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Brassinosteroids: A New Way to Define the Structural Requirements

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Dedicated in memoriam of Prof. Felix Serratosa Petit.

Abstract: The synthesis of four new brassinosteroid analogs is reported. Two of them elicited high activity as plant growth promoters. Also a new way to define the structural requirements, that can explain the relative high activity of these compounds, is presented.

Brassinosteroids represent a new class of plant growth regulators that are widely distributed in the plant kingdom. Since the discovery of brassinolide (**1**) many efforts have been made in different areas such as analytical studies, chemistry, biochemistry, physiology and molecular action in order to broaden the knowledge of this kind of phytohormone for further application in agriculture.[1] Several bioassays have been developed to evaluate the activity of natural and synthetic brassinosteroids and to study their interaction with other hormones and related growth substances.[2] Although different qualitative structure-activity relationships have been established,[3,4] two of the structural requirements pointed out (2α,3α-diol and A/B *trans* ring junction) are unconvincing because of the lack of data. No brassinosteroid with A/B *cis* junction nor with 2β,3β-diol were examined.

An approach by molecular modelling has been recently reported.[5] However a more complete study should be carried out in order to know the brassinosteroid conformation which interacts with the receptor. This will help us to search for a quantitative structure-activity relationship (QSAR) to predict the activity of new analogs and to design the brassinosteroid with the best synthetic cost-activity ratio for further application in agriculture. Also, this will help to increase the knowledge about what the binding site of brassinosteroids receptors could be like.

With these points in mind we report the synthesis of new brassinosteroid analogs with a 2β,3β-diol and a A/B *cis* and *trans* ring junction. We also report their activity as well as the results obtained on our molecular modelling studies which allowed us to establish a preliminary QSAR correlation. Moreover we propose a new way to define the structural requirement for active brassinosteroids.

Results and Discussion

A typical scheme was followed to get a QSAR which is summarized in Figure 1. Thus, a broad number of brassinosteroid analogs were selected for which strictly homogeneous activity data was required. A systematic molecular modelling study was developed to find the conformation of each brassinosteroid which should interact with the receptor binding site named "*active conformation*". With this conformation, several parameters were calculated and correlated with the activity to find a QSAR.

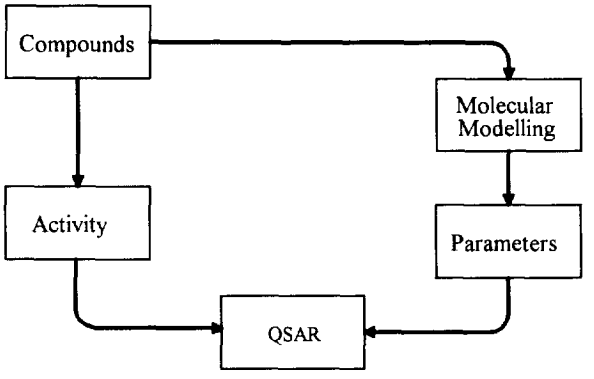


Figure 1: QSAR scheme

Compounds.

The compounds used in this study are shown in Figure 2. All of them, except brassinolide (**1**), have either been synthesized from our previously reported syntheses,[6-8] are described here (**8, 9, 15, 17**) or will be reported elsewhere (**18-23**). They possess different alkyl substituents at C-24 (R-methyl, S-methyl or S-ethyl), different hydroxyl configuration at C-22 and C-23 (RR or SS), as well as at C-2 and C-3 (α,α or β,β), and have different A/B ring junction (*trans* or *cis*). They also present several functionalities in the B ring such as: lactone, ketone, hydroxyketone and ether. The number and structural modifications of brassinosteroid analogs involved in this study is considered to be large enough to establish a QSAR.

The compounds **8, 9, 15, 17** are obtained following the procedure outlined in Scheme 1. The diene **28** was obtained starting from stigmasterol (**24**) with a global yield of 53% following the strategy previously described.[6] Thus, the mesylation of stigmasterol (**24**) followed by solvolysis and oxidation with Jones' reagent gave the cyclopropylketone **27**, which was isomerized to **28** by treatment with pyridine hydrobromide and lithium bromide. The diols **29** and **30** were obtained by Woodward-Prevost method[9] followed by isomerization in basic media and chromatographic separation.

