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Synthesis of aromatic stigmastanes: application to the synthesis of aromatic analogs of brassinosteroids

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ABSTRACT

In this paper, we report the first synthesis of aromatic analogs of brassinosteroids. In order to accomplish this task, we explored two different synthetic approaches, which involved demethylation of the C-19 of stigmasterol to yield A-ring aromatic 3-hydroxystigmastanes. One of the new aromatic analogs showed a reduced but significant bioactivity when compared to the natural hormones.

1. Introduction

In 1979, Groove et al. showed that the unique growth-promoting activity of *Brassica* pollen extracts was conferred by brassinolide (**1**), a steroid with an unusual lactone B-ring structure and a dihydroxylated side chain.[1] Since then, brassinolide and more than 60 related compounds have been isolated from a wide variety of plant species, and the biological activity of this class of molecules—known as brassinosteroids (BRs)—has been studied in great detail.[2–4] Their exogenous application leads to a spectrum of growth responses, such as stem elongation, inhibition of root growth, leaf epinasty, and xylem differentiation, which are partly brought about by changes in enzyme activity and gene expression. BRs are able to increase yield and stress resistance in a number of commercially important crops, particularly when grown under conditions of biotic or abiotic stress.[5]

Brassinolide (**1**) is a C28 brassinosteroid bearing an *S*-methyl group at C-24 in the side chain of its 5α-ergostane structure. Although C28 brassinosteroids are the most ubiquitous in nature, other BRs with a different steroidal side chain are known.[6] Among them, 28-homobrassinolide (HBL) (**2**) and 28-homocastasterone (HCS) (**3**), which have a 5α-stigmastane structure, are the most active C29 BRs (Fig. 1).

In the past few years, important progress has been made in understanding how BRs are perceived and how the information is transduced to promote genomic responses. In contrast to animal steroid signals, BRs are perceived by a plasma membrane-localized receptor kinase. This kinase is encoded by the BRI1 gene, which is part of a large, plant-specific family of leucine-rich repeat receptor-like kinases (LLR-LRK).[7] The structure of the *Arabidopsis thaliana* BRI1 ligand-binding domain has been recently determined by X-ray diffraction, which revealed the molecular mechanisms underlying the recognition of BRs.[8,9] Both the alkyl side chain of the hormones and the C and D rings fit into a pocket formed by hydrophobic residues. A few polar interactions with the diol moiety at C22 and C23 of brassinolide are established with a tyrosine residue in the binding domain and are mediated by water molecules. Interestingly, the 2α,3α-diol moiety, which is known to be important for biological activity, is exposed to the solvent. On the other hand, the A and B rings make hydrophobic contacts through their β face with a concave surface of the receptor.

We are interested in exploring the effect that a radical structural change would have upon the binding ability of the BRs. Thus, we designed two new analogs in which the alicyclic A-ring was replaced by an aromatic ring. It is well known from the mammalian steroid hormones that such a change has profound biological effects (e.g., androgens vs estrogens) that are associated to the binding affinities to the respective cellular receptors. The synthesis of ring-A aromatic analogs of brassinosteroids (**4** and **5**, Fig. 1), implied achieving the first chemical synthesis of aromatic 3-hydroxystigmastanes.

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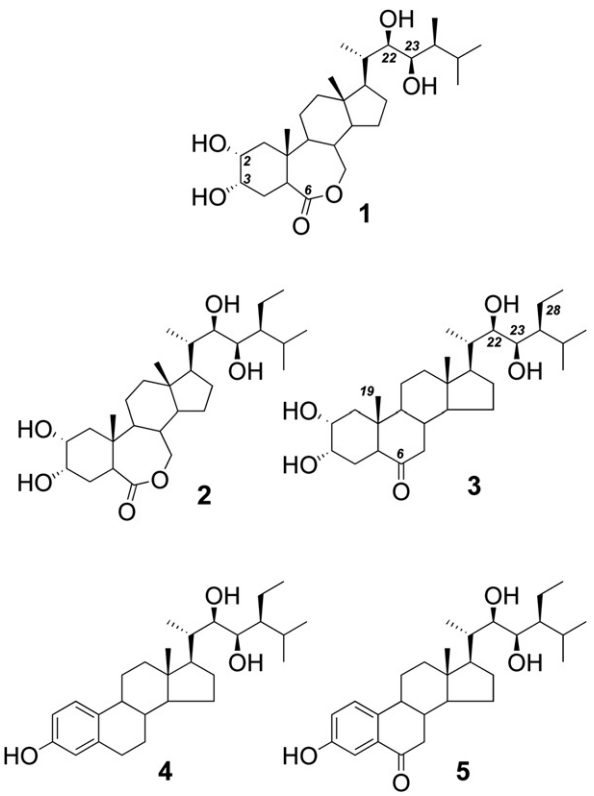


Fig. 1. Structure of natural brassinosteroids (**1**–**3**) and synthesized aromatic analogs.

2. Results and discussion

Any partial synthesis of ring-A aromatic steroids starting from available natural sources must encompass demethylation of the C-19 at some stage. A bibliographic search yielded two previously reported methodologies that could be suitable for our task.

