Pergamon

Tetrahedron 54 (1998) 12337–12348

TETRAHEDRON

New Synthetic Brassinosteroids: a 5α-Hydroxy-6-ketone Analog with Strong Plant Growth Promoting Activity

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Received 3 June 1998; revised 22 July 1998; accepted 30 July 1998

Keywords: Brassinosteroids, Plant growth regulators, Rice lamina inclination test, Molecular modeling.

Abstract: *Five 5-hydroxy-6-ketone brassinosteroid analogs have been synthesized from stigmasterol (1) and their plant growth promoting activity has been evaluated using rice lamina inclination test. 3 has elicited high activity as plant growth regulator.*

Brassinosteroids are potent plant growth regulators that can be considered as good candidates to improve crop yields as well as to overcome environmental stress and herbicidal injury, and to control pathogenic diseases.[1,2] Much synthetic effort has been devoted not only to find brassinosteroids with high activity for further application in agriculture but also to delve into the knowledge of them from the structural, physiological, and biogenetic point of view. In this sense, several new analogs have been synthesized and promising results have been obtained with some of them.[3-7]

Due to the synthetic difficulty and the resulting high cost of such compounds, the use of brassinosteroids (mainly the more active natural ones) is at present uneconomic, limiting the scope of applicability in agriculture. In this sense, our aim is to design other brassinosteroids with a good activity/synthetic cost ratio, suited for their profitable use in agriculture. We planned to achieve this goal by means of establishing a quantitative structure/activity relationship (QSAR), which should provide further knowledge about the structural requirements for a brassinosteroid to be active.

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0040-4020/98/$ - see front matter
PII: S0040-4020(98)00743-1

A preliminary QSAR approach, as well as the methodology used to establish it, has been recently reported,[5,6,8] and a more extensive study will be published soon.[9] This procedure opens a new way to explain the activity of different brassinosteroids from the structural point of view and it has shown to be appropriate to predict the activity of new analogs. Studies to illustrate which are the main functional groups of the molecule involved in the brassinosteroid-receptor junction and the kind of binding to the receptor that may take place are currently in progress. To stablish the QSAR, an extensive training set has been employed embodying new structural variations not considered by previous structure-activity correlations (SAR).[10-12] Some of our target molecules are brassinosteroids having a 5-hydroxy group and with different configuration at the diols on A ring and side chain as well as different A/B ring junction.

In this paper we report the synthesis of such 5-hydroxy analogs, the evaluation of their bioactivity using the rice lamina inclination test and a preliminary result on agricultural application of one of them (**3**) on winter barley. Moreover, the effect of these structural features on the bioactivity is analyzed in terms of the information obtained from molecular mechanics calculations.[6,8,9]