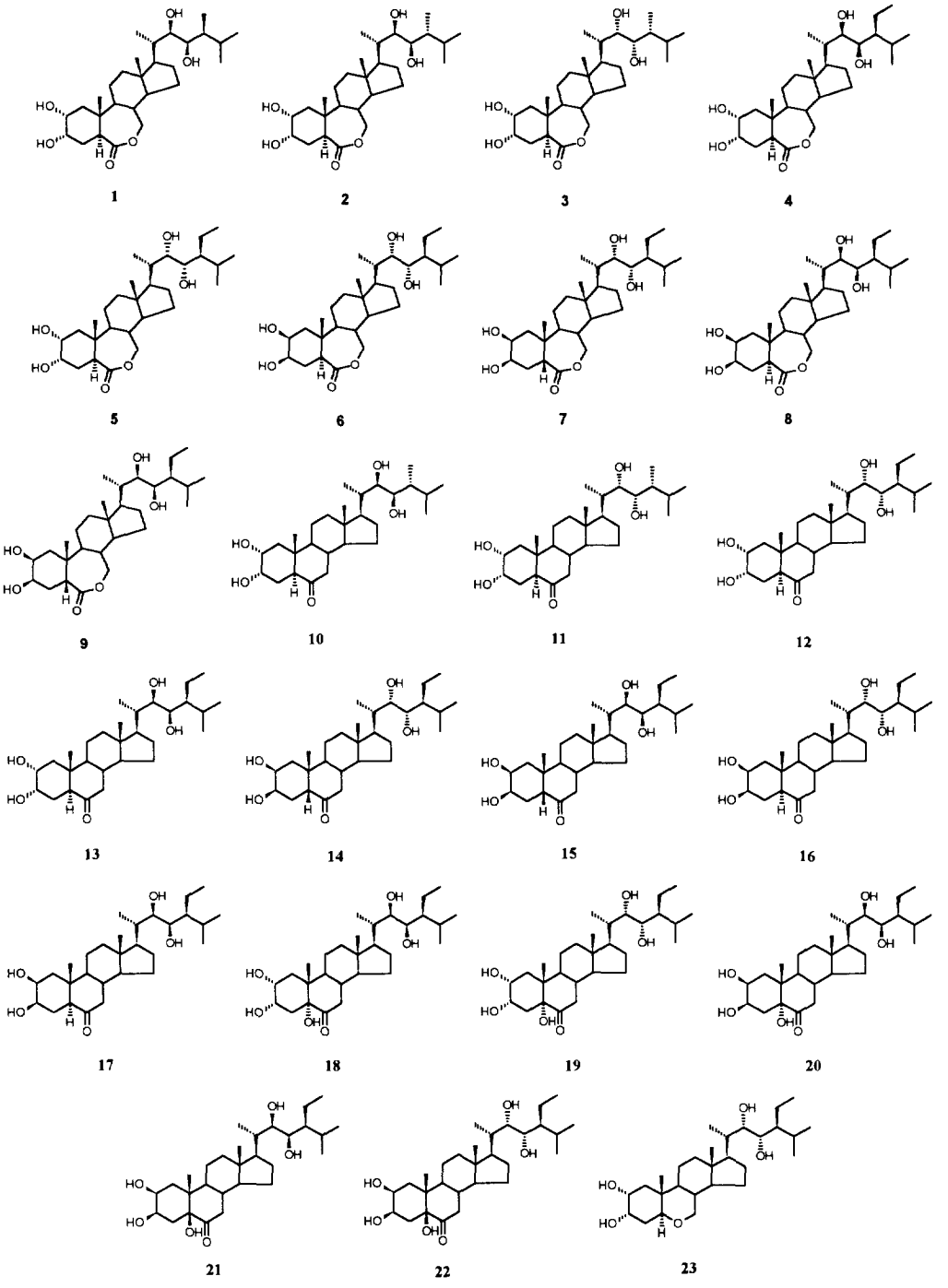
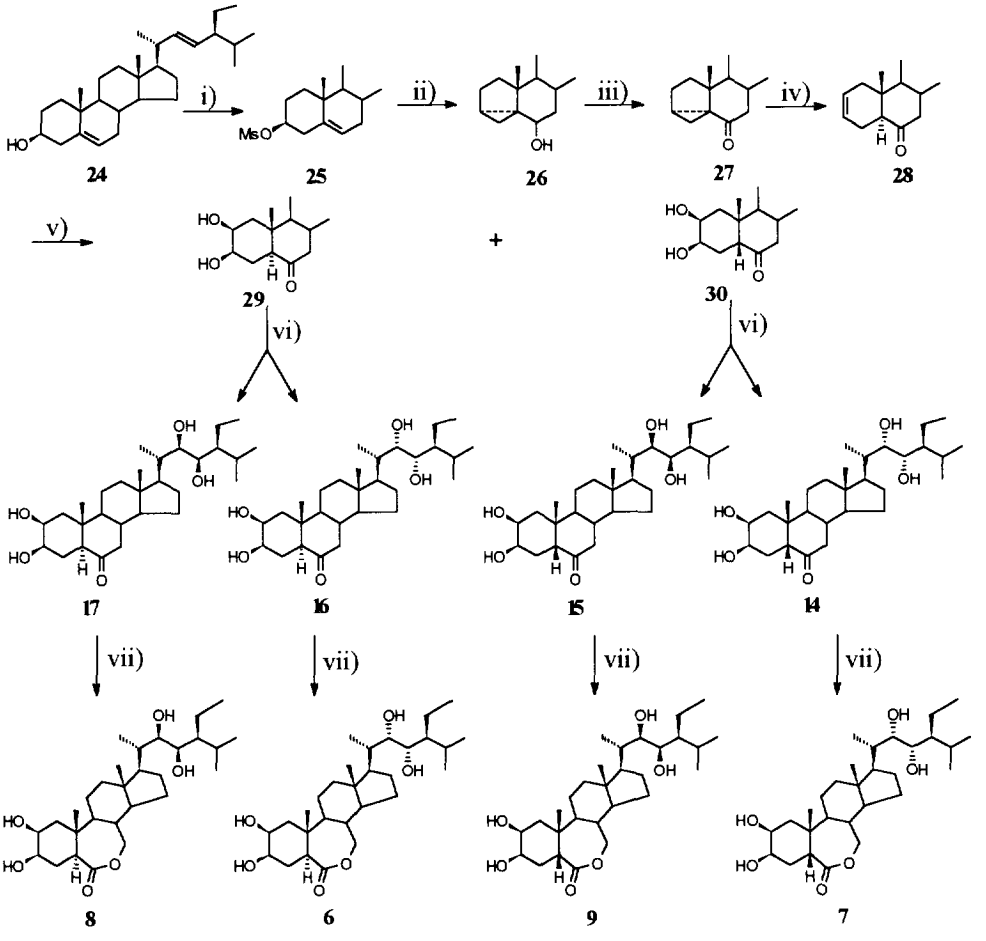


Figure 2: Brassinosteroid analogs

Glycolization of the side chain double bond of **29** by osmium-catalyzed asymmetric dihydroxylation using dihydroquinidine 9-*O*-(9'-phenanthryl) ether (DHQD PHN) as chiral agent, following the method previously described,[7] afforded a mixture of isomers **17** and **16** with a yield of 63 % and (22R,23R):(22S,23S) ratio of 1.8 : 1. Using the same procedure, glycolization of **30** gave a mixture of **15** and **14** with a yield of 56 % and (22R,23R):(22S,23S) ratio 1.5 : 1.

Baeyer-Villiger oxidation of each ketone **17**, **16**, **15** and **14** lead to the lactones **8**, **6**, **9** and **7** respectively, with a yield of 33%, 66%, 23% and 63% respectively.



Reagents: i) $MsCl/Et_3N/0°C$; ii) $MeCOMe/KHCO_3/H_2O$; iii) Jones Reagent; iv) Py.HBr/DMF; v) v.a) $I_2/AcOAg/H_2O$, v.b) $KOH/MeOH$; vi) OsO_4/THF/DHQD PHN/NMO/$Et_4N^+ACO^-$; vii) CF_3COOOH.

Scheme 1: Synthesis of **17**, **15**, **9** and **8**.

Biological activity

The bioassay employed to determine homogeneous activity was the rice lamina inclination test using the cultivar *Bahia.*[10] The relative activity data obtained in bending the rice lamina by applying 1μg/plant are presented in Table 1.