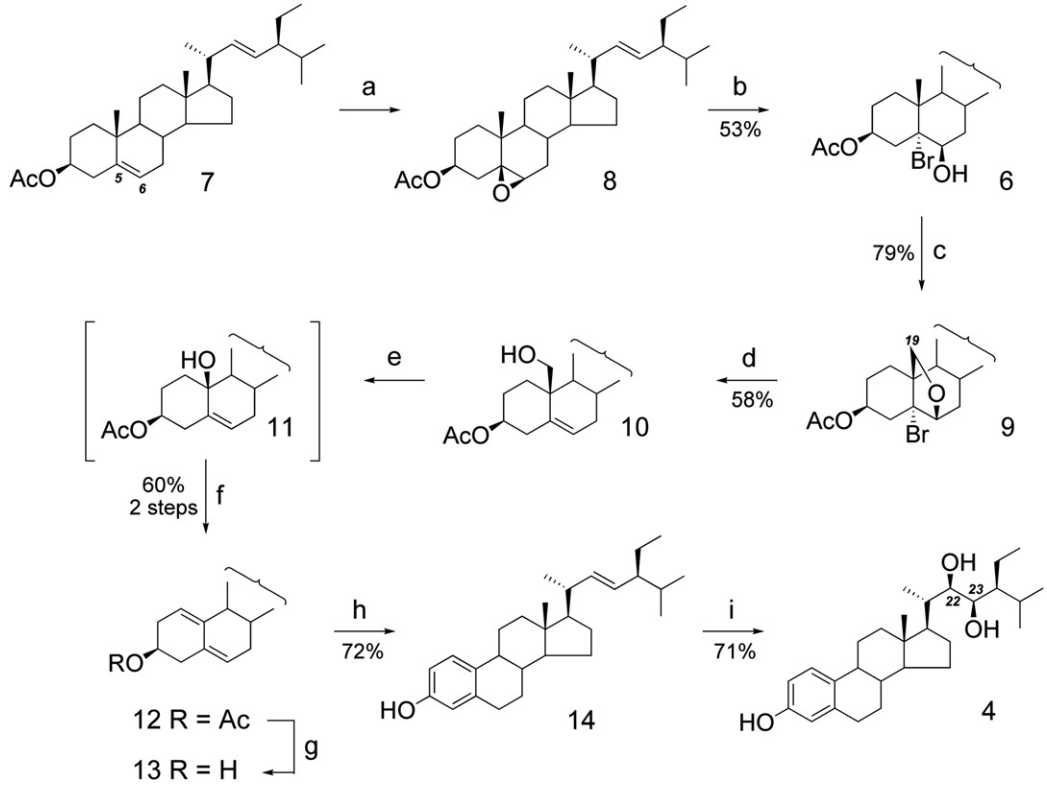
The first involves the thallium(III)-mediated degradation of a 19-hydroxy steroid,[10] which could be prepared from stigmasterol, the common starting material for the synthesis of 28-homobrassinosteroid. This approach is depicted in Scheme 1.

Functionalization of C-19 was achieved adapting the classical strategy,[11,12] which involves the remote oxidation of a 5α-bromo-6β-hydroxy intermediate **6**. In our case, the usual preparation of the bromohydrine using *N*-bromosuccinimide was not feasible due to the presence of two double bonds in the starting steroid. Thus, stigmasteryl acetate (**7**) was regio- and stereoselectively epoxidated at the Δ^5 double bond with a mixture of $KMnO_4$ and $Fe(NO_3)_3$, affording the known epoxide **8**.[13] Treatment of **8** with HBr yielded 53% of the desired intermediate **6** (41% yield in three steps from stigmasterol).

The photochemically induced oxidation of the axial 6β-hydroxyl of compound **6** generates an oxy radical[10] that attacks the axial methyl and leads to the 6,19 cyclic ether **9**, which was hydrolytically opened to the 19-hydroxysteroid **10**.

When **10** reacted with thallium(III) nitrate in dioxane, a rapid disappearance of the steroid was observed by TLC, with the concomitant formation of a more polar product. NMR analysis of the crude reaction suggested the formation of the 10-hydroxysteroid **11** (the signals for the H-19 were absent in the ^{1}H NMR and a quaternary carbon at 77.1 ppm was observed). Nevertheless, attempts to isolate this product by silica chromatography failed and the elimination product **12** was obtained instead, with 60% yield.

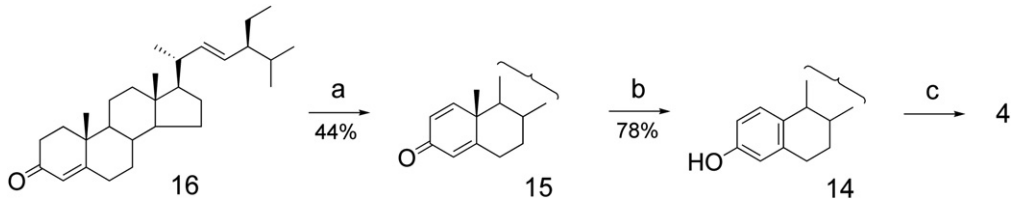
Further hydrolysis of the 3β-acetate followed by an Oppenauer oxidation of the corresponding alcohol yielded the desired



Scheme 1. Synthesis of aromatic brassinosteroid **4**. Reagents and conditions: (a) $KMnO_4/Fe(NO_3)_3/t$-BuOH/H_2O/CH_2Cl_2, rt; (b) HBr/AcOH/THF, rt; (c) $Pb(AcO)_4/CaCO_3/I_2$/cyclohexane/hν, reflux; (d) Zn/AcOH/*i*-PrOH, reflux; (e) $Tl(NO_3)_3/HClO_4/H_2O$/dioxane, rt; (f) K_2CO_3/H_2O/methanol, rt; (g) Al(*i*-OPr)$_3$/1-benzyl-4-piperidone/toluene, reflux; (h) $K_2OsO_4/K_3Fe(CN)_6/(DHQD)_2PhAl/K_2CO_3/t$-BuOH/$H_2O/CH_3SO_3NH_2$, rt.

aromatized 19-norstigmastane **14**, which was stereoselectively dihydroxylated to give **4** using the Sharpless methodology with $(DHQD)_2Phal$, seeking to favor the formation of the diol with 22*R*,23*R* configuration, which is characteristic of natural BRs. The configuration of the resulting side chain diol was established by comparison with chemical shifts and coupling constants of known closely related structures.[14]

The second aromatization strategy was adapted from the work by Dryden Jr. et al., in which a reductive demethylation is involved[15] (Scheme 2).



Scheme 2. Alternative synthesis of compound **4**. Reagents and conditions: (a) DDQ/dioxane, reflux; (b) biphenyl/CH_2Ph_2/Li/THF, reflux; (c) $K_2OsO_4/K_3Fe(CN)_6/(DHQD)_2Phal/K_2CO_3/$*t*-BuOH/$H_2O/CH_3SO_3NH_2$, rt.

The steroidal dienone **15**[16] was obtained from the known enone **16** and treated with lithium/biphenyl/diphenylmethane to give the aromatic steroid **14** in good yield. This second synthetic approach resulted to be much more shorter and efficient than that shown in Scheme 1, but implies harsher reaction conditions.

The synthesis of the 6-oxo analog **5** is depicted in Scheme 3. The 6,19 cyclic ether **9** was opened with zinc in acetic acid/water to afford the 3β,19-diacetate. Selective hydrolysis of the 3β-acetate **17** was achieved with K_2CO_3, and the resulting alcohol **18** was oxidized with Jones reagent to the corresponding conjugated diketone **19**. It is known that this type of steroidal structure can aromatize in basic media. Then, treatment of **19** with KOH in methanol yielded the desired aromatic 6-oxo-19-norstigmastane **20**, which was further dihydroxylated as previously described to give compound **5**.