Results and discussion

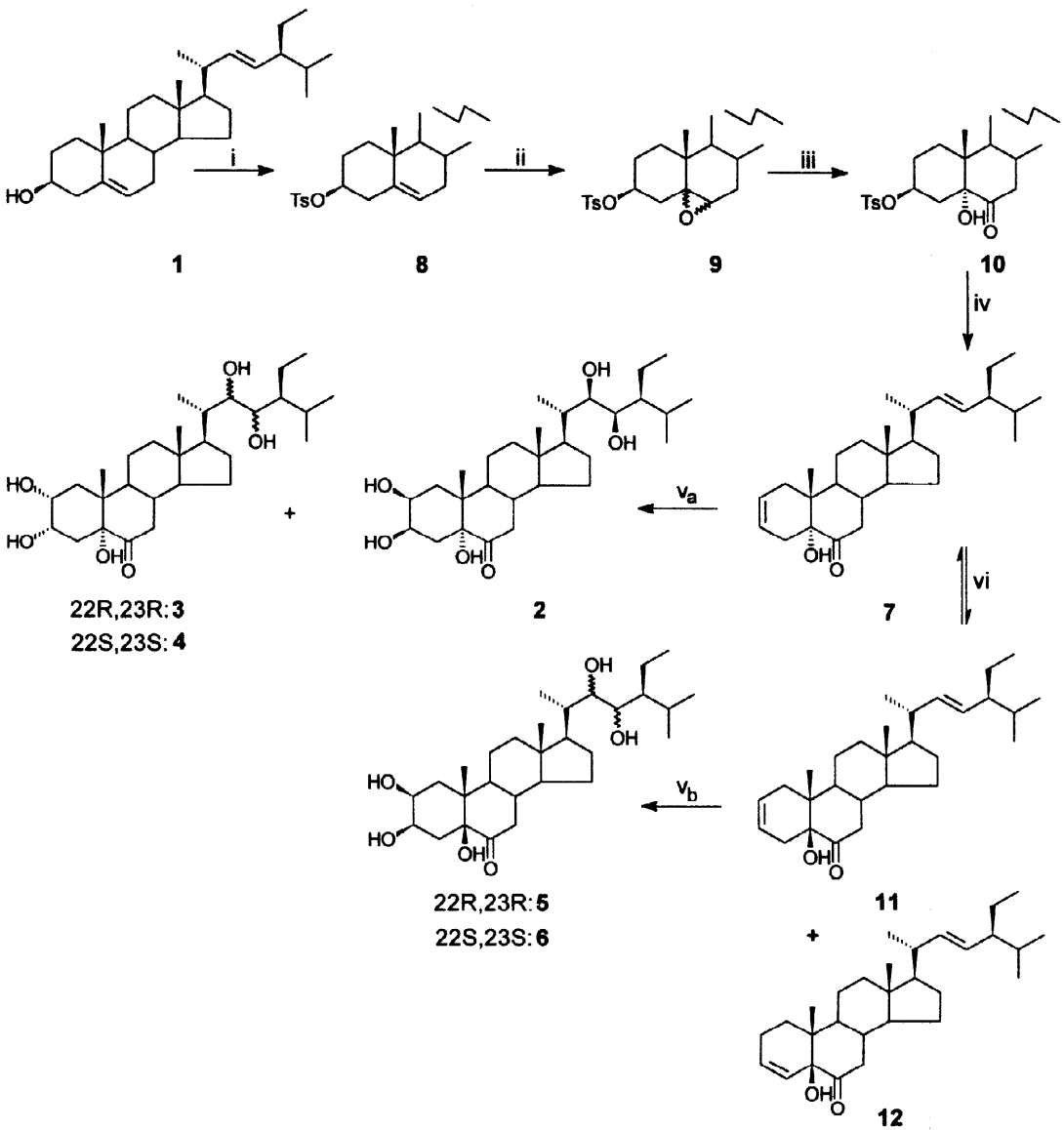
a) Synthesis and structural elucidation

A retrosynthetic analysis indicates that the 5-OH function could result from the oxidative opening of an epoxide, readily available from the double bond Δ^5 of stigmasterol (**1**), and the A/B *cis* junction could result from the epimerization of the 5α-hydroxy-6-ketone thus formed. Moreover, the presence of the 5-OH group could modify the stereoselectivity of the Δ^2 dihydroxylation giving a mixture of the corresponding 2α,3α and 2β,3β diasteroisomeric glycols.

Following this procedure, five compounds (**2**, **3**, **4**, **5**, and **6**) were obtained from stigmasterol (**1**) through the diene **7** (Scheme 1). All these analogs are new compounds, except **4** that has been previously obtained by Kovganko *et al.* following another strategy.[13]

The diene **7** was synthesized in four steps, without purification of intermediates. Thus, tosylation of stigmasterol (**1**) with TsCl/Py, followed by regioselective epoxidation of the Δ^5 double bond of **8** with *m*-chloroperbenzoic acid (MCPBA) in CH_2Cl_2 afforded a mixture of the monoepoxides **9**. The highly regioselective epoxidation was achieved by performing the reaction at -78°C and by adding a 5% Na_2SO_3 solution at -5°C to the reaction mixture before the usual work-up, to destroy the excess of peracid. Following this procedure, no compounds with epoxy function at C-22,C-23 were detected. Subsequent oxidation of **9** with Jones reagent ($CrO_3/H_2SO_4/H_2O$) and dehydrotosylation of the crude product with LiBr/DMF afforded, after chromatographic purification, **7** with an overall yield of 33% from stigmasterol (**1**).

Osmium-catalyzed asymmetric dihydroxylation[14] of the two double bonds of **7**, using 4-methylmorpholine-4-oxide monohydrate (NMO) as cooxidant and dihydroquinidine 9-O-(9'-phenanthryl) ether (DHQD PHN) as chiral ligand, gave after chromatographic purification (22R,23R) 2β,3β,5α,22,23-pentahydroxystigmastan-6-one (**2**), (22R,23R) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**3**) and (22S,23S) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**4**), with yields of 16%, 31%, and 15%, respectively.



Reagents and conditions: **i**: ClTs, Py, 22 h, rt. **ii**: MCPBA/CH_2Cl_2, Ar, 18 h, -78°C. **iii**: $CrO_3/H_2SO_4/H_2O$, MeCOMe, 30 min., rt. **iv**: LiBr/DMF, Ar, 2 h at reflux. **v**: OsO_4, NMO, DHQD PHN, $(Et)_4N^+(AcO^-).4H_2O$, THF/H_2O/t-butanol, a = 87 h, b = 163 h, 0°C. **vi**: KOH/MeOH 10%, Ar, 67 h at reflux.

Scheme 1

Alternatively, epimerization at C-5 by means of basic treatment of **7** with KOH/MeOH afforded a mixture of **11** and **7** in a 1:1,6 ratio (determined by HPLC). The corresponding Δ^3 analog of **11** (**12**) was also isolated as a by-product in a 10 % after chromatographic purification over $SiO_2/AgNO_3$.

Dihydroxylation of the diene **11** under the same conditions as those described for **7** gave (22R,23R) 2β,3β,5β,22,23-pentahydroxystigmastan-6-one (**5**) and (22S,23S) 2β,3β,5β,22,23-pentahydroxystigmastan-6-one (**6**) with 27% and 17% yield, respectively.

The results obtained on the dihydroxylation of the dienes **7** and **11** show that the diastereoisomeric 2α,3α/2β,3β ratio is highly dependent on the configuration of the hydroxy group at C-5. Thus, the glycols

obtained from the diene **7**, having a 5α-OH group, present a 2α,3α (**3** + **4**) to 2β,3β (**2**) ratio of 3:1. This result can be explained due to the higher steric hindrance of the C-19 methyl group compared to the 5α-OH group. In the case of the diene **11** with a 5β-OH, only 2β,3β-dihydroxy analogs (**5** and **6**) have been detected due to the greater β face accessibility of the A ring.

NMR experiments were performed on compounds **2** to **6** allowing the unequivocal assignment of the configuration at C-2, C-3 and C-5. Table 1 and 2 show the NMR data of all them.

	2	3	4	5	6
H-C2	4,08-3,96	3,77-3,70	3,82-3,72	4,00-3,92	4,05-3,95
H-C3	4,08-3,96	4,08	4,14-4,08	4,00-3,92	4,05-3,95
αH-C7	2,78	2,67	2,70		
18-CH_3	0,65	0,66	0,67	0,66	0,68
19-CH_3	1,01	0,75	0,76	0,68	0,68
21-CH_3	0,89	0,88	1,02	0,89	1,02
H-C22	3,53	3,49	3,58-3,50	3,52	3,59-3,52
H-C23	3,66	3,63	3,58-3,50	3,64	3,59-3,52
26-CH_3	0,98-0,92	0,97-0,89	0,98-0,84	0,97-0,92	0,93
27-CH_3	0,98-0,92	0,97-0,89	0,98-0,84	0,97-0,92	0,87
29-CH_3	0,94	0,97-0,89	0,94	0,97-0,92	0,96

Tabla 1. ^{1}H-NMR data.