The high activity elicited by **9** was similar to that for 28-homobrassinolide (**4**), and the relatively high activity elicited by **15** highlights the weakness of the requirements postulated previously.[2] Both compounds have a 2β,3β-diol and a A/B *cis* ring junction.

Compost	Activ.(%)	SD*	Compost	Activ.(%)	SD*
1	100	2,35	**5**	14	0,53
13	97	0,75	**19**	11	1,32
3	97	3,70	**16**	11	0,64
9	87	5,10	**20**	8	0,76
4	87	1,66	**6**	7	0,84
2	87	1,85	**14**	7	0,59
10	75	2,13	**23**	6	0,55
18	66	3,47	**7**	6	0,66
15	51	4,40	**8**	6	1,60
17	27	2,13	**21**	0,00	0,93
12	17	0,71	**22**	0,00	0,5
11	17	1,60			

*SD: Standard deviation

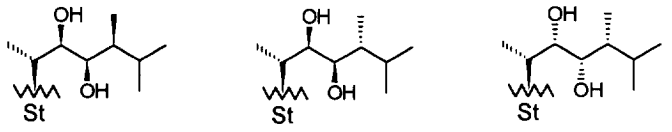
Table 1: Relative activity of brassinosteroids in rice lamina inclination test.

Molecular modeling

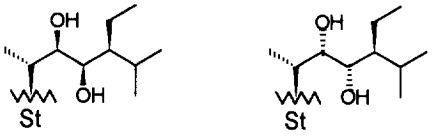
This study has been carried out in a Risk 6000 workstation using the program MAD[11] for molecular optimization and conformational analysis.

Brassinosteroids present two structural points with higher conformational movement that should be considered: the seven-membered B ring and the side chain. A conformational analysis has to be made to find the possible conformations within a range of 3 Kcal/mol. One of them, named *active conformation,* should be that which interacts with the binding site of the brassinosteroids receptors. In this *active conformation* the atoms involved in the brassinosteroid-receptor junction ought to have the same spatial situation in all molecules.

In our previous study (unpublished results) two possible conformations for the 7-membered B ring, with energy differences of approximately 5 Kcal/mol were found. Conformational analysis of the different side chains has been performed considering the lower energy conformer encountered for the 7-membered B ring. Since the number of degrees of freedom is enormous for an exhaustive analysis, a systematic study was planned for five different side chains showed in Figure 4 . As a result, a different number of possible conformers within a range of 3 Kcal/mol has been found: seven conformers for the side chain Brasin, six for R-Epi, nine for S-Epi, twelve for R-Homo and a large range of possible orientations for the side chain S-Homo. In each side chain, although the energy conformation encountered for each conformer is similar, the relative position of each atom differs, in some cases, significantly.



Brasin R-Epi S-Epi



R-Homo S-Homo

Figure 4: Types of brassinosteroid side chain studied.

On the other hand, a Free-Wilson analysis[12] was performed to show which functionalities are important for the activity. As a conclusion of this analysis, the configuration R, R for the side chain hydroxyl groups and α,α for A ring diols are shown to be the more significant groups, participating with a 35% and 25% respectively in relation to the total activity. These findings are in accordance with the qualitative structure-activity relationships described previously by Takatsuto *et al.*[4] but, on the contrary, they considered the affinity of the A ring stronger than that of the side chain. Although the contribution of functionalities in the B ring is less important, it should be considered for a complete description of the activity, due to the decrease observed when the ketone function is changed to an ether.

Taking into account these considerations, the *active conformation* for each compound has been found by comparing the spatial position of the oxygen atoms at C-2, C-3, C-22, C-23 and C-6 in all possible conformers encountered. The conformer of brassinolide (**1**) which presents more similarity to the conformers of the other compounds was taken as its *active conformation* and as the reference to select the *active conformation* of the other compounds.

Parameters and QSAR

For the *active conformation* found for each compound, several parameters have been calculated with the program TSAR.[13] A linear correlation has been obtained with the same program using a multivariable regression method (Figure 5).

$$-\log A = 0{,}135\ X_1 - 0{,}296\ X_2 + 0{,}13\ X_3 + 0{,}1775\ X_4 - 0{,}085\ X_5 - 1{,}295$$

Statistics parameters: $r = 0{,}922$; $s = 0{,}212$; $F = 15{,}96$ ($F_{95\%} = 3$); $r(cv)^2 = 0{,}652$

$$\text{Where: } X_i = \sum_{j=1}^{n} Y_j\, Y_j$$

X_1 : Y_j= absolute atomic charges
n = atoms pairs between 7 and 8 Å.

X_2 : Y_j= negative atomic charges
n = atoms pairs between 6 and 7 Å.

X_3 : Y_j= negative atomic charges
n = atoms pairs between 8 and 9 Å.

X_4 : Y_j= Van der Waals Radios
n = atoms pairs between 12 and 13 Å.

X_5 : Y_j= Van der Waals Radios
n = atoms pairs between 14 and 15 Å.

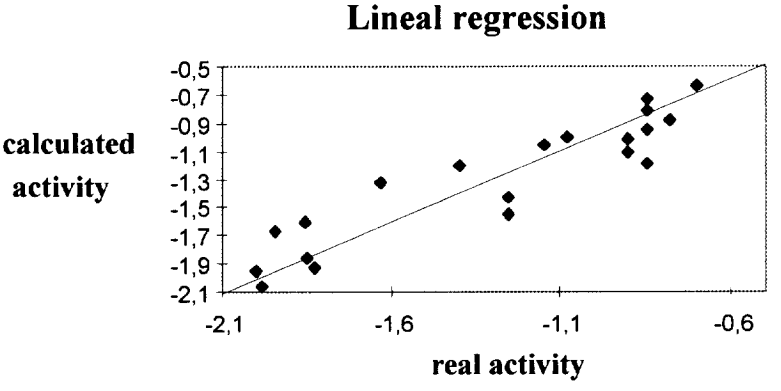


Figure 5: Structure-activity correlation.

In this preliminary QSAR only five parameters correlate well with the activity, depending on distances of atomic charges (X_1, X_2, X_3) and Van der Waals radios (R_{vdW}) (X_4, X_5). The pairs of atoms involved in the parameters X_1, X_2 and X_3 are formed between: the oxygen atoms at C-2, C-3 or C-6 and the hydrogen or carbon atoms at the D ring (non shadow area, Figure 6), as well as the oxygen atoms at the C-22 or C-23 and the hydrogen or carbon atoms at the C ring (shadow area, Figure 6). With regard to X_4 and X_5 the pairs are formed between the A ring and side chain atoms.