The bioactivity of the new analogs as plant growth promoters was evaluated using a modified version of the rice lamina inclination test (RLIT), which has been widely used as a sensitive and specific bioassay for BRs.[17]

In order to assess the influence of the aromatization of the A-ring on the bioactivity, the natural brassinosteroid 28-homoteasterone[18] (**21**, Scheme 4) and its putative biosynthetic precursor 6-deoxo-28-homoteasterone **22** (whose synthesis from the known steroid **23**[19] is depicted in Scheme 4) were co-evaluated.

Fig. 2 shows the bioactivity of the compounds tested at a 1 nmol/plant dose. First, 6-deoxo-28-homoteasterone **22** and its aromatic counterpart **5** were inactive, thus confirming that an oxygenated moiety at C-6 is essential for activity.[2]

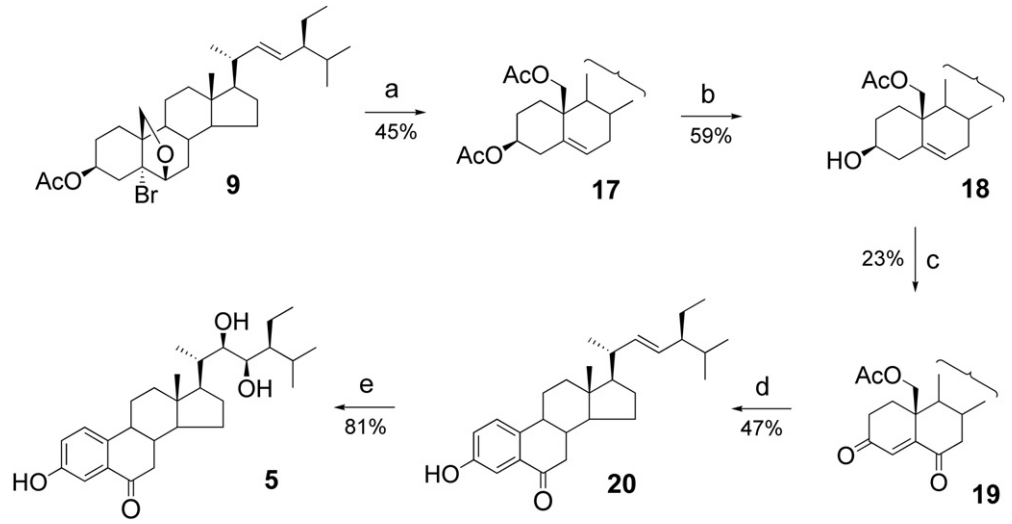
On the other hand, the aromatic analog **4** showed a significant bioactivity, only 30% lower than that of the natural compound 28-homotasterone **21**, suggesting that the binding to BRI1 is not substantially affected by the aromatization of the A-ring on the steroidal structure.

3. Conclusions

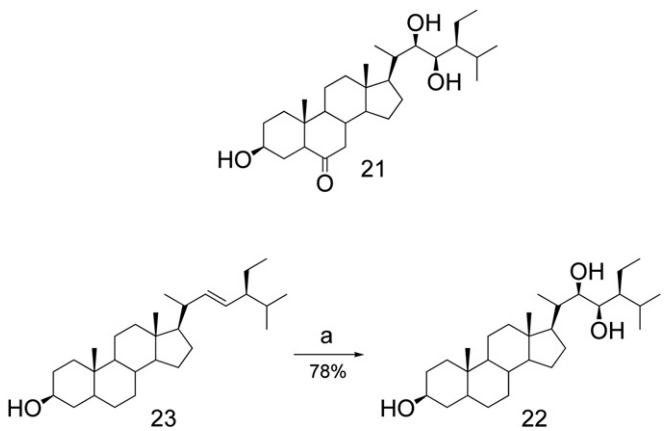
Although several aromatic stigmastanes are known in nature, mainly as diagenetic products from plant-derived sitosterol,[20] there are few studies on the chemical synthesis of this class of compounds. Since aromatic steroids are a biologically important group (e.g., estrogens), their synthesis constitutes an interesting task.

In the case of aromatic 3-hydroxystigmastanes, the only previous report used a microbial transformation as the aromatization step.[21] In this paper, we showed the feasibility of two alternative chemical approaches to obtain these compounds that might enrich the efforts directed to the discovery of new biological active steroids.

In fact, we applied these approaches to the synthesis of the first aromatic analogs of brassinosteroids. The preliminary results shown on their biological activity could not only give new insights into the structural mechanism of BRs recognition by BRI1, but also open new perspectives to design nonsteroidal mimetics of BRs, in



Scheme 3. Synthesis of aromatic brassinosteroid **5**. Reagents and conditions: (a) Zn/AcOH/H_2O, reflux; (b) K_2CO_3/H_2O/THF/methanol, rt; (c) Jones reagent/acetone, 0 °C; (d) KOH/MeOH/H_2O, rt; (e) $K_2OsO_4/K_3Fe(CN)_6/(DHQD)_2Phal/K_2CO_3$/t-BuOH/$H_2O/CH_3SO_3NH_2$, rt.



Scheme 4. Structure of the natural brassinosteroid 28-homoteasterone (**21**) and synthesis of its 6-deoxo analog **22**. Reagents and conditions: (a) $K_2OsO_4/K_3Fe(CN)_6/(DHQD)_2PhAl/K_2CO_3/t$-BuOH/$H_2O/CH_3SO_3NH_2$, rt.

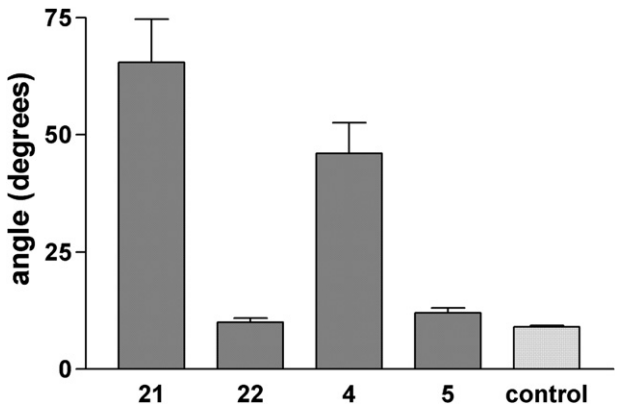


Fig. 2. Bioactivity of the new analogs in the rice lamina inclination test. Mean values from 30 replicates. Control (ethanol): 11±3°. Error bars indicate the standard error.

which the ring A of the natural compounds might be mimicked by aromatic moieties without loss of activity.