	2	3	4	5	6		2	3	4	5	6
C1	30,4	29,6	30,1	35,3*	35,6*	**C16**	27,3	26,5	27,5	27,0	27,7
C2	67,7*	66,3	67,2	66,9	67,3	**C17**	52,2	51,5	52,2	51,9	52,2
C3	68,7*	68,8	69,5	68,9	69,4	**C18**	11,6	10,9	11,6	11,3	11,6
C4	36,1	33,5	34,2	35,1*	35,3*	**C19**	15,7	13,7	14,4	15,9	16,3
C5	80,0	78,5	79,2	81,4	81,8	**C20**	36,3*	36,1	42,1	36,5	42,2
C6	213,0	210,4	211,2	212,8	213,1	**C21**	11,7	11,1	13,7	11,5	13,8
C7	41,4	40,5	41,2	41,4	41,7	**C22**	73,9	72,8	71,5	73,4	71,7
C8	36,8*	38,5	36,8	37,2	37,5	**C23**	71,9	70,9	69,7	71,5	69,9
C9	44,3	43,5	44,2	44,8	45,3	**C24**	46,2	45,6	49,4	46,0	49,5
C10	42,6*	44,2*	43,2*	43,8*	44,2*	**C25**	28,7	28,0	26,5	28,4	26,6
C11	21,1	20,1	20,8	21,7	22,1	**C26**	19,3	18,7	21,7	19,1	21,7
C12	39,2	--	39,2	38,9	39,2	**C27**	20,9	20,2	17,5	20,7	17,6
C13	41,6*	41,8*	45,0*	42,2*	43,3*	**C28**	18,6	18,0	18,2	18,4	18,4
C14	55,8	55,2	55,6	56,1	56,2	**C29**	13,3	12,7	14,1	13,1	14,2
C15	23,5	22,8	23,9	23,3	24,1						

*: Assignments that can be interchanged.

Table 2. ^{13}C-NMR data

Thus, the configuration of the A/B junction for the dienes **7** and **11** has been deduced by performing two n.O.e. DIFF experiments. A n.O.e. enhancement of H-C-2 (1.5%), H-C-3 (1.5%) and βH-C-4 (8.1%) upon irradiation of H-C-19 (0.71 ppm) of **7** indicates an A/B *trans* junction. On the other hand, the only n.O.e.

enhancement observed at OH-C-5 proton upon irradiation of H-C-19 (0,77 ppm) of **11** agrees with an A/B *cis* junction. This was also corroborated by the downfield shift of C-19 protons (0,77 *vs* 0,71 ppm) and carbon (16,5 *vs* 14,5 ppm) of **11** with respect to **7**.

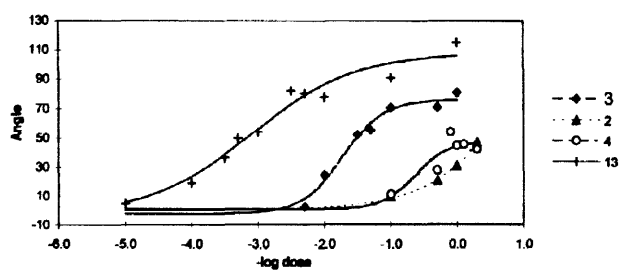
The C-2 and C-3 stereochemistry of **2**, **3** and **4** was deduced from ^{1}H-NMR and NOESY experiments. Thus, a deshielding of C-19 methyl protons, observed in compound **2** (1,01 ppm) *versus* **3** and **4** analogs (0,75, 0,76), due to the 1,3-diaxial interaction between C-2β-OH and C-19 methyl group, indicates the 2β,3β and the 2α,3α-dihydroxy configuration, respectively. Moreover, a NOESY experiment of compound **3**, shows a n.O.e at βH-C2 upon irradiation of H-C-19, confirming a 2α,3α-diol structure.

A HETCOR experiment of **5** shows a correlation between the assigned C-3 (68,9 ppm) and a H signal at 4,00-3,92 ppm (m, $w_{1/2}$ = 3Hz). The low value of $w_{1/2}$ confirmed the 2β,3β-diol stereochemistry. The comparison of ^{1}H-NMR spectrum of compound **6** with those of **5** clearly established that the 2,3-diol has the same stereochemistry in both cases.

b) Molecular modeling and bioactivity

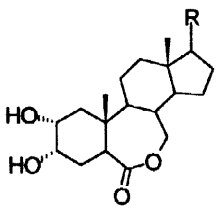
The activity data of the new compounds synthesized have been evaluated by means of a highly sensitive modified rice lamina inclination test using *Bahia* as cultivar[15] based on the procedure developed by Takeno and Pharis.[16] Figure 1 shows the dose-dependent activity curves obtained for **2**, **3**, **4**, **5** and **6** employing this bioassay and compared with that of homobrassinolide (**13**) (Figure 2) which also presents a stigmastan side chain.

Following the methodology developed by us, the molecular electrostatic potential (MEP) of each compound has been used as an alignment rule to stablish a QSAR. The electrostatic Carbó similarity index (CI)[17] has been used to quantify the similarity between each compound and brassinolide (**14**) (Figure 2). This compound has been chosen as reference because it is the most active natural brassinosteroid found.[8,9] We have found that, for a CI greater than 0.6, the higher the electrostatic CI the more active is the compound. Figure 1 shows the CI obtained for **2**, **3**, **4**, **5**, **6**, **13**, and **14** as well as the activity data expressed as -log $(dose)_{50\%}$ to be compared and the significance level associated with them.