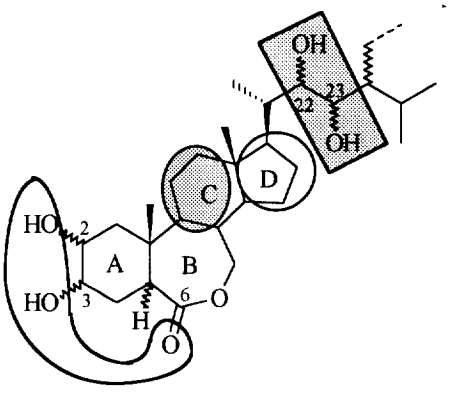


Figure 6: Pairs of atoms involved in the parameters X_1,X_2,X_3.

Because there are no structural modifications on the C and D rings in all the brassinosteroids studied, the activity will depend only on the spatial situation of the oxygen atoms concerned. This could be the reason for the high activity of **9** and **15** (A/B *cis* junction and 2β,3β diol), because the distances between the D ring and the oxygen atoms at C-2, C-3 are similar to **4** and **13** (A/B *trans* junction and 2α,3α diol). This was confirmed by the low RMS (Root Mean Square) index (0.285 Å, 0.177 Å) obtained by superposing the oxygen atoms of **9** and **15** with **4** and **13** respectively.

Table 2 shows the distance between the oxygen atoms for every compound, setting them in decreasing activity order. In the last column, "DIF" indicates the sum of the absolute value differences for each distance between the *active conformation* of brassinolide (**1**) and the one of the rest of compounds. Therefore, the lower the "DIF" value, the more similar the compound will be to brassinolide (**1**). It can be observed that the compounds that show an activity higher than 50% have a "DIF" value lower than 5 except for compound **18**. This points out the importance of these oxygen atoms in the activity. The low activity shown by (22S,23S)-28-homobrassinolide (**5**), although its "DIF" value, will be discussed later.

In conclusion, the activity of brassinosteroids depends on the oxygen atoms spatial situation. Therefore the structural requirements should not be indicated as the presence or not of a specific functional group in the molecule,[3,4] but as the spatial distribution of all the functionalities present in it. This spatial orientation can be indicated as distances or angles between the oxygen atoms present in a brassinosteroid. In this sense, we propose a new way to define the structural requirements for a brassinosteroid to be active that can be represented as in Figure 7. Thus, a compound will be more active the closer its values in the unshaded area are to the ones for brassinolide (**1**) in the shadowed area. In Figure 7 there are two brassinosteroids examples: one presenting high activity (24-epibrassinolide (**2**)), and another one **20** with much lower response. While for the first one the values are quite similar to those of brassinolide (**1**), for the second they differ significantly.

dist.	O-2	O-3	Ocarb	O-22	O-23
O-2	-	2.7	6.1	11.1	12.7
O-3	2.7	-	4.8	11.1	12.9
Ocarb	6.1	4.8	-	10.8	12.6
O-22	11.3	11.1	10.9	-	2.4
O-23	12.9	13.1	12.7	2.4	-

24-Epibrassinolide (**2**)

dist.	O-2	O-3	Ocarb	O-22	O-23
O-2	-	2.7	6.1	11.1	12.7
O-3	2.8	-	4.8	11.1	12.9
Ocarb	5.4	5.2	-	10.8	12.6
O-22	11.5	12.7	9.9	-	2.4
O-23	12.7	14.3	10.8	2.7	-

20

Figure 7: Distances between oxygen atoms at C2, C3, C6, C22, C23 for **2** and **20** (white areas) and for brassinolide (**1**) (shadowed area).

	O_2 - O_3	O_2 - O_{carb}	O_2 - O_{22}	O_2 - O_{23}	O_3 - O_{carb}	O_3 - O_{22}	O_3 - O_{23}	O_{carb} - O_{22}	O_{carb} - O_{23}	O_{22} - O_{23}	DIF
1	2.7	6.1	11.1	12.7	4.8	11.1	12.9	10.8	12.6	2.4	0
13	2.7	6.3	11.3	12.8	4.9	11.0	12.9	10.0	11.7	2.6	2.6
3	2.7	6.1	11.6	13.0	4.8	11.1	12.9	10.3	11.5	2.6	2.6
9	2.7	6.4	9.9	11.4	4.5	11.2	13.0	11.1	12.9	2.7	4.2
4	2.7	6.1	11.8	12.7	4.8	11.7	13.0	10.3	12.2	2.6	2.5
2	2.7	6.1	11.3	12.9	4.8	11.1	13.1	10.9	12.7	2.4	0.8
10	2.6	6.3	11.2	12.8	4.9	10.8	12.9	9.8	11.7	2.5	2.9
18	2.7	6.4	12.1	13.0	5.0	12.0	13.3	9.6	11.6	2.6	5.5
15	2.8	6.6	10.2	11.8	4.9	11.2	13.2	9.9	11.8	2.7	4.9
17	2.8	5.4	12.1	12.4	5.2	13.7	13.9	10.9	10.3	2.9	9.0
12	2.7	6.4	9.3	10.1	4.9	9.4	10.8	9.2	10.4	2.7	12.7
11	2.7	6.3	11.8	12.7	4.9	11.2	12.9	9.4	11.3	3.6	5.0
5	2.8	6.2	11.9	12.3	4.9	11.2	12.4	10.7	12.7	3.6	3.5
19	2.7	6.5	11.3	12.3	4.9	11.1	12.3	9.7	9.4	2.6	6,2
16	2.6	5.6	11.6	10.7	5.0	12.8	11.8	9.9	9.7	2.5	10.0
20	2.8	5.4	11.5	12.7	5.2	12.7	14.3	9.9	10.8	2.7	7.6
6	2.8	4.8	11.6	12.0	4.9	12.9	13.9	10.6	13.9	3.7	8.3
14	2.7	6.4	10.2	11.9	4.6	11.3	12.3	9.1	10.1	2.5	7.3
23	2.7	-	11.6	13.0	-	11.8	12.8	-	-	2.6	-
7	2.7	6.4	10.7	11.1	4.6	11.6	12.7	9.9	12.2	3.6	5.7
8	2.8	4.7	11.3	12.1	4.9	12.5	13.6	11.0	12.5	2.4	4.8
21	2.8	5.3	11.6	12.5	5.1	13.2	14.4	9.7	11.6	2.7	7.9
22	2,8	6.6	11,2	12,1	4,9	11,8	13,2	9,4	11,3	3,1	5,8

Table 2: Distances between oxygen atoms.