4. Experimental

4.1. Synthesis

4.1.1. General. All the solvents and reagents were purchased from Sigma–Aldrich Chemical Co and were of analytical grade. ESI-HRMS were measured on a Bruker micrOTOF-Q II. Melting points were determined on a Fisher Johns apparatus and are uncorrected. NMR spectra were recorded in $CDCl_3$ either on a Bruker AM-500 (500 MHz for ^1H and 125.1 MHz for ^{13}C) or on a Bruker AC-200 (200 MHz for ^1H and 50 MHz for ^{13}C). Chemical shifts (δ) are given in parts per million downfield from TMS as the internal standard. Coupling constant (J) values are in hertz. All new compounds gave satisfactory combustion analysis data on an Exeter CE 440 Elemental Analyzer, and their structures were confirmed by 1-D and 2-D NMR spectroscopic analyses. ESI-HRMS of the final compounds were measured on a Bruker micrOTOF-Q II.

*4.1.2. (22E)-3β-Acetoxy-5α-bromostigmast-22-en-6β-ol (**6**).* 3β-Acetoxy-5β,6β-epoxystigmast-22-ene[13] (**8**) (1.4 g, 2.97 mmol) was dissolved in THF (150 mL) and a solution of 0.1% bromhydric acid in acetic acid was added dropwise until the reaction was completed. Then, the reaction mixture was washed with brine and a $NaHCO_3$ saturated solution. The organic layer was dried with sodium sulfate and evaporated under reduced pressure. The crude product was separated by silica column chromatography (hexane/EtOAc gradient) to yield bromohydrine **6** (863 mg, 53%). Mp: 109–110 °C. ^1H NMR ($CDCl_3$): 0.70 (H-18, 3H, s); 0.79 (H-26, 3H, d, J=6.4); 0.80 (H-29, 3H, t, J=7.1); 0.85 (H-27, 3H, d, J=6.4); 1.01 (H-21, 3H, d, J=6.6); 1.32 (H-19, 3H, s); 2.03 (CH_3CO–, 3H, s); 2.51 (H-4β, 1H, dd, J=10.5 and 13.5); 4.18 (H-6α, 1H, m); 5.00 (H-23, 1H, dd, J=8.1 and 15.2); 5.16 (H-22, 1H, dd, J=8.1 and 15.2); 5.47 (H-3α, 1H, m). ^{13}C NMR ($CDCl_3$): 12.3 and 12.4 (C-18 and C-29); 18.0 (C-19); 19.0 (C-26); 21.1 and 21.4 (C-21 and C-27); 21.2 (*C*H_3CO–); 21.3 (C-11); 24.1 (C-15); 25.4 (C-28); 26.4 (C-2); 28.9 (C-16); 30.6 (C-8); 31.9 (C-25); 34.6 (C-7); 35.1 (C-1); 38.4 (C-4); 39.6 (C-12); 40.4 (C-10); 40.5 (C-20); 42.6 (C-13); 47.5 (C-9); 51.2 (C-24); 55.8 and 55.9 (C-14 and C-17); 72.2 (C-3); 75.7 (C-6); 86.8 (C-5); 129.3 (C-23); 138.2 (C-22); 170.5 (CH_3CO–). Anal. Calcd for $C_{31}H_{51}BrO_3$: C, 67.50; H, 9.32. Found: C, 67.56; H, 9.44.

*4.1.3. (22E)-3β-Acetoxy-5α-bromo-6,19-epoxystigmast-22-ene (**9**).* A mixture of compound **6** (863 mg, 1.56 mmol), lead(IV) acetate (3.67 g, 8.27 mmol), $CaCO_3$ (1.15 g, 11.47 mmol), I_2 (711 mg, 2.80 mmol), and cyclohexane (150 mL) was heated under reflux for 30 min and with irradiation by a 300-W tungsten lamp. The mixture was then filtered through a bed of silica gel, which was washed with ethyl ether. The filtrate was sequentially washed with $NaHSO_3$ 10% and brine and then dried with Na_2SO_4. Evaporation of the solvent gave an oil, which was purified by column chromatography (hexane/EtOAc=99:1) to afford compound **9** (680 mg, 79%). Mp: 113–115 °C. ^1H NMR ($CDCl_3$): 0.72 (H-18, 3H, s); 0.78 (H-26, 3H, d, J=6.2); 0.81 (H-29, 3H, *t*, J=6.0); 0.84 (H-27, 3H, d, J=7.1); 1.00 (H-21, 3H, d, J=6.6); 2.03 (CH_3CO–, 3H, s); 3.75 (H-19a, 1H, d, J=8.3); 3.93 (H-19b, 1H, d, J=8.3); 4.07 (H-6α, 1H, d, J=4.2); 5.00 (H-23, 1H, dd, J=8.1 and 15.2); 5.15 (H-22, 1H, dd, J=7.8 and 15.1); 5.21 (H-3α, 1H, m). ^{13}C NMR ($CDCl_3$): 12.3 and 12.6 (C-18 and C-29); 19.0 (C-26); 21.1 (*C*H_3CO–); 21.1 and 21.3 (C-21 and C-27); 22.7 (C-11); 23.3 (C-1); 23.5 (C-15); 25.4 (C-28); 26.9 (C-2); 29.0 (C-16); 31.9 (C-25); 32.8 (C-7); 33.3 (C-8); 39.6 (C-4); 40.5 (C-20); 41.3 (C-12); 43.1 (C-13); 45.9 (C-10); 48.7 (C-9); 51.2 (C-24); 54.5 (C-14); 55.8 (C-17); 67.5 (C-19); 70.0 (C-3); 74.5 (C-5); 82.3 (C-6); 129.4 (C-23); 138.2 (C-22); 170.4 (CH_3CO–). Anal. Calcd for $C_{31}H_{49}BrO_2$: C, 69.77; H, 9.26. Found: C, 69.70; H, 9.20.

*4.1.4. (22E)-3β-Acetoxystigmasta-5,22-dien-19-ol (**10**).* Zinc powder (116 mg) and acetic acid (0.1 mL) were added to a solution of **9** (300 mg, 91 μmol) in recently distilled *i*-PrOH (7 mL) and heated under reflux. The mixture was vigorously stirred for 24 h, filtered through a bed of silica gel, which was washed with EtOAc and the solvent evaporated. The resulting residue was taken with dichloromethane, washed with $NaHCO_3$ and water, and dried with Na_2SO_4. Purification by column chromatography (hexane/EtOAc=93:7) gave compound **10** (150 mg, 58%).