	CI	-log $(dose)_{45}$	Significance level
14	1	-5.25	0,21
13	0.82	-3.11	0.28
3	0.76	-1.73	0.22
4	0.55	-0.63	0.15
5	0.55	-0.025	----
2	0.61	1.63	1.00
6	0.60	> 0	----

Figure 1. Activity data and electrostatic Carbó similarity index (CI)



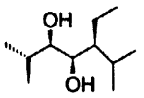
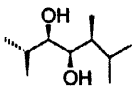
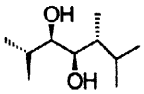
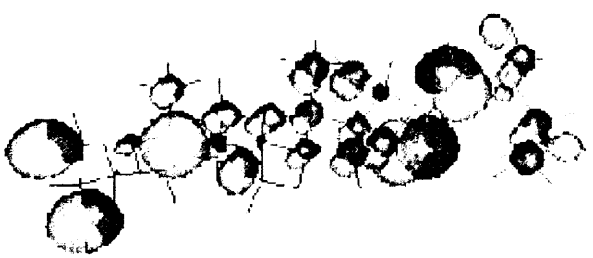
	Homobrassinolide (13)	Brassinolide (14)	Epibrassinolide (15)
R			

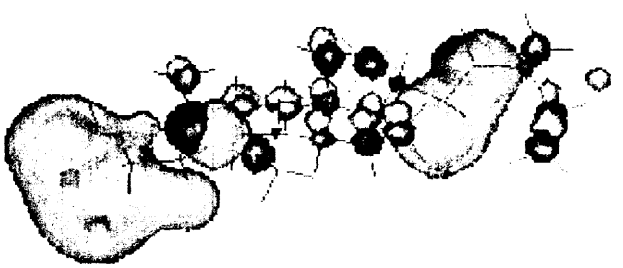
Figure 2

The high activity elicited by **3** is clearly in accordance with its electrostatic Carbó similarity index (CI). Figure 3 shows, as an example, the MEP maps overlay between brassinolide (**14**) and the new compounds **3** (the highly active one) and **5** (one of the less active ones) and compared with the one for homobrassinolide (**13**). **3**, **5** and **13** present the same functionality on the side chain. Graphically one can observe that there is a good overlay for the electrostatic sites of the hydroxy groups both of the A ring and the side chain between brassinolide (**14**) and homobrassinolide (**13**) in accordance with its high CI (0.816) and its high activity (-3.11). In the case of **3** there is also a good overlay for all the electrostatics sites with those of **14** but the shape and size are quite different. The area corresponding to the electrostatic potential of the A ring is larger for **3** than brassinolide (**14**). Similarly occurs, but in a less extension, with the area of the side chain thus decreasing its CI (0.756) and also its activity (-1.73). In the case of **5**, the lack of activity can also been explained looking at the overlay MEP maps. As one can see, there is no overlay between A ring nor B ring. Only the area corresponding to the electrostatic potential of hydroxy function at C_{22} overlay with that of **14** thus with a low CI (0.55).

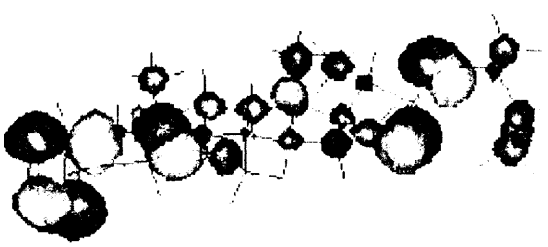
This is another example of the good correlation encountered between the CI and the bioactivity which allows us to assess the methodology followed to design other suitable BRs for further application in agriculture.



14 (black) / 13 (grey)



14 (black) / 3 (grey)



14 (black) / 5 (grey)

Figure 3

d) Practical application.

With respect to practical applications, an important response on field trials should be expected for **3**, which has elicited the higher activity of all the compounds synthesized and presented in this communication. In order to be compared, the field trials experiments have also been done with 24-epibrassinolide (**15**), a natural active brassinosteroid, which has been shown to improve the yield, quality and stress ressistance of different crops in field experiments[18,19]. Thus, **3** and **15** were assayed on winter barley by applying different concentrations (0.01; 0.1 and 1 ppm) at different growth stages. Although the results are preliminary and need more replicates, an improvement of crop yield of 15% has been obtained by spraying a solution of 0.1 ppm in water of compound **3** at vegetative stage. Similar results have been obtained by spraying the same concentration of 24-epibrassinolide (**15**) (16%).

A more detailed explanation of the procedure followed and the parameters measured to evaluate the yield of crops will be published elsewere.

Conclusions.

The high activity elicited by **3** both in the rice lamina inclination bioassay and field trials on barley, as well as its relative easiness of preparation compared to other brassinosteroid analogs, make this new brassinosteroid a very good candidate for further application in agriculture.

Studies to obtain more information about the influence on the activity of the large site observed on the molecular electrostatic potential of **3** in the region of the B ring are currently in progress.

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 as well as n.O.e experiments were recorded on a Varian-Gemini-300 (300 MHz) spectrometer and HETCOR and NOESY spectra were run on a Varian XL-500 spectrometer, using TMS as internal standard. The multiplicity of the signals in the ^{13}C-NMR was determined using the sequence Distorsionless Enhancement Polarization transfer (D.E.P.T.). Mass spectra (chemical ionization, (CIMS) m/z) were run on a Hewlett-Packard 5988-A using methane as the carrier gas. The progress of all reactions and column chromatography was monitored by TLC on silica gel $60F_{254}$ microplates (Macherey-Nagel (MN), Art 804023) and spots were detected by spraying with 50 % sulfuric acid, followed by heating. Flash column chromatography was performed on 230-400 mesh MN silica gel. Column chromatography was performed on MN silica gel (70-230 mesh) alone or coated with $AgNO_3$ 0,1N. HPLC chromatography was run on a Waters 600E chromatograph, an ultraviolet Waters detector (λ=210 nm), a Waters-Millipore Nova-Pak RP-18 (ϕ_i=3.9 mm, μ=4 μm, L=15 m) column and CH_3CN/H_2O 96:4 as mobile phase. **8**, **9** and **10** were identified from crude products by TLC comparison with authentic samples.