Moreover, looking at the results obtained in the conformational analysis for different side chains, there are a similar number of possible conformers in a range of 3 kcal/mol for the side chain Brasin, R-Epi, S-EPi and R-Homo (Figure 4). In these cases, compounds differing only in the side chain and having the same functionalities in the skeleton (i.e.: **1**, **2**, **3** and **4**) showed more or less the same activity (100, 87, 97 and 87 respectively). Nevertheless, for the side chain S-Homo (i.e.: **5**) the number of possible conformers increases and the activity falls (14%).

Therefore, the number of possible conformers also seems to be related to the activity. Due to the high flexibility of the S-Homo side chain, the *active conformation* which fits the receptor could be "diluted" into the other ones. This could be one of the reasons why the compounds with this kind of side chain such as **5** are much less active than the corresponding ones with other side chains. In accordance with its "DIF" value (3.5 Å) the activity of **5** is lower than it could be expected, but the lower population (4,5 %) of the *active conformation* compared to the other side chains (Table 3), decrease the probability that it interacts with the receptor.

Compounds	**% *active conformation***
Brassinolide (**1**)	13,5
24-Epibrassinolide (**2**)	14,5
(22S,23S)-24-Epibrassinolide (**3**)	14
28-Homobrassinolide (**4**)	11,5
(22S,23S)-28-Homobrassinolide (**5**)	4,5

Table 3: Percentage of *the active confotmation* calculated by the Boltzman distribution

So we suggest two independent factors to explain the activity: an entropic one that is related to the flexibility of the side chains (that is currently being studied) and an enthalpic one that is related to the oxygen atoms spatial situation involved in the interaction brassinosteroids-receptor.

Experimental Section:

Melting points were determined on a Gallenkamp instrument and are uncorrected. IR spectra were obtained on a Perkin-Elmer 683 spectrometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian-Gemini-300 (300 MHz) and Varian XL-200 (200 MHz) spectrometer, using TMS as internal standard. The multiplicity of the signals in the ^{13}C-NMR spectra was determined using the sequence Distorsionless Enhancement Polarization Transfer (D.E.P.T.). Mass spectra (electron impact, (EIMS) m/z) were run on a Hewlett-Packard 5995-A spectrometer and mass spectra (chemical ionization, (CIMS) m/z) on a Hewlett-Packard 5988-A using methane as the carring gas. The progress of all reactions and column chromatography was monitored by TLC on silica gel $60F_{254}$ microplates (Macherey-Nagel, Art 804023) and spots were detected by spraying with 50 % sulfuric acid, followed by heating. Flash column chromatography was performed on silica gel 60 (230-400 mesh) (Merck). Medium-pressure chromatography was run on a Lichroprep RP-18 (230-400 mesh) (Merck). "Usual work-up" refers to dilution with water, extraction with an organic solvent, washing the extract to neutrality, drying ($MgSO_4$) and removal of the solvent under reduced pressure.

• ***(22R,23R)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one(15) and (22S,23S)-2β,3β,22,23-tertrahydroxy-5β-stigmastan-6-one(14).*** A solution of 1.007 g (2.26 mmol) of (22E)-2β,3β-dihydroxi-5β-stigmast-22-en-6-one (**30**)[6] in 20 mL of THF, free of peroxides, was treated with a mixture of 63 mg (0.24 mmol) of OsO_4, 1.22 g (2.43 mmol) of dihydroquinidine 9-*O*-(9'-phenanthryl) ether (DHQD PHN), 9.8 g (72.5 mmol) of N-methylmorpholine N-oxide (NMO), 1.265 g (4.84 mmol) of $Et_4N^+AcO^-$ hydrated in 20 mL of THF, free of peroxides, and 5 mL of water in 23 mL of t-BuOH. The mixture was stirred at 0°C in argon and protected from light for six days. After addition of 200 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (CH_2Cl_2) afforded 1.223 g of a crude, which were chromatographed (Hexane/AcOEt 1:6) and recrystallized from methanol to give (22R,23R)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (**15**) (348 mg, 32 %) and (22S,23S)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (**14**) (226 mg, 25 %).

(22R,23R)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (15):
m.p.(MeOH/H_2O) : 225.4 - 226 °C; IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600-3100, 1700, 1450, 1430, 1370, 1030, 740; ^{1}H-RMN (300 MHz, $CDCl_3$ + DMSO) : δ 4.03-3.98 (1H, m, αH-C-3), 3.77-3.73 (1H, m, αH-C-2), 3.72-3.66 (1H, m, H-C-23), 3.58-3.52 (1H, m, H-C-22), 2.43 (1H, dd, J=4.8 Hz, J=12.7 Hz, βH-C-5), 0.99-0.86 (12H, m, 21, 26, 27, 29-CH_3), 0.89 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C-RMN (75 MHz, $CDCl_3$ + DMSO) : δ 214.9 (s, C-6), 74.0(d) + 72.2(d) (C-22 and C-23), 67.7(d) + 66.9(d) (C-3 and C-2), 56.5 (d, C-14), 53.4 (d, C-9), 52.3 (d, C-17), 46.4 (d, C-24), 42.7 (s, C-13), 42.6 (t, C-7), 40.3 (s, C-10), 40.2 (d, C-5), 39.4 (t, C-12), 36.9 (d, C-8), 36.9 (t, C-1), 36.8 (d, C-20), 31.8 (t, C-4), 28.7 (d, C-25), 27.4 (t, C-16), 23.6 (t, C-15), 23.4 (q, C-19), 21.1 (t, C-11), 21.0 (q, C-27), 19.3 (q, C-26), 18.7 (t, C-28), 13.4 (q, C-29), 11.8 (q, C-21), 11.6 (q, C-18); APT (75 MHz, $CDCl_3$ + DMSO) : δ 214.9 (s, C-6), 42.7 (s, C-13), 40.3 (s, C-10); EIMS 70 eV, m/z (rel.

int.) : 365 [M - $C_7H_{17}O$]$^+$ (23), 364 [M - $C_7H_{16}O$]$^+$ (48), 363 [M - $C_7H_{15}O$]$^+$ (28), 346 [M - $C_7H_{17}O_2$]$^+$ (23), 334 [M - $C_7H_{19}O_3$]$^+$ (4), 277 (33); CIMS (methanol), 70 eV, m/z (rel. int) : 519 [(M+1) - C_3H_5]$^+$ (6), 507 [(M+1) - C_2H_5]$^+$ (12), 479 [M+1]$^+$ (66), 461 [(M+1) - H_2O]$^+$ (100), 443 [(M+1).- 2×H_2O]$^+$ (30), 425 [(M+1)-3×H_2O]$^+$ (8), 407 [(M+1) - 4×H_2O]$^+$ (1); CIMS (h.r.) : 479.3681 [M]$^+$ (calc. 479.7189), 461.3618 [M-OH]$^+$ (calc. 461.7037), 443.3508 [M-(2×OH)]$^+$ (calc. 443.6885).