Mp: 109–110 °C. ^1H NMR ($CDCl_3$): 0.77 (H-18, 3H, s); 0.81 (H-26, 3H, d, J=6.6); 0.82 (H-29, 3H, t, J=7.5); 0.86 (H-27, 3H, d, J=6.1); 1.04 (H-21, 3H, d, J=6.6); 2.05 (CH_3CO–, 3H, s); 2.44 (H-4α, 1H, ddd, J=2.2, 5.1 and 13.2); 3.64 (H-19a, 1H, m); 3.85 (H-19b, 1H, d, J=11.6); 4.66 (H-3α, 1H, m); 5.03 (H-23, 1H, dd, J=8.7 and 15.2); 5.17 (H-22, 1H, dd, J=8.7 and 15.2); 5.79 (H-6, 1H, m). ^{13}C NMR ($CDCl_3$): 12.3 and 12.4 (C-18 and C-29); 19.0 (C-26); 21.1 and 21.2 (C-21 and C-27); 21.4 (*C*H_3CO–); 21.7 (C-11); 24.2 (C-15); 25.4 (C-28); 28.1 (C-2); 28.9 (C-16); 31.2 (C-7); 31.9 (C-25); 33.1 (C-1); 33.4 (C-8); 38.2 (C-4); 39.9 (C-12); 40.5 (C-20); 41.6 (C-10); 42.4 (C-13); 50.3 (C-9); 51.2 (C-24); 55.9 (C-14); 57.7 (C-17); 62.7 (C-19); 73.4 (C-3); 128.3 (C-6); 129.3 (C-23); 134.5 (C-5); 138.3 (C-22); 170.5 (CH_3CO–). Anal. Calcd for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found: C, 79.13; H, 10.75.

*4.1.5. (22E)-3β-Acetoxy-19-norstigmasta-1(10),5,22-triene (**12**).* Water (10 μL), 10% aqueous perchloric acid (1 μL), and thallium(III) nitrate trihydrate (29 mg, 65 μmol) were successively added to a solution of **10** (100 mg, 51 μmol) in dioxane (4 mL) and the mixture then stirred at room temperature for 30 min. The mixture was then diluted with ether and filtered through silica gel, and the filtrate was washed with NaCl and $NaHCO_3$ saturated solutions and dried with Na_2SO_4. Purification by column chromatography (hexane/EtOAc 97:3) gave compound **12** (48 mg, 60%). Mp: 132 °C. ^{1}H NMR ($CDCl_3$): 0.70 (H-18, 3H, s); 0.80 (H-26, 3H, d, *J*=6.4); 0.81 (H-29, 3H, t, *J*=7.3); 0.85 (H-27, 3H, d, *J*=6.6); 1.04 (H-21, 3H, d, *J*=6.7); 2.04 (CH_3CO–, 3H, s); 3.67 (H-3α, 1H, m); 5.02 (H-23, 1H, dd, *J*=8.7 and 15.2); 5.16 (H-22, 1H, dd, *J*=8.7 and 15.2); 5.38 (H-6, 1H, m); 5.48 (H-1, 1H, d, *J*=5.5). ^{13}C NMR ($CDCl_3$): 12.1 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1 and 21.2 (C-21 and C-27); 21.5 (CH_3CO–); 24.0 (C-11); 24.5 (C-15); 25.4 (C-28); 29.0 (C-16); 31.4 (C-2); 31.6 (C-7); 31.9 (C-25); 35.9 (C-4); 37.9 (C-8); 39.3 (C-12); 40.5 (C-20); 42.5 (C-13); 42.6 (C-9); 51.3 (C-24); 56.0 (C-14); 56.4 (C-17); 69.5 (C-3); 115.3 (C-1); 124.6 (C-6); 129.3 (C-23); 130.8 (C-5); 138.3 (C-10); 138.4 (C-22); 170.9 (CH_3CO–). Anal. Calcd for $C_{30}H_{46}O_2$: C, 82.14; H, 10.57. Found: C, 82.06; H, 10.53.

*4.1.6. (22E)-19-Norstigmasta-1(10),5,22-trien-3β-ol (**13**).* The acetate **12** (48 mg, 0.11 mmol) in methanol (7 mL) was treated with K_2CO_3 saturated solution at room temperature overnight. The mixture was poured on NH_4Cl saturated solution (20 mL) and then concentrated in vacuo to about 1/5, the residue was diluted with EtOAc and NaCl saturated solution. The organic layer was washed with brine and dried with Na_2SO_4. The crude product was purified by column chromatography (hexane/EtOAc 9:1) to obtain compound **13** (43 mg; 99%). Mp: 107–110 °C. ^{1}H NMR ($CDCl_3$): 0.70 (H-18, 3H, s); 0.80 (H-26, 3H, d, *J*=6.6); 0.81 (H-29, 3H, t, *J*=7.2); 0.85 (H-27, 3H, d, *J*=6.6); 1.03 (H-21, 3H, d, *J*=6.6); 4.05 (H-3α, 1H, m); 5.02 (H-23, 1H, dd, *J*=8.7 and 15.0); 5.16 (H-22, 1H, dd, *J*=8.7 and 15.0); 5.35 (H-6, 1H, m); 5.55 (H-1, 1H, d, *J*=4.7). ^{13}C NMR ($CDCl_3$): 12.1 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1 and 21.2 (C-21 and C-27); 23.9 (C-11); 24.5 (C-15); 25.4 (C-28); 29.0 (C-16); 31.7 (C-7); 31.9 (C-25); 34.8 (C-2); 38.2 (C-8); 39.1 (C-4); 39.3 (C-12); 40.5 (C-20); 42.5 (C-13); 42.5 (C-9); 51.3 (C-24); 56.0 (C-14); 56.5 (C-17); 66.0 (C-3); 115.3 (C-1); 126.0 (C-6); 129.3 (C-23); 130.2 (C-5); 138.2 (C-10); 138.3 (C-22). Anal. Calcd for $C_{28}H_{44}O$: C, 84.79; H, 11.18. Found: C, 84.59; H, 11.25.