Stigmasterol tosylate (8). A solution of 10.100 g (24.47 mmol) stigmasterol (**1**) in 104 mL pyridine (dried over CaH_2) was treated with 200 g (59.30 mmol) p-toluene-sulfonyl chloride (ClTs) previously recrystallized from petroleum ether and stirred at room temperature in the dark for 22 h. The mixture was poured on 1L of a

cold aqueous solution of 4 % $KHCO_3$ and kepet at 0-5°C for 1 h. The solid was filtered, washed with cold water and dried affording 13.100 g of crude product **8**.

(22E) 5α,6α-Epoxy-3β-tosyloxystigmast-22-ene and 5β,6β-epoxy-3β-tosyloxystigmast-22-ene (9). A solution of 4.950 g (28.70 mmol) MCPBA in 50 mL CH_2Cl_2 was added dropwise under argon to a solution of 6.510 g crude **8** in 25 mL CH_2Cl_2 at -77 °C in the dark. The reaction was stirred at this temperature for 6 h and kept at -34°C overnight. The mixture was poored on 3L of a cold (4-5°C) solution of 5 % Na_2SO_3 and was kept at this temperature for 4 h. The reaction mixture was extracted several times with $CHCl_3$. The organic phase was washed with water, dried over anhydrous $MgSO_4$ and evaporated to dryness yieldind 6.23 g of a crude mixture of epoxides **9**.

(22E) 5-Hydroxy-3β-tosyloxy-5α-stigmast-22-en-6-one (10). Approximately 6 mL of Jones reagent was added dropwise to a stirred solution of 6.180 g of the crude **9** in 563 mL of acetone (distilled over $KMnO_4$). 30 min after the last addition, isopropyl alchohol was added to stop the reaction. The reaction mixture was filtered over Celite and extracted with AcOEt. The green solution obtained was concentrated to 25% initial volume, poured into 1L of an aqueous solution of NaCl and extracted with AcOEt. The organic phase was washed with brine, dried over $MgSO_4$ and evaporated to dryness affording 6.300 g of crude product **10**.

(22E) 5-Hydroxy-5α-stigmasta-2,22-dien-6-one (7). A solution of 6.300 g crude **10** in 500 mL of DMF (dried over BaO) was treated with 2.479 g (28.50 mmol) LiBr under argon and refluxed for 2 h 15 min in the dark. The reaction mixture was poured into 2 L of water and extracted with AcOEt. The organic phase was washed with HCl 2N, 5% $NaHCO_3$ and water, dried over $MgSO_4$ and evaporated to dryness yielding 5.520 g of a brown oil. 5.480 g of which was chromatographed by flash column chromatography (hexane/AcOEt 8:1) to give 1.735g of **7** (33% from **1**). IR ν^{CHCl_3} cm^{-1}: 3600-3300, 3020, 2940, 2860, 1705, 1455, 1380, 1200, 970; ^{1}H-NMR (300 MHz, Cl_3CD): δ 5.72-5.58 (2H, m, H-C-2, H-C-3), 5.15 (1H, dd, J=8.4 Hz, 15.2 Hz, H-C-22), 5.02 (1H, dd, J=8.4 Hz, 15.2 Hz, H-C-23), 2.70 (dd, J=12.6 Hz,12.6 Hz, αH-C-7), 2.66 (m, βH-C-4), 1.02 (3H, d, J=6.6 Hz, 21-CH_3), 0.85 (3H, d, J=6.6 Hz, 26-CH_3), 0.79 (3H, d, J=6.0 Hz, 27-CH_3), 0.80 (3H, t, J=7.2Hz, 29-CH_3), 0.71 (3H, s, 19-CH_3), 0.67 (3H, s,18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD): δ 211.4 (s, C-6), 138.0 (d, C-22), 129.5 (d, C-23), 125.5 (d, C-2), 122.4 (d, C-3), 77.9 (s, C-5), 56.5 (d, C-14), 55.8 (d, C-17), 51.2 (d, C-24), 45.2 (d, C-9), 42.8 (t, C-7), 42.6(s) + 42.3(s) (C-10 and C-13), 40.4 (d, C-20), 39.4 (t, C-12), 37.5 (d, C-8), 34.5(t) + 30.1(t) (C-1 and C-4), 31.9 (d, C-25), 28.7 (t, C-16), 25.4 (t, C-28), 24.0(t, C-15), 21.2(q) + 21.1(q) (C-21 and C-26), 21.0 (t, C-11), 19.0 (q, C-27), 14.5 (q, C-19), 12.2 (q) + 12.1(q) (C-29 and C-18).

(22R,23R) 2β,3β,5α,22,23-pentahydroxystigmastan-6-one (2), (22R,23R) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (3) and (22S,23S) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (4). A solution of 1.000 g (2,34 mmol) **7** in 20 mL of THF, free of peroxides, was slowly added dropwise over 8 h to a solution of 0.190 g (0.75 mmol) OsO_4, 2.410 g (4.78 mmol) dihydroquinidine 9-*O*-(9'-phenanthryl) ether (DHQD PHN), 19.060 g (0.14 mol) N-methylmorpholine N-oxide (NMO) and 2.440 g (15.26 mmol) Et_4N^+ AcO^- hydrated in 37.5 mL of THF, free of peroxides, 9.5 mL of water and 43 mL of t-BuOH. The mixture was

stirred, under argon atmosphere, at 0 °C in the dark for 87 h. The mixture was treated with 300 mL of a saturated solution of $Na_2S_2O_5$ and stirred at room temperature for 1 h. The crude mixture was extracted with CH_2Cl_2, evaporated to dryness and disolved in 500 mL of ethyl acetate. This organic phase was washed with 1N H_2SO_4, saturated solution of $NaHCO_3$ and brine, dried with $MgSO_4$ and evaporated to dryness yielding 1.180 g of crude material, which was chromatographed (AcOEt/$CHCl_3$ 5:2, 7:2, 13:2, 20:2, 30:2, 1:0) to give **2** (181mg, 16%), **3** (361 mg, 31%) and **4** (174 mg, 15%).