(22S,23S)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (14):
m.p.(MeOH) : 117 - 119 °C; IR $\nu_{max.}^{p.KBr}$ cm^{-1}: 3600-3100, 1700, 1465, 1442, 1380, 1060, 755; ^{1}H-RMN (300 MHz, $CDCl_3$) : δ 4.04 (1H, s.a., αH-C-3), 3.82-3.58 (3H, m, αH-C-2, H-C-22, H-C-23), 2.41 (1H, dd, J=10 Hz, J=5 Hz, βH-C-5), 1.03 (3H, d, J=6.9 Hz, 21-CH_3), 1.0-0.86 (m, 26, 27, 29-CH_3), 0.90 (3H, s, 19-CH_3), 0.70 (3H, s, 18-CH_3); ^{13}C-RMN (50 MHz, $CDCl_3$) : δ 215.3 (s, C-6), 72.1 (d) + 70.5 (d) (C-22 and C-23), 67.8 (d) + 67.1 (d) (C-2 and C-3), 56.4 (d, C-24), 53.2 (d, C-9), 52.5 (d, C-17), 49.5 (d, C-24), 43.3 (s, C-13), 42.7 (t, C-7), 42.2 (d, C-20), 40.3 (d, C-5), 39.4 (t, C-12), 39.2 (s, C-10), 37.0/t C-1, 36.8/d C-8, 31.8/t C-4, 27.7/t C-16, 26.7/d C-25, 24.0/t C-15, 23.4/q C-19, 21.6 (q, C-26), 21.1 (t, C-11), 18.3 (t, C-28), 17.4 (q, C-27), 14.3 (q, C-29), 13.9 (q, C-21), 11.7 (q, C-18); EIMS, 70 eV, m/z (rel. int.) : 365 [M - $C_7H_{17}O$]$^+$ (34), 364 [M - $C_7H_{16}O$]$^+$ (64), 363 [M - $C_7H_{15}O$]$^+$ (40), 346 (37), 334 (16).

• ***(22R,23R)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (17) and (22S,23S)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (16)*** A solution of 1.009 g (2.27 mmol) of (22E)-2β,3β-dihydroxy-5α-stigmastan-22-en-6-one (**29**)[6] in 20 mL of THF, free of peroxides, was treated with a mixture of 614 mg (0.24 mmol) of OsO_4, 1.227 g (2.44 mmol) of dihydroquinidine 9-*O*-(9′-phenantryl) ether (DHQD PHN), 9.815 g (73 mmol) of N-methylmorpholine N-oxide (NMO), 1.267 g (4.84 mmol) of $Et_4N^+AcO^-$ hydrated in 20 mL of THF, free of peroxides and 5 mL of water in 24 mL of t-BuOH. The mixture was stirred at O°C in argon and protected from light for seven days. After addition of 200 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (CH_2Cl_2) afforded 1.142 g of a crude, which were chromatographed (Hexane/AcOEt 1:6) and recrystallized from methanol to give (22R,23R)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (**17**) (369 mg, 34 %) and (22S,23S)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (**16**) (206 mg, 19 %).

(22R,23R)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (17):
m.p.(MeOH/H_2O) : 227.9 - 228.8 °C; IR $\nu_{max.}^{p.KBr}$ cm^{-1}: 3700-3100, 1700, 1460, 1380, 1050, 950, 750; ^{1}H-RMN (300 MHz, $CDCl_3$) : δ 3.99 (1H, $w_{1/2}$=6 Hz, αH-C-2), 3.71-3.52 (3H, m, αH-C-3, H-C-22, H-C-23), 2.28 (1H, dd, J=4.2 Hz, J=13 Hz, βH-C-7), 2.22 (1H, dd, J=3 HZ, J=11.9 Hz, αH-C-5), 2.10 (1H, dd, J=2.7 Hz, J=14.4 Hz, βH-C-1), 0.99-0.88 (12 H, m, 21, 26, 27, 29-CH_3), 0.89 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C-RMN (75 MHz, $CDCl_3$ + DMSO) : δ 210.7 (s, C-6), 74.0 (d) + 72.1 (d) (C-22 and C-23), (71.3 (d) + 68.9 (d) (C-3 + C-2), 57.0 (d, C-14), 56.3 (d, C-5), 54.4 (d, C-9), 52.3 (d, C-17), 46.3 (t, C-7), 46.2 (d, C-24), 42.5 (s, C-13), 42.2 (t, C-1), 40.4 (s, C-10), 39.3 (t, C-12), 37.1 (d, C-8), 36.8 (d, C-20), 28.7 (d, C-4), 27.4 (t, C-16), 23.9 (t, C-25), 23.6 (t, C-15), 21.3 (t, C-11), 21.0 (q, C-27), 19.3 (q, C-26), 18.7(t, C-28), 15.0 (q, C-19), 11.8 (q, C21), 11.6 (q, C-18); EIMS, 70 eV, m/z (rel. int): 365 [M - $C_7H_{17}O$]$^+$ (10), 364 [M - $C_7H_{16}O$]$^+$ (40), 363 [M - $C_7H_{15}O$]$^+$ (20), 362 (17), 346 [M - $C_7H_{17}O_2$]$^+$ (20); CIMS (methanol), 70 eV, m/z (rel. int) : 519 [(M+1) - C_3H_5]$^+$ (3), 507 [(M+1) - C_2H_5]$^+$ (5), 479 [M+1]$^+$ (40), 461 [(M+1) - H_2O]$^+$ (100),

443 [(M+1) - 2 × H_2O]$^+$ (18), 425 [(M+1) - 3 × H_2O]$^+$ (3), 407 [(M+1) - 4 × H_2O]$^+$ (1); CIMS (h.r.) : 477.3528 [M]$^+$ (calc. 477.7031), 461.3559 [M-OH]$^+$ (calc. 461.7037), 443.3487 [M-(2×OH)]$^+$ (calc. 443.6885).