*4.1.7. (22E)-19-Norstigmasta-1,3,5(10),22-tetraen-3-ol (**14**).* An aliquot of ca. 3 mL was distilled off from a mixture of **13** (43 mg, 0.11 mmol) and 1-bencyl-4-piperidone (0.1 mL) in toluene (30 mL). Aluminum isopropoxide (22 mg, 0.11 mmol) was then added and the mixture was refluxed for 5 h. After cooling, the mixture was diluted with ether, washed successively with ice-cold 5% aqueous HCl (three times) and water (three times), and dried with Na_2SO_4. The solvent was evaporated, and the crude product purified by column chromatography (hexane/EtOAc 97:3) to obtain compound **14** (31 mg; 72%). Mp: 129–130 °C. ^{1}H NMR ($CDCl_3$): 0.71 (H-18, 3H, s); 0.81 (H-29, 3H, t, *J*=7.5); 0.81 (H-26, 3H, d, *J*=7.2); 0.86 (H-27, 3H, d, *J*=6.4); 1.05 (H-21, 3H, d, *J*=6.6); 2.81 (H-6, 1H, m); 4.78 (HO–, 1H, br s); 5.03 (H-23, 1H, dd, *J*=8.7 and 15.2); 5.17 (H-22, 1H, dd, *J*=8.7 and 15.2); 6.55 (H-4, 1H, d, *J*=2.6); 6.61 (H-2, 1H, dd, *J*=2.6 and 8.3); 7.14 (H-1, 1H, d, *J*=8.3). ^{13}C NMR ($CDCl_3$): 12.2 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1 and 21.2 (C-21 and C-27); 24.0 (C-15); 25.4 (C-28); 26.7 (C-11); 27.6 (C-7); 29.0 (C-16); 29.7 (C-6); 31.9 (C-25); 38.8 (C-8); 39.8 (C-12); 40.5 (C-20); 42.7 (C-13); 43.7 (C-9); 51.2 (C-24); 55.5 (C-14); 56.2 (C-17); 112.5 (C-2); 115.2 (C-4); 126.5 (C-1); 129.3 (C-23); 133.1 (C-10); 138.3 (C-22); 138.4 (C-5); 153.1 (C-3). Anal. Calcd for $C_{28}H_{42}O$: C, 85.22; H, 10.73. Found: C, 85.19; H, 10.69.

*4.1.8. (22R,23R)-19-Norstigmasta-1,3,5(10)-triene-3,22,23-triol(**4**).* A mixture of **14** (175 mg, 0.44 mmol), *tert*-butanol/water (1:1, 8 mL), $(DHQD)_2$Phal (138 mg, 0.18 mmol), methansulfonamide (84 mg, 0.89 mmol), potassium ferricyanide (876 mg, 2.66 mmol), potassium carbonate (368 mg, 2.66 mmol), and potassium osmate dihydrate (16 mg, 44 μmol) was stirred at room temperature for 7 days. An excess of $NaHSO_3$ was added until no evolution of bubbles was observed. Layers were separated and the aqueous phase was thoroughly extracted with CH_2Cl_2/MeOH (95:5). Combined organic layers were washed with 0.25 M H_2SO_4 and 2% NaOH. Purification by column chromatography (hexane/EtOAc gradient) yielded compound **4** (135 mg; 71%). Mp: 181–182 °C. ^{1}H NMR ($CDCl_3$/CD_3OD 9:1): 0.75 (H-18, 3H, s); 0.94 (H-21, 3H, d, *J*=6.1); 0.96 (H-26, 3H, d, *J*=6.3); 0.97 (H-27, 3H, d, *J*=7.6); 0.97 (H-29, 3H, t, *J*=6.9); 2.83 (H-6, 1H, m); 3.60 (H-22, 1H, dd, *J*=1.0 and 8.6); 3.70 (H-23, 1H, dd, *J*=1.5 and 8.5); 4.29 (HO–, 1H, br s); 6.54 (H-4, 1H, d, *J*=2.6); 6.60 (H-2, 1H, dd, *J*=2.6 and 8.5); 7.11 (H-1, 1H, d, *J*=8.5). ^{13}C NMR ($CDCl_3$): 11.1 (C-18); 11.2 (C-21); 12.9 (C-29); 18.4 (C-28); 18.7 (C-26); 20.3 (C-27); 23.2 (C-15); 26.2 (C-11); 27.1 (C-7); 27.2 (C-16); 28.5 (C-25); 29.1 (C-6); 36.7 (C-20); 38.5 (C-8); 39.5 (C-12); 42.1 (C-13); 43.2 (C-9); 46.2 (C-24); 52.3 (C-17); 54.9 (C-14); 71.8 (C-23); 73.8 (C-22); 112.0 (C-2); 114.5 (C-4); 125.6 (C-1); 131.3 (C-10); 137.3 (C-5); 153.8 (C-3). Anal. Calcd for $C_{28}H_{44}O_3$: C, 78.46; H, 10.35. Found: C, 78.49; H, 10.48.

*4.1.9. (22E)-Stigmasta-1,4,22-trien-3-one (**15**).* A solution of Δ^4-3-keto-steroid **16** (700 mg, 1.71 mmol) and DDQ (1.16 g, 5.12 mmol), in 100 mL of dry dioxane, was heated under reflux for 1 day. The suspension was concentrated in vacuo to about 1/5 and the residue was diluted with EtOAc (100 mL). The organic layer was consecutively washed with 10% $NaHSO_3$ (2×100 mL), 5% NaOH (2×100 mL), and brine, and then dried with Na_2SO_4. The solvent was evaporated and the crude product purified by column chromatography (hexane/EtOAc 92:8) to obtain compound **15** (306 mg; 44%). Mp: 101–102 °C (lit.: 100–101 °C.)[14]

*4.1.10. Alternative synthesis of (22E)-19-norstigmasta-1,3,5(10),22-tetraen-3-ol (**14**).* Biphenyl (325 mg, 211 mmol) and diphenylmethane (246 mg, 1.46 mmol) were dissolved in dry tetrahydrofuran (50 mL), and lithium metal (40 mg, 5.70 mmol) was added under nitrogen. The mixture was refluxed with rapid stirring until a deep green color persisted. A solution of the ketone **15** (306 mg, 0.75 mmol) in dry tetrahydrofuran (5 mL) was added dropwise to the mixture and reflux was continued for another 5 min. The mixture was cooled to 0 °C and methanol was added slowly to destroy the excess of lithium, after which it was diluted with EtOAc, washed successively with brine, $NaHCO_3$ saturated solution and brine, and dried with Na_2SO_4. The solvent was evaporated and the crude product purified by column chromatography (hexane/EtOAc 95:5) to obtain compound **14** (230 mg; 78%).