(22R,23R) 2β,3β,5α,22,23-pentahydroxystigmastan-6-one (**2**): m.p. (AcOEt (dec.)): 266.0-268.0 °C; IR ν^{CHCl_3} cm^{-1}: 3600-3200, 2960, 2860, 1700, 1465, 1385, 1245, 1010, 980; ^{1}H-NMR (300 MHz, Cl_3CD + d_6-DMSO): δ 4.08-3.96 (2H, m, αH-C-2, αH-C-3), 3.66 (1H, dd, J=1.2 Hz, 8.6 Hz, H-C-23), 3.53 (1H, dd, J1 Hz, 8.6 Hz, H-C-22), 2.78 (1H, dd, J=12.6 Hz,12.6 Hz, αH-C-7), 1.01 (3H, s, 19-CH_3), 0.98-0.92 (6H, m, 26-CH_3, 27-CH_3), 0.94 (3H, t, J=8.1 Hz, 29-CH_3), 0.89 (3H, d, J=6.3 Hz, 21-CH_3), 0.65 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD + d_6-DMSO): δ 213.0 (s, C-6), 80.0 (s, C-5), 73.9 (d, C-22), 71.9 (d, C-23), 68.7 (d) + 67.7 (d) (C-2 and C-3), 55.8 (d, C-14), 52.2 (d, C-17), 46.2 (d, C-24), 44.3 (d, C-9), 42.6 (s) + 41.6 (s) (C-10 and C-13), 41.4 (t, C-7), 39.2(t, C-12), 36.8 (d) + 36.3 (d) (C-20 and C-8), 36.1 (t, C-4), 30.4 (t, C-1), 28.7 (d, C-25), 27.3 (t, C-16), 23.5 (t, C-15), 21.1 (t, C-11), 20.9 (q, C-27), 19.3 (q, C-26), 18.6 (t, C-28), 15.7 (q, C-19), 13.3 (q, C-29), 11.7 (q, C-21), 11.6 (q, C-18); CIMS, 70 eV, m/z (rel. int.): 495 $[M+1]^+$ (100), 477 $[(M-H_2O)+1]^+$ (84), 459 $[(M-2H_2O)+1]^+$ (92), 441 $[(M-3H_2O)+1]^+$ (59), 423 $[(M-4H_2O)+1]^+$ (18); CIMS (h.r.) m/z: 495.3639 $[M+1]^+$ (calc. 495.3686).

(22R,23R) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**3**): m.p. (AcOEt): 250.0-251.0 °C; IR ν^{CHCl_3} cm^{-1}: 3600-3100, 2940, 2910, 1715, 1460, 1380, 1075, 980; ^{1}H-NMR (500 MHz, Cl_3CD): δ 4.08 (1H, dd, J=6.0 Hz, 3 Hz, βH-C-3), 3.77-3.70 (1H, m, βH-C-2), 3.63 (1H, dd, J=1.5 Hz, 8.5 Hz, H-C-23), 3.49 (1H, dd, J1 Hz, 8.5 Hz, H-C-22), 2.67 (1H, dd, J=12.5 Hz, 12.5 Hz, αH-C-7), 0.97-0.89 (m, 26, 27, 29-CH_3), 0.88 (3H, d, J=7.0 Hz, 21-CH_3), 0.75 (3H, s, 19-CH_3), 0,66 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD + d_6-DMSO): δ 210.4 (s, C-6), 78.5 (s, C-5), 72.8 (d, C-22), 70.9 (d, C-23), 68.8 (d, C-3), 66.3 (d, C-2), 55.2 (d, C-14), 51.5 (d, C-17), 45.6 (d, C-24), 44.2 (s) + 41.8 (s) (C-10 and C-13), 43.5 (d, C-9), 40.5 (t, C-7), 38.5 (t, C-8), 36.1 (d, C-20), 33.5 (t, C-4), 29.6 (t, C-1), 28.0 (d, C-25), 26.5 (t, C-16), 22.8 (t, C-15), 20.2 (q, C-27), 20.1 (t, C-11), 18.7 (q, C-26), 18.0 (t, C-28), 13.7 (q, C-19), 12.7 (q, C-29), 11.1 (q, C-21), 10.9 (q, C-18); CIMS 70 eV, m/z (rel. int.): 495 $[M+1]^+$ (3), 493 $[M-1]^+$ (3), 477 $[(M-H_2O)+1]^+$ (95), 459 $[(M-2H_2O)+1]^+$ (100), 441 $[(M-3H_2O)+1]^+$ (72), 423 $[(M-4H_2O)+1]^+$ (20); CIMS (h.r.) m/z: 477.3544 $[M-H_2O+1]^+$ (calc. 477.3580).

(22S,23S) 2α,3α,5α,22,23-pentahydroxystigmastan-6-one (**4**): m.p.(MeOH / H_2O, AcOEt): 189.0-191.0 °C; IR ν^{CHCl_3} cm^{-1}: 3650-3100, 2940, 2870, 1720, 1465, 1445, 1385, 1130, 1075, 1020,1005, 990; ^{1}H-NMR (300 MHz, Cl_3CD+ d_6-DMSO): δ 4.14-4.08 (1H, m, βH-C-3), 3.82-3.72 (1H, m, βH-C-2), 3.58-3.50 (2H, m, H-C-22, H-C-23), 2.70 (1H, dd, J=12.3Hz, 12.3 Hz, αH-C-7), 1.02 (3H, d, J=6.9 Hz, 21-CH_3), 0.94 (3H, t, J=3.3 Hz, 29-CH_3), 0.98-0.84 (m, 26, 27-CH_3), 0.76 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD): δ 211.2 (s, C-6), 79.2 (s, C-5), 71.5(d, C-22), 69.7 (d, C-23), 69.5 (d, C-3), 67.2 (d, C-2), 55.6 (d, C-14), 52.2 (d, C-17), 49.4 (d, C-24), 45.0 (s) + 43,2 (s) (C-10 and C-13), 44.2 (d, C-9), 42.1 (d, C-20), 41.2 (t, C-7), 39.2 (t, C-12), 36.8 (d, C-8), 34.2 (t, C-4), 30.1 (d, C-1), 27.5 (t, C-16), 26.5 (t, C-25), 23.9 (t, C-15),