(22S,23S)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (16) :

m.p.(MeOH) : 163 - 165 °C; IR $\nu_{max.}^{p.KBr}$ cm^{-1}: 3600-3100, 1700, 1460, 1380, 1055; ^{1}H-NMR (300 MHz, $CDCl_3$) : δ 4.03 (1H, $w_{1/2}$=9 Hz, αH-C-2), 3.68-3.65 (3H, m., αH-C-3, H-C-22, H-C-23), 2.31 (1H, dd, J=4.2 Hz, J=13 Hz, βH-C-7) 2.21 (1H, dd, J=3 Hz, J=11.7 Hz, αH-C-5), 1.03 (3H, d, J=6.6 Hz, 21-CH_3), 0.99-0.93 (9H, m., 29, 19, 26-CH_3), 0.87 (3H, d, J=7 Hz, 27-CH_3), 0.70 (3H, s, 18-CH_3); ^{13}C-NMR (50 MHz, $CDCl_3$) : δ 211.2 (s, C-6), 72.1 (d) + 71.7 (d) (C-22 and C-23), 70.6 (d) + 69.0 (d) (C-3 and C-2), 57.0 (d, C-14), 56.1 (d, C-5), 54.5 (d, C-9), 52.5 (d, C-17), 49.4 (d, C-24), 46.3 (t, C-7), 43.4 (s, C-13), 42.3 (t, C-1), 42.1 (d, C-20), 40.4 (s, C-10), 39.3 (t, C-12), 37.1 (d, C-8), 29.5 (t, C-4), 27.6 (t, C-16), 26.7 (d, C-25), 23.9 (t, C-15), 21.6 (q, C-26), 21.3 (t, C-11), 18.3 (t, C-28), 17.4 (q, C-27), 15.0 (q, C-19), 14.3 (q, C-29), 13.9 (q, C-21), 11.7 (q, C-18); EIMS, 70 eV, m/z (rel. int) : 364 [M - $C_7H_{16}O$]$^+$ (100), 363 [M - $C_7H_{15}O$]$^+$ (45), 346 [M - $C_7H_{17}O_2$]$^+$ (31), 345 [M - $C_7H_{16}O_2$]$^+$ (29).

• ***(22S,23S)-2β,3β,22,23-tetrahydroxy-6,7-seco-5α-stigmastan-6,7-lactone (6).*** A solution of 210 mg (0.287 mmol) of (22S,23S)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (**16**) in 6 mL of $CHCl_3$ at 0°C was treated with 1.5 mL of a solution of 7.4 mL of $(CF_3CO)_2O$ and 1 mL of 30 % H_2O_2 at 0°C in argon for six hours. After addition of 10 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (AcOEt) afforded a crude, which was purified by medium pressure chromatography (RP-18, CH_3CN) to give (22S,23S)-2β,3β ,22,23-tetrahydroxy-6,7-seco-5α-stigmastan-6,7-lactone (**6**) (128 mg, 66%). m.p. (MeOH): 118-119 °C ; IR $\nu_{max.}^{p.KBr}$ cm^{-1}: 3650-3150, 1720, 1470, 1060 ; ^{1}H-NMR (200 MHz, $CDCl_3$): δ 4.40-3.90 (4H, m, H-C-7, αH-C-3, αH-C-2), 3.65-3.58 (2H, m, H-C-22, H-C-23), 3.20 (1H, dd, J= 4 Hz, J= 12 Hz, αH-C-5), 1.16-0.91 (m, 19, 21, 26, 27, 29-CH_3), 0.86 (3H, s, 18-CH_3); ^{13}C-NMR (50 MHz, $CDCl_3$) : δ 179.3 (s, C-6), 72.9 (d) + 71.3 (d) (C-22 and C-23), 71.8 (t, C-7), 71.2 (d, C-3), 70.2 (d, C-2), 59.9 (d) + 53.9 (d) (C-14 and C-9), 52.2 (d, C-17), 52.2 (d, C-17), 50.7 (d, C-24), 45.9 (t, C-1), 44.2 (s) (C-10 and C-13), 43.7 (d, C-20), 41.0 (t, C-12), 40.2 (d) + 36.9 (d) (C-5 and C-8), 28.9 (t, C-4), 28.7 (t, C-16), 27.9 (d, C-25), 25.9 (t, C-15), 23.6 (t, C-11), 22.2 (q, C-26), 19.6 (t, C-28), 18.0 (q, C-19), 17.7 (q, C-27), 14.8 (q, C-29), 14.4 (q, C-21), 12.0 (q, C-18); CIMS (methanol), 70 eV, m/z (rel. int) : 512 [M + 18]$^+$ (20), 495 [M + 1]$^+$ (15).

• ***(22R,23R)-2β,3β,22,23-tetrahydroxy-6,7-seco-5α-stigmastan-6,7-lactone (8).*** A solution of 354 mg (0.74 mmol) of (22R,23R)-2β,3β,22,23-tetrahydroxy-5α-stigmastan-6-one (**17**) in 12 mL of $CHCl_3$ at 0°C was treated with 5.5 mL of a solution of 7.4 of mL $(CF_3CO)_2O$ and 1 mL of 30 % H_2O_2 at 0°C in argon for 12 hours. After addition of 25 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (AcOEt), afforded a crude which were purified by Flash chromatography (Cy/AcOEt 1:8) to give (22R,23R)-2 β,3β,22,23-tetrahydroxy-6,7-seco-5α-stigmastan-6,7-lactone (**8**) (196 mg, 33 %).IR $\nu_{max.}^{CHCl_3}$ cm^{-1}: 3600-3100, 2960, 1720, 1470, 1060; ^{1}H-RMN (300 MHz, CD_3OD) : δ 4.3-3.8 (2H, m, H-C-7), 3.7-3.5 (2H, m, H-C-22, H-C-23), 3.10 (1H, dd, J=3.9 Hz, J=12.3 Hz, αH-C-5), 1.1-0.85 (15H, m, 19, 21, 26, 27, 29-CH_3), 0.75 (3H, s, 18-CH_3); ^{13}C-RMN (75 MHz, CD_3OD) : δ 178.8 (s, C-6), 75.5 (d) + 73.6 (d) (C-22 and C-23) , 71.8 (t, C-