*4.1.11. (22E)-3β,19-Diacetoxystigmasta-5,22-diene (**17**).* Zinc powder (1.1 g) was added to a solution of **9** (300 mg, 0.55 mmol) in acetic acid/water (15:1, 48 mL), and heated to 120 °C. The mixture was vigorously stirred for 3 days at that temperature, filtered through a bed of silica gel, which was washed with EtOAc and the solvent evaporated. The resulting residue was taken with dichloromethane, washed with $NaHCO_3$ and NaCl saturated solutions, and dried with Na_2SO_4. Purification by column chromatography (hexane/EtOAc=97:3) gave *19-acetate steroid* ***17*** (126 mg, 45%). Mp: 180–182 °C. ^{1}H NMR ($CDCl_3$): 0.71 (H-18, 3H, s); 0.78 (H-26, 3H, d, *J*=6.2); 0.81 (H-29, 3H, *t*, *J*=6.0); 0.84 (H-27, 3H, d, *J*=7.1); 1.01 (H-21, 3H, d, *J*=6.6); 2.02 (CH_3CO–, 3H, s); 2.05 (CH_3CO–, 3H, s); 3.98 (H-19a, 1H, d, *J*=11.7); 4.46 (H-19b, 1H, d, *J*=11.7); 4.63 (H-3α, 1H, m); 5.00 (H-23, 1H, dd, *J*=8.1 and 15.2); 5.16 (H-22, 1H, dd, *J*=8.1 and 15.2); 5.62 (H-6, 1H, d, *J*=4.6). ^{13}C NMR ($CDCl_3$): 12.1 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1, 21.1, 21.2 and 21.4 (2×CH_3CO–, C-21 and C-27); 21.7 (C-11); 24.3 (C-15); 25.4 (C-28);

27.9 (C-2); 28.9 (C-16); 31.3 (C-7); 31.9 (C-25); 32.9 (C-8); 33.4 (C-1); 38.2 (C-4); 39.7 (C-10); 39.8 (C-12); 40.5 (C-20); 42.3 (C-13); 50.1 (C-9); 51.2 (C-24); 55.9 (C-14); 57.3 (C-17); 64.5 (C-19); 73.4 (C-3); 126.8 (C-6); 129.3 (C-23); 134.5 (C-5); 138.2 (C-22); 170.6 and 170.8 (CH_3CO–). Anal. Calcd for $C_{33}H_{52}O_4$: C, 77.30; H, 10.22. Found: C, 77.47; H, 10.32.

*4.1.12. (22E)-19-Acetoxystigmasta-5,22-dien-3β-ol (**18**).* A mixture of compound **17** (120 mg, 0.23 mmol), K_2CO_3 (49 mg, 0.35 mmol), MeOH (15 mL), THF (10 mL), and water (2 mL) was stirred at room temperature for 6 h. The mixture was then poured on 30 mL of NH_4Cl saturated solution and extracted with EtOAc (50 mL). The organic layer was washed with brine and dried (Na_2SO_4). The crude product was purified by column chromatography (hexane/EtOAc 9:1) to obtain compound **18** (65 mg, 59%). Mp: 97–98 °C. ^{1}H NMR ($CDCl_3$): 0.71 (H-18, 3H, s); 0.80 (H-26, 3H, d, *J*=6.2); 0.81 (H-29, 3H, *t*, *J*=6.0); 0.84 (H-27, 3H, d, *J*=7.3); 1.02 (H-21, 3H, d, *J*=6.6); 2.05 (CH_3CO–, 3H, s); 3.55 (H-3α, 1H, m); 3.98 (H-19a, 1H, d, *J*=11.7); 4.45 (H-19b, 1H, d, *J*=11.7); 5.00 (H-23, 1H, dd, *J*=8.1 and 15.2); 5.16 (H-22, 1H, dd, *J*=8.1 and 15.2); 5.59 (H-6, 1H, d, *J*=4.9). ^{13}C NMR ($CDCl_3$): 12.1 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1, 21.1 and 21.2 (*C*H_3CO–, C-21 and C-27); 21.7 (C-11); 24.3 (C-15); 25.4 (C-28); 28.9 (C-16); 31.4 (C-7); 31.8 (C-2); 31.9 (C-25); 33.0 (C-8); 33.7 (C-1); 39.6 (C-10); 39.8 (C-12); 40.5 (C-20); 42.3 (C-13); 42.4 (C-4); 50.2 (C-9); 51.2 (C-24); 55.9 (C-14); 57.4 (C-17); 64.7 (C-19); 71.4 (C-3); 125.8 (C-6); 129.3 (C-23); 135.6 (C-5); 138.2 (C-22); 170.9 (CH_3*C*O–). Anal. Calcd for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71. Found: C, 79.09; H, 10.69.

*4.1.13. (22E)-19-Acetoxystigmasta-4,22-dien-3,6-dione (**19**).* Compound **18** (65 mg, 0.14 mmol) was dissolved in 25 mL of acetone and cooled in an ice bath. Jones reagent was added dropwise until a persistent orange color was obtained. The reaction mixture was stirred (30 min) at 0 °C. The reaction mixture was allowed to warm up to room temperature and then quenched with methanol, producing a dark green solution, the solvent from which it was then removed in vacuo. The steroid was extracted with EtOAc and washed with water and brine and dried with Na_2SO_4. The organic solvent was then evaporated in vacuo. The crude product was purified by column chromatography (hexane/EtOAc 95:5) to obtain compound **19** (25 mg, 23%). Mp: 117–118 °C. ^{1}H NMR ($CDCl_3$): 0.74 (H-18, 3H, s); 0.78 (H-26, 3H, d, *J*=7.3); 0.81 (H-29, 3H, t, *J*=6.4); 0.84 (H-27, 3H, d, *J*=6.4); 1.03 (H-21, 3H, d, *J*=6.6); 1.97 (CH_3CO–, 3H, s); 4.28 (H-19a, 1H, d, *J*=11.5); 4.36 (H-19b, 1H, d, *J*=11.5); 5.03 (H-23, 1H, dd, *J*=7.7 and 15.2); 5.15 (H-22, 1H, dd, *J*=7.7 and 15.0); 6.36 (H-4β, 1H, m). ^{13}C NMR ($CDCl_3$): 12.2 and 12.2 (C-18 and C-29); 19.0 (C-26); 21.1; 21.2 and 21.2 (*C*H_3CO–, C-21 and C-27); 20.7 (C-11); 24.0 (C-15); 25.4 (C-28); 28.7 (C-16); 31.8 (C-25); 32.8 and 34.1 (C-1 and C-2); 34.9 (C-8); 39.2 (C-12); 40.3 (C-20); 42.4 and 42.6 (C-10 and C-13); 46.3 (C-7); 51.2 and 51.2 (C-9 and C-24); 55.7 (C-14); 57.2 (C-17); 65.1 (C-19); 128.1 (C-4); 129.8 (C-23); 137.7 (C-22); 156.0 (C-5); 170.2 (CH_3CO–); 198.7 (C-3); 200.9 (C-6). Anal. Calcd for $C_{31}H_{46}O_4$: C, 77.14; H, 9.61. Found: C, 77.06; H, 9.49.

