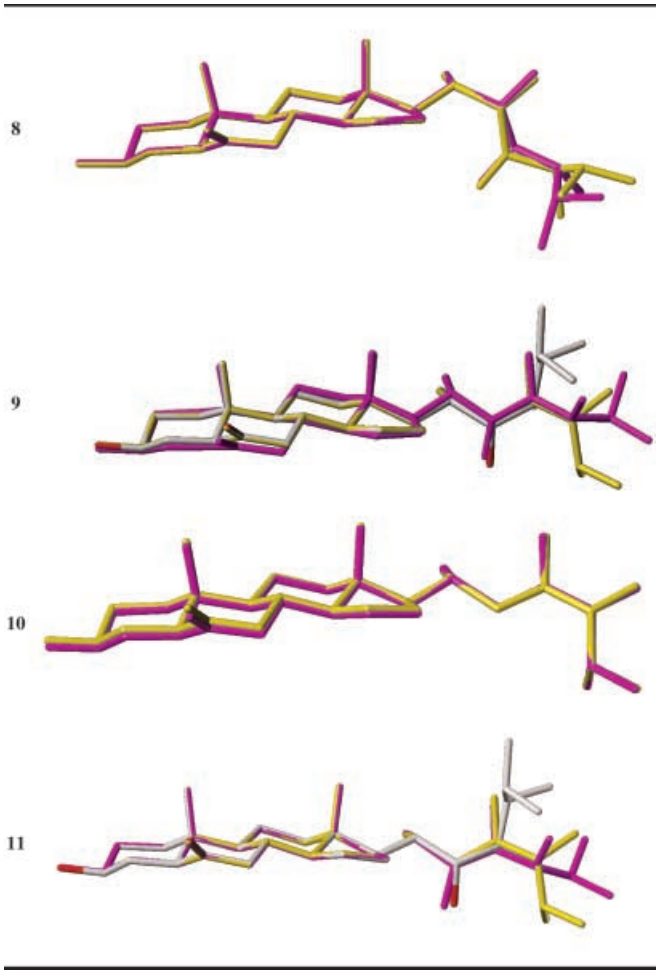
Simulated annealing calculations for **5** resulted in one family with a high probability (f1 79%). The side-chain is β-oriented as in brassinolide (**1**) (Scheme 5). Two other families were obtained with probabilities of 7% and 6% with a α- and another β-oriented side-chain conformation, respectively. In the course of the determination of the solution conformation, higher flexibility was found at the end of the side-chain than for the brassinosteroids that had only two methyl groups attached to C-25.

24-Epi-25-methylcastasterone (**6**) was not available for NMR investigations. However, simulated annealing calculations were carried out for this brassinosteroid. Two families having similar probabilities were found (Scheme 5). In one of these families (f1) the side-chain is extended, whereas it is α-oriented in another (f2). The behavior of the side-chain conformations of **5** and **6** is similar to that of brassinolide (**1**) and 24-epi-brassinolide (**2**) except that in the latter case an additional probable conformation with a β-oriented side-chain was found.

For 25-hydroxy-castasterone (**7**), four simulated annealing families were obtained with nearly the same probability and energy (Scheme 5). This finding suggests

**Scheme 5** The three most probable conformation families, with their energies and probabilities, in %, for **5**–**7**






**Scheme 6** Comparison of the most probable simulated annealing conformation (*yellow*) with one solution conformation (*magenta*) and the X-ray structure (varicoloured) of **8**–**11** (for clarity protons are not shown)

that different side-chain conformations might also occur in solution. The solution conformation is poorly defined, because of a lack of suitable NOE information, for the same reasons as described above for **5**. However, the possible solution conformations of **7** have more or less β-oriented side-chains (Scheme 4).

For 22,24-diepiteasterone (**8**) the most probable family shows close correlation to the solution conformation (rms=0.6 Å) (Scheme 6). In contrast to this, distinct differences were found for the side-chain conformations of 23,24-diepiteasterone (**9**) obtained. There are five families with a probability of more than 10% showing stretched and β-oriented side-chains (Scheme 7). The family with the best correlation to the solution conformation (rms=0.7 Å) has a probability of 10% and has a more stretched side-chain. The family with the best correlation to the X-ray structure [4] (rms=1.1 Å) has a probability of only 0.01% with a β-oriented side-chain conformation (for **8** no X-ray structure was available).