7), 71.1 (d, C-3), 70.1 (d, C-2), 59.9 (d, C-9), 53.8 (d, C-14) , 52.5 (d, C-17), 48.3 (d) + 48.0 (d) (C-24 and C-20), 45.9 (t, C-1), 43.6 (s, C-10), 41.1 (t, C-12), 40.3 (d) + 38.6 (d) (C-5 and C-8)], 36.9 (s, C-13), 30.4 (d, C-25), 28.8 (t, C-4), 28.6 (t, C-16), 25.6 (t, C-15), 23.8 (t, C-11), 21.6 (q, C-26), 20.1 (q, C-19), 20.1 (t, C-28), 18.1 (q, C-27), 14.2(q, C-29), 12.5 (q, C-21), 12.1 (q, C-18); EIMS, 70 eV, m/z (rel. int) : 380 [M - $C_7H_{14}O$]$^+$ (5), 379 [M - $C_7H_{13}O$]$^+$ (5), 362 [M - $C_7H_{16}O_2$]$^+$ (4), 361 [M - $C_7H_{15}O_2$]$^+$ (4), 350 [M - $C_8H_{16}O_2$]$^+$ (4), 349 [M - $C_8H_{15}O_2$]$^+$ (2), 343 (4), 285 (4).

• ***(22S,23S)-2β,3β,22,23-tetrahydroxy-6,7-seco-5β-stigmastan-6,7-lactone (7).*** A solution of 550 mg (0.38 mmol) of (22S,23S)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (**14**) in 23 mL of $CHCl_3$ at 0°C was treated with 4.1 mL of a solution of 7.4 mL of $(CF_3CO)_2O$ and 1 mL of 30 % H_2O_2 at 0°C in argon for eight hours. After addition of 10 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (AcOEt) afforded a crude, which were purified by medium pressure chromatography (RP-18, CH_3CN/H_2O 1:3 and CH_3CN) to give (22S,23S)-2β,3β,22,23-tetrahydroxy-6,7-seco-5β-stigmastan-6,7-lactone (7) (319 mg, 63 %) .m.p. 109-110 °C; IR $\nu_{max.}^{CHCl_3}$ cm^{-1}: 3550-3200, 1715,1470, 1055, 760; ^{1}H-RMN (200 MHz, CD_3OD): δ 4.30- 4.05 (3H, m, H-C_7, αH-C-3), 2.82 (1H, d, J=12 Hz, αH-C-2), 3.70-3.56 (2H, m, H-C-22, H-C-23), 3.21 (1H, d, J= 12.5 Hz, βH-C-5), 1.18-0.92 (m, 21, 26, 27, 29-CH_3), 1.05 (3H, m, 19-CH_3), 0.88 (3H, s, 18-CH_3) ; ^{13}C-RMN (125 MHz, $CDCl_3$) : δ 179.4 (s, C-6), 73.0 (d) + 71.3 (d) (C-22 and C-23), 72.7 (t, C-7), 68.7 (d) + 67.6 (d) (C-3 + C-2), 53.9 (d, C-9), 52.9 (d, C-17), 51.7 (d, C-14), 50.9 (d, C-24), 44.2 (d) + 40.0 (d) (C-5 and C-8), 43.7 (d, C-20), 38.7 (s, C-13), 30.7 (t, C-1), 29.0 (t, C-4), 28.5 (s, C-10), 27.9 (d, C-25), 26.6 (t, C-16), 26.1 (t, C-15), 23.5 (t, C-11), 23.2 (q, C-19), 22.3 (q, C-26), 19.7 (t, C-28), 17.9 (q, C-27), 14.7 (q, C-29), 14.6 (q, C-21), 12.0 (q, C-18) ; CIMS, 70 eV, m/z (rel. int): 512 [M + 18]$^+$ (4).

• ***(22R,23R)-2β,3β,22,23-tetrahydroxy-6,7-seco-5β-stigmastan-6,7-lactone (9).*** A solution of 275 mg (0.57 mmol) of (22R,23R)-2β,3β,22,23-tetrahydroxy-5β-stigmastan-6-one (**15**) in 8.5 mL of $CHCl_3$ at 0°C was treated with 4.4 mL of a solution of 7.4 mL of $(CF_3CO)_2O$ and 1 mL of 30 % H_2O_2 at 0°C in argon for six hours. After addition of 19.5 mL of a saturated solution of $Na_2S_2O_5$ and stirring for one hour, the work-up (AcOEt) afforded 273 mg of a crude, 273 mg of which were purified by Flash chromatography (AcOEt) to give (22R,23R)-2β,3β,22,23-tetrahydroxy-6,7-seco-5β-stigmastan-6,7-lactone (**9**) (63 mg, 23 %). IR $\nu_{max.}^{p.KBr}$ cm^{-1} : 3700-3100, 1710, 1470, 1230, 1080; ^{1}H-RMN (300 MHz, CD_3OD) : δ 4.22- 3.98 (3H, m, H-C-7, α H-C-3), 3.78-3.50 (3H, m, αH-C-2, H-C-22, H-C-23), 3.16-3.06 (1H, m, βH-C-5), 1.10-0.93 (12H, m, 19, 21, 26, 29-CH_3), 0.91 (3H, d, J=6.9 Hz, 27-CH_3), 0.76 (3H, s, 18-CH_3); ^{13}C-RMN (75 MHz, CD_3OD) : 179.5 (s, C-6), 75.5 (d) + 73.6 (d) (C-22 and C-23), 59.5 (d, C-9), 53.8 (d, C-14), 52.9 (d, C-17), 51.9 (d, C-24), 48.2 (d, C-20), 44.3 (d) + 38.6 (d) (C-5 + C-8), 43.6 (s, C-10), 42.3 (t, C-1), 42.2 (t, C-12), 40.7 (s, C-13), 30.7 (t, C-4), 30.4 (d, C-25), 28.6 (t, C-16), 25.7 (t, C-15), 23.5 (t, C-11), 23.2 (q,C-26), 21.6 (q, C-19), 20.1 (t, C-28), 20.1 (q, C-27), 14.2 (q, C-29), 12.5 (q, C-21), 11.9 (q, C-18); EIMS, 70 eV, m/z (rel. int) : 350 [M - $C_7H_{11}O_3$]$^+$ (14), 362 (12), 380 [M - $C_7H_{15}O$]$^+$ (20), 423 (5).

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