20.8 (t, C-11), 21.7 (q, C-26), 18.2 (t, C-28), 17.5 (q, C-27), 14.4 (q, C-19), 14.1 (q, C-29), 13.7 (q, C-21), 11.6 (q, C-18); CIMS 70 eV, m/z (rel. int.): 495 $[M+1]^+$ (2), 493 $[M-1]^+$ (4), 477 $[(M-H_2O)+1]^+$ (78), 459 $[(M-2H_2O)+1]^+$ (100), 441 $[(M-3H_2O)+1]^+$ (52), 423 $[(M-4H_2O)+1]^+$ (14); CIMS (h.r.) m/z: 477.3557 $[M-H_2O+1]^+$ (calc. 477.3580).

(22E) 5-Hydroxy-5β-stigmasta-2,22-dien-6-one (11). A solution of 0.329 g (0.77 mmmol) **7** in 118 mL 10 % KOH/MeOH was refluxed for 67 h under argon. The reaction mixture was poured into 600 mL water and extracted with 3 x 500 mL AcOEt. The organic phase was washed with water, dried over $MgSO_4$ and evaporated to dryness yielding 0.484 g of yellow material; 0.465g of which were chromatographed ($SiO_2/AgNO_3$, hexane/AcOEt 12:1) to give **11** (89 mg, 28%), **7** (139 mg, 44%) and **12** (30 mg, 10%).

(22E) 5-Hydroxy-5β-stigmasta-2,22-dien-6-one (**11**): IR ν^{CHCl_3} cm^{-1}: 3600-3350, 2960, 2870, 1715, 1470, 1440, 1380, 1005, 630; ^{1}H-NMR (300 MHz, Cl_3CD): δ 5.80-5.70 (1H, m, H-C-2), 5.67-5.58 (1H, m, H-C-3), 5.14 (1H, dd, J=8.4 Hz, 15.2 Hz, H-C-22), 5.02 (1H, dd, J=8.4 Hz,15.2 Hz, H-C-23), 4.07 (1H, m, OH-C-5), 2.70 (1H, d, J=18.3 Hz, βH-C-4), 1.01 (3H, d, J=6.6 Hz, 21-CH_3), 0.84 (3H, d, J=6.6 Hz, 26-CH_3), 0.80 (3H, t, J=7.2 Hz, 29-CH_3), 0.79 (3H, d, J=7.5 Hz, 27-CH_3), 0.77 (3H, s, 19-CH_3), 0.68 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD): δ 214.2 (s, C-6), 137.9 (d, C-22), 129.6 (d, C-23), 125.1 (d) + 121.8 (d) (C-2 and C-3), 79.7 (s, C-5), 57.0 (d, C-14), 55.9 (d, C-17), 51.2 (d, C-24), 43.4 (d, C-9), 43.2 (s) + 43.1 (s) (C-10 and C-13), 41.2 (t, C-7), 40.4 (d, C-20), 39.4 (t, C-12), 37.2 (d, C-8), 35.8 (t) + 30.9 (t) (C-1 and C-4), 31.8 (d, C-25), 28.7 (t, C-16), 25.4 (t, C-28), 24.0 (t, C-15), 22.2 (t, C-11), 21.1 (q) + 21.0 (q) (C-26 and C-21), 19.0 (q, C-27), 16.5 (q, C-19), 12.2 (q, C-29), 12.2 (q, C-18).

(22E) 5-Hydroxy-5β-stigmasta-3,22-dien-6-one (**12**): IR ν^{CHCl_3} cm^{-1}: 3500-3350, 2960, 2900, 2870, 1705, 1455, 1355, 1060, 1045; ^{1}H-NMR (300 MHz, Cl_3CD): δ [6.05-5.97 (1H, m) + 5.42 (1H, m), H-C-3 and H-C-4)], 5.14 (1H, dd, J=8.4 Hz, 15.2 Hz, H-C-22), 5.02 (1H, dd, J=8.4 Hz,15.2 Hz, H-C-23), 2.39 (1H, dd, J=3.3 Hz, 12.5 Hz, βH-C-7), 2.21 (1H, dd, J=12.5 Hz, 12.5 Hz, αH-C7), 1.02 (3H, d, J=6.6 Hz, 21-CH_3), 0.84 (3H, d, J=6.3 Hz, 26-CH_3), 0.80 (3H, t, J=7.2 Hz, 29-CH_3), 0.79 (3H, d, J=6.6 Hz, 27-CH_3), 0.78 (3H, s, 19-CH_3), 0.67 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD): δ 213.4 (s, C-6), 137.9 (d, C-22), 129.7 (d, C-23), 131.2 (d) + 126.7 (d) (C-3 and C-4), 78.4 (s, C-5), 56.8 (d, C-14), 55.9 (d, C-17), 51.2 (d, C-24), 44.4 (s) + 42.9 (s) (C-10 and C-13), 43.1 (d, C-9), 43.0 (t, C-7), 40.3 (d, C-20), 39.4 (t, C-12), 38.1 (d, C-8), 31.8 (d, C-25), 28.6 (t, C-16), 27.6 (t) + 23.0 (t) (C1 and C2), 25.4 (t, C-28), 24.1 (t, C-15), 22.5 (t, C-11), 21.2 (q) + 21.1(q) (C-26 and C-21), 19.0 (q, C-27), 16.0 (q, C-19, 12.2 (q) + 12.1 (q) (C-29 and C-18).