*4.1.14. (22E)-19-Nor-3-hydroxystigmasta-1,3,5(10),22-tetraen-6-one (**20**).* A solution of KOH (890 mg, 16 mmol) in 1.6 mL of water was added to a solution of compound **19** (25 mg, 51.8 μmol) in 10 mL of MeOH and the mixture was stirred at room temperature for 3.5 h. After this time, the reaction mixture was neutralized with 5% HCl and then condensed to a small volume and extracted with EtOAc. The organic layer was washed with brine and dried with Na_2SO_4. After evaporation of the solvent, the residue obtained was purified by column chromatography (hexane/EtOAc 9:1) to obtain compound **20** (10 mg, 47%). Mp: 125–127 °C. ^{1}H NMR ($CDCl_3$): 0.71 (H-18, 3H, s); 0.80 (H-26, 3H, d, *J*=6.5); 0.81 (H-29, 3H, t, *J*=7.3); 0.86 (H-27, 3H, d, *J*=6.4); 1.05 (H-21, 3H, d, *J*=6.6); 2.73 (H-7α, 1H, dd, *J*=3.4 and 16.9); 5.04 (H-23, 1H, dd, *J*=8.7 and 15.2); 5.17 (H-22, 1H, dd, *J*=8.7 and 15.2); 7.06 (H-2, 1H, dd, *J*=2.7 and 8.4); 7.31 (H-1, 1H, d, *J*=8.4); 7.52 (H-4, 1H, d, *J*=2.7). ^{13}C NMR ($CDCl_3$): 12.0 and 12.3 (C-18 and C-29); 19.0 (C-26); 21.1 and 21.2 (C-21 and C-27); 23.7 (C-15); 25.4 (C-28); 25.9 (C-11); 28.9 (C-16); 31.9 (C-25); 39.3 (C-12); 40.1 (C-8); 40.5 (C-20); 42.5 (C-13); 42.8 (C-9); 44.5 (C-7); 51.3 (C-24); 55.3 (C-14); 55.9 (C-17); 112.7 (C-4); 121.3 (C-2); 126.9 (C-1); 129.6 (C-23); 133.4 (C-5); 138.0 (C-22); 140.0 (C-10); 154.2 (C-3); 198.7 (C-6). Anal. Calcd for $C_{28}H_{40}O_2$: C, 82.30; H, 9.87. Found: C, 82.51; H, 9.93.

*4.1.15. (22R,23R)-19-Nor-3,22,23-trihydroxystigmasta-1,3,5(10)-triene-6-one(**5**).* Compound **20** (10 mg, 24.5 μmol) was dihydroxylated as described above for compound **14** to yield compound **5** (7 mg; 81%). Mp: 173–174 °C. ^{1}H NMR ($CDCl_3$): 0.73 (H-18, 3H, s); 0.96 (H-21, 3H, d, *J*=6.6); 0.96 (H-29, 3H, *t*, *J*=7.7); 0.96 (H-26, 3H, d, *J*=7.1); 0.98 (H-27, 3H, d, *J*=6.0); 2.73 (H-7α, 1H, dd, *J*=3.4 and 17.0); 3.62 (H-22, 1H, d, *J*=8.8); 3.73 (H-23, 1H, d, *J*=8.8); 7.06 (H-2, 1H, dd, *J*=2.9 and 8.4); 7.29 (H-1, 1H, dd, *J*=0.7 and 8.4); 7.46 (H-4, 1H, d, *J*=2.9). ^{13}C NMR ($CDCl_3$): 11.6 (C-18); 11.8 (C-21); 13.5 (C-29); 18.8 (C-28); 19.3 (C-26); 21.1 (C-27); 23.4 (C-15); 25.8 (C-11); 27.6 (C-16); 28.9 (C-25); 36.9 (C-20); 39.3 (C-12); 40.2 (C-8); 42.4 (C-13); 42.5 (C-9); 44.4 (C-7); 46.4 (C-24); 52.5 (C-17); 54.9 (C-14); 72.4 (C-23); 74.3 (C-22); 112.2 (C-4); 121.8 (C-2); 126.6 (C-1); 132.9 (C-5); 139.0 (C-10); 155.3 (C-3); 199.8 (C-6). Anal. Calcd for $C_{28}H_{42}O_4$: C, 75.98; H, 9.56. Found: C, 75.90; H, 10.05.

*4.1.16. (22R,23R)-Stigmasta-3β,22,23-triol(6-deoxo-28-homoteasterone, **22**).* (22E)-Stigmast-22-en-3β-ol[19] (52 mg, 0.13 mmol) was dihydroxylated as described above for compound **14** to yield compound **22** (44 mg; 78%). Mp: 199–200 °C. ^{1}H NMR ($CDCl_3$): 0.67 (H-18, 3H, s); 0.81 (H-19, 3H, s); 0.87–0.98 (H-21, H-26, H-27 and H-29, 12H, m); 3.56 (H-22, 1H, d, *J*=9.0); 3.68 (H-23, 1H, d, *J*=8.4). ^{13}C NMR ($CDCl_3$): 11.8 (C-18); 11.8 (C-21); 12.2 (C-19); 13.5 (C-29); 18.8 (C-28); 19.3 (C-26); 21.1 (C-11); 21.2 (C-27); 24.0 (C-15); 27.7 (C-16); 28.6 (C-25); 28.9 (C-6); 29.6 (C-7); 31.0 (C-2); 31.9 (C-10); 35.5 (C-8); 36.9 (C-1); 36.9 (C-4); 37.7 (C-20); 40.0 (C-12); 42.4 (C-13); 44.7 (C-5); 46.3 (C-24); 52.6 (C-17); 54.2 (C-9); 56.4 (C-14); 70.9 (C-3); 72.4 (C-23); 74.4 (C-22). Anal. Calcd for $C_{29}H_{52}O_3$: C, 77.62; H, 11.68. Found: C, 77.57; H, 11.59.

4.2. Rice lamina inclination test (RLIT)

Rice seedlings (*Oryza sativa*, cv Chui) were washed with ethanol (1 min) and water and then left in water at 30 °C for 2 days (with a 16 h photoperiod). Germinated seeds were cultivated in agar under the same growing conditions for 4 days. Intact seedlings (4–5 cm tall) were inoculated with 0.5 μL of the test compound solution (in ethanol) just under the second leaf joint. The magnitude of the induced angle between the leaf and the sheath was measured after 48 h in the dark (at 30 °C).

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Supplementary data

Supplementary data related to this article can be found in the online version, at doi:10.1016/j.tet.2012.03.082.

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