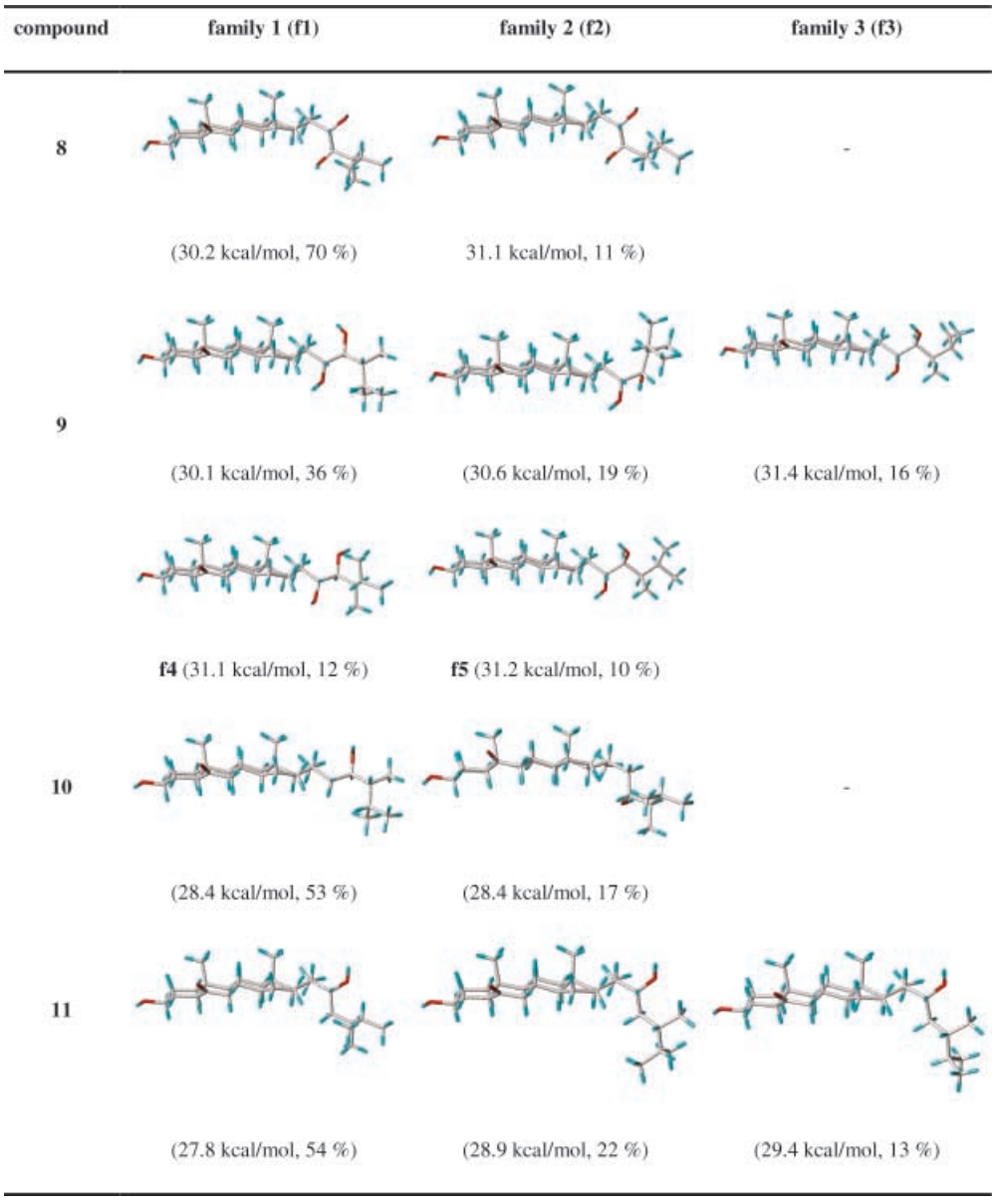
For 22-desoxy-23,24-diepiteasterone (**10**) and 23-desoxy-22,24-diepiteasterone (**11**) the determination of a single well-defined solution conformation suffered from an insufficient amount of NOE correlations and from signal overlapping. A pro-*R*/pro-*S* assignment was possible for neither the geminal methyl groups Me-26 and Me-27 nor for the geminal protons attached to C-22 (**10**) and C-23 (**11**). However, all solution conformations obtained resembled the side-chain conformation up to C-24. For comparison with simulated annealing results shown in Scheme 6, an arbitrary selected solution conformation was used. One of the possible solution conformations of **10** shows a close correlation with a conformation of the most probable family (rms=0.05 Å). X-ray data were available only for **11**. The X-ray structure of **11** [22] resembles that of a conformation of the most probable family obtained by simulated annealing and Boltzmann distribution determination (rms=0.0004 Å) (Scheme 6).

In general, results from simulated annealing calculations and NMR-supported molecular dynamics simulations are in good agreement. For all the compounds investigated the spatial position of the methyl group Me-21 is constant with regard to the steroid skeleton, irrespective of the medium. All conformations from simulated annealing calculations lie in an energy range of 2–3 kcal $mol^{-1}$ with respect to the global minimum. For compounds **2**–**5** and **8** only one family with a high probability of 60–80% was found, in contrast with compounds **6** and **7** for which two and four families with almost equal probability were obtained. For all other compounds investigated (**1**, **9**–**11**), several families were found with different probabilities, all greater than 10%.

Higher flexibility was found towards the end of the side-chain for all brassinosteroids investigated. This effect is most pronounced for those compounds with 25-methyl or 25-hydroxy substitution (**5**, **6**, **7**), which can be explained by the similar steric demands of all three substituents.

In brassinolide (**1**), the most bioactive brassinosteroid, most conformations have a side-chain bent towards the β-face of the steroid skeleton. This behavior of the brassinolide conformation is independent of the medium [20]. In all other (less active) brassinosteroids the side-chains prefer a stretched or α-faced conformation, with relatively few of the low-energy conformations adopting a brassinolide-like β-oriented position. Because the bioactive conformation should be included in the populations of the low-energy conformations, it seems likely that brassinolide in its bioactive conformation has a β-faced side-chain. The bioactivity of the other brassinosteroids could therefore be proposed to depend on the ability to adopt such a β-oriented side-chain. More detailed analyses of structure activity relationships based on the data evaluated in this paper will be feasible once a receptor for brassinosteroids has been discovered and more reliable bioactivity data are available.

**Scheme 7** The three most probable conformation families with their energies and probabilities, in %, for compounds **8–11**

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**Supplementary material** $^{1}H$ and $^{13}C$ NMR data of compounds **4**, **5**, **10** and **11** are shown in Tables 1–4, the corresponding data of **1–3** and **7–9** can be found in references [3], [4], [18] and [19].

The NOE correlations obtained by 2D NOESY and 1D NOE difference experiments of compounds **3–5**, **7**, **10** and **11** are listed in Tables 5–10. For **1**, **2**, **8** and **9** these data have already been published [3, 4].

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