(22R,23R) 2β,3β,5β,22,23-pentahydroxystigmastan-6-one (5) and (22S,23S) 2β,3β,5β,22,23-pentahydroxystigmastan-6-one (6). A solution of 0.707 g (1.66 mmol) **11** in 14 mL THF, free of peroxides, was slowly added dropwise to a solution of 0.085 g (0.33 mmol) OsO_4, 1.670 g (3.31 mmol) dihydroquinidine 9-*O*-(9'-phenanthryl) ether (DHQD PHN), 13.460 g (0.10 mol) N-methylmorpholine N-oxide (NMO) and 1.730 g (10.82 mmol) $Et_4N^+AcO^-$ hydrated in 27 mL THF, free of peroxides, 7 mL water and 30.5 mL t-BuOH. The mixture was stirred under argon atmosphere at 0 °C in the dark for 163 h. The mixture was treated with 212 mL of a saturated solution of $Na_2S_2O_5$ and stirred at room temperature for 1 h. The crude was extracted with 3

x 600 mL CH_2Cl_2, evaporated to dryness and disolved in 500 mL of AcOEt. The organic phase was washed with 1N H_2SO_4, a saturated solution of $NaHCO_3$ and brine, dried with $MgSO_4$ and evaporated to dryness yielding 0.957 g of crude product, 0.863 g of which were chromatographed (AcOEt/$CHCl_3$ 1:1, 4:3, 10:1, 1:0) to give **5** (222 mg, 27 %) and **6** (141 mg, 17 %).

(22R,23R) 2β,3β,5β,22,23-Pentahydroxystigmastan-6-one (**5**): m.p. (AcOEt): 237.0-239.0 °C; IR ν^{CHCl_3} cm^{-1}: 3640-3200, 2960, 2940, 2900, 2860, 2840, 1700, 1385, 1065, 1035, 1015, 1005; ^{1}H-NMR (500 MHz, Cl_3CD+d_6-DMSO): δ 4.00-3.92 (2H, m, αH-C-2, αH-C-3), 3.64 (1H, dd, J=1.5 Hz, 8.8 Hz, H-C-23), 3.52 (1H, dd, J=1.0 Hz, 8.8 Hz, H-C-22), 0.97-0.92 (m, 26, 27, 29-CH_3), 0.89 (3H, d, J=7.0 Hz, 21-CH_3), 0.68 (3H, s, 19-CH_3), 0.66 (3H, s, 18-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD+d_6-DMSO): δ 212.8 (s, C-6), 81.4 (s, C-5), 73.4 (d, C-22), 71.5 (d, C-23), 68.9 (d, C-3), 66.9 (d, C-2), 56.1 (d, C-14), 51.9 (d, C-17), 46.0 (d, C-24), 44.8 (d, C-9), 43.8 (s) + 42,2 (s) (C-10 and C-13), 41.4 (t, C-7), 38.9 (t, C-12), 37.2 (d, C-8), 36.5 (d, C-20), 35.3 (t) + 35.1 (t) (C-1 and C-4), 28.4 (d, C-25), 27.0 (t, C-16), 23.3 (t, C-15), 21.7 (t, C-11), 20.7 (q, C-27), 19.1 (q, C-26), 18.4 (t, C-28), 15.9 (q, C-19), 13.1 (q, C-29), 11.5 (q, C-21), 11.3 (q, C-18); CIMS 70 eV, m/z (rel. int.): 495 $[M+1]^+$, (9), 477 $[(M-H_2O)+1]^+$, (56), 459 $[(M-2H_2O)+1]^+$, (100), 441 $[(M-3H_2O)+1]^+$, (51), 423 $[(M-4H_2O)+1]^+$, (17); CIMS (h.r.) m/z: 477.3481 $[M-H_2O+1]^+$ (calc. 477.3580).

(22S,23S) 2,3,5,22,23-Pentahydroxystigmastan-6-one (**6**): m.p. (AcOEt): 133.0-135.0 °C; IR ν^{CHCl_3} cm^{-1}: 3650-3150, 2950, 2870, 1705, 1465, 1385, 1055, 1030, 1010, 990; ^{1}H-NMR (300 MHz, $CDCl_3$+ d_6-DMSO): δ 4.05-3.95 (2H, m, αH-C-2, αH-C-3), 3.59-3.52 (2H, m, H-C-22, H-C-23), 1.02 (3H, d, J=6.9 Hz, 21-CH_3), 0.96 (3H, t, J=7.2 Hz, 29-CH_3), 0.93 (3H, d, J=7.2 Hz, 26-CH_3), 0.87 (3H, d, J=6.9 Hz, 27-CH_3), 0.68 (6H, s, 18-CH_3, 19-CH_3); ^{13}C-NMR (75 MHz, Cl_3CD+ d_6-DMSO): δ 213.1 (s, C-6), 81.8 (s, C-5), 71.7 (d, C-22), 69.9 (d, C-23), 69.4 (d, C-3), 67.3 (d, C-2), 56.2 (d, C-14), 52.2 (d, C-17), 49.5 (d, C-24), 45.3 (d, C-9), 44.2 (s) + 43.3 (s) (C-10 and C-13), 42.2 (d, C-20), 41.7 (t, C-7), 39.2 (t, C-12), 37.5 (d, C-8), 35.6 (t) + 35.3 (t) (C-1 and C-4), 27.7 (t, C-16), 26.6 (d, C-25), 24.1 (t, C-15), 22.1 (t, C-11), 21.7 (q, C-26), 18.4 (t, C-28), 17.6 (q, C-27), 16.3 (q, C-19), 14.2 (q, C-29), 13.8 (q, C-21), 11.6 (q, C-18); CIMS 70 eV, m/z (rel. int.): 495 $[M+1]^+$, (10), 477 $[(M-H_2O)+1]^+$, (64), 459 $[(M-2H_2O)+1]^+$, (100), 441 $[(M-3H_2O)+1]^+$, (52), 423 $[(M-4H_2O)+1]^+$, (12); CIMS (h.r.) m/z: 477.3550 $[M-H_2O+1]^+$ (calc. 477.3580).

Acknowledgments:

We wish to thank Dr. F. Lafont from the Universidad de Córdoba for the high resolution mass spectra. Lidia Soca gratefully thanks the Generalitat de Catalunya, Spain, for a doctoral fellowship.

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