Synthesis of (22R,23R)-22,23-epoxy-3β,5α-dihydroxystigmastan-6-one from stigmasterol

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A new (22R,23R)-22,23-epoxy-3β,5α-dihydroxy-stigmastan-6-one brassinosteroid analogue has been synthesised from stigmasterol **1** and its activity as a plant growth promoter has been tested.

Keywords: plant growth regulators, brassinosteroids, epoxide, steroids, brassinolide

Brassinosteroids (BS) are a widely accepted new group of phytohormones, which play an important role in the growth and development of plants.[1] BS are a unique class of plant hormones but structurally similar to other well-documented animal and insect steroids. When applied exogenously to plants, BS trigger variety of physiological processes, including cell division, cell elongation, vascular differentiation, root growth inhibition, biotic and abiotic stress tolerance, reproductive development, and modulation of gene expression.[2]

Natural brassinosteroids are integral components of the plant hormone spectrum influencing the phytohormone balance[3] and are sensed by the plasma membrane-localised leucine-rich-repeat-receptor kinase BRI1.[4-7]

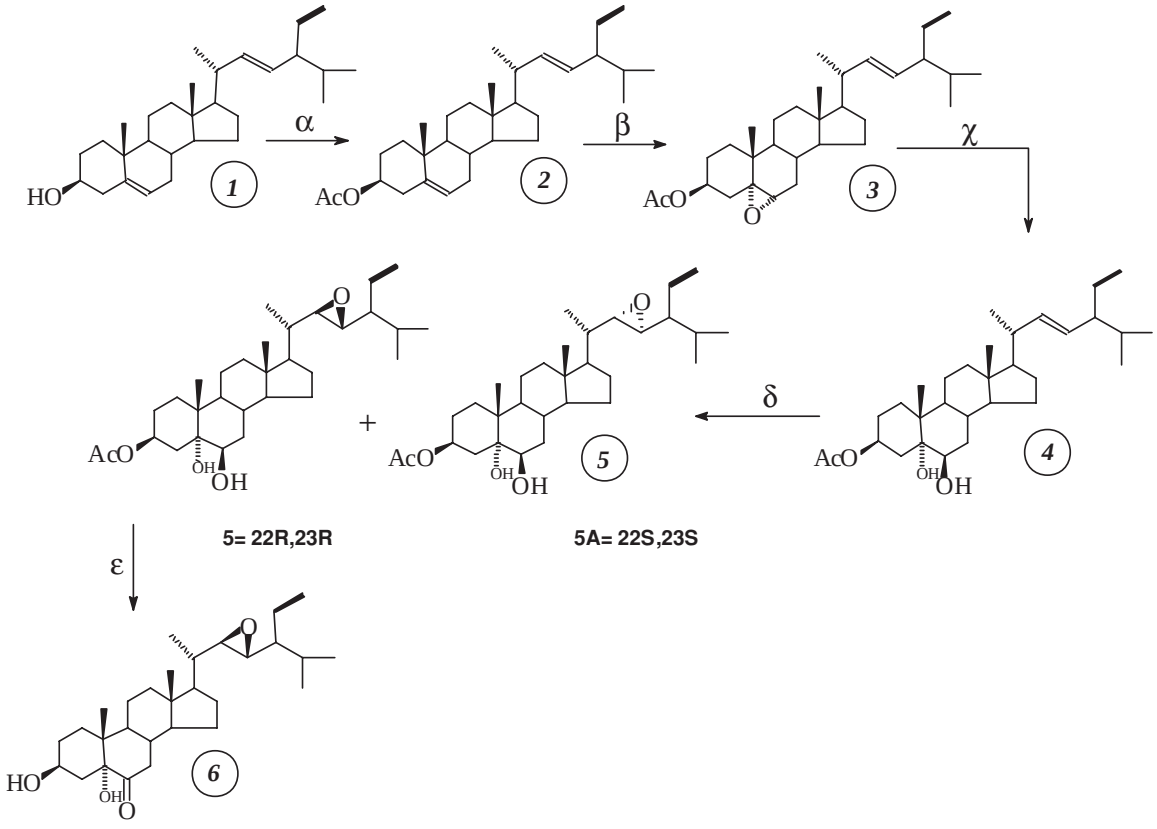
The preparation of BS analogues been developed simultaneously with the isolation of BS from plant sources and with the synthesis of natural BS. It is known that analogues frequently exhibit a high and selective type of activity that makes them most suitable for practical applications. Up to now several analogues have been synthesised and promising results have been obtained, not only in laboratory bio-tests but also in field conditions.[8-13]

Some brassinosteroid analogues with 22,23-epoxide have been synthesised and showed higher growth-promoting activity in field conditions than in test systems.[14-15] These results suggest that these epoxy steroids are transformed in plants to more active compounds after some days. Some 5α-hydroxy-6-ketone brassinosteroid analogues have also been synthesised and show high activity as plant growth regulators.[16-18]

The synthesis, characterisation and bioactivity evaluation of a steroid containing both the 22R,23 R epoxy ring and the 5α-hydroxy-6-ketone functions is described here.

The synthetic strategy used to obtain the 22R,23R-epoxy-5α-hydroxy-6-ketone analog is summarised in Scheme 1.

Selective epoxidation of the double bond of the ring B of stigmasterol acetate **2** with magnesium monoperoxyphthalate



Scheme 1 Reagent and conditions: (a) $(CH_3CO)_2O$/Pyr; (b) MMPP/TEBA/CH_2Cl_2–H_2O; (c) $HClO_4$/H_2O/acetone; (d) MCPB/bencene; (e) i, Jones/Acetone; ii, KOH/EtOH.

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hexahydrate (MMPP),[19-21] in a mixture of dichloromethane and water, using triethyl-*t*-buthylammonium chloride, as a phase transfer catalyst, yields (22E)-3β-acetoxy-5α,22-stigmasten-5,6α-epoxide **3** as a major product of this reaction. By using this procedure, no compounds with an epoxide function at C_{22},C_{23} were detected.

Nucleophilic *trans*-diaxial opening using percloric acid (aq), of a mixture of epoxide **3** and its isomer with 5α,6β-epoxy function, afforded the 5α,6β-dihydroxylated steroid **4**. Independently of the stereochemistry of the starting 5,6-epoxide, the acidic opening always yields a *trans* diol by means of a unimolecular nucleophilic substitution.

Treatment of compound **4** with *m*-chloroperoxybenzoic acid (MCPBA) at room temperature for 24h yielded a mixture of the (22R,23R)-3β-acetoxy-22,23-epoxy-5α-stigmastan-5,6β-diol **5** and (22S,23S)-3β-acetoxy-22,23-epoxy-5α-stigmastan-5,6β-diol **5A**. A small portion of this mixture was chromatographed and both isomers were separated in a ratio of 2:1.

The rest of this crude mixture of both steroisomers **5** and **5A** was oxidised using Jones reagent at 5ºC to produce the 5α-hydroxy-6-ketone. The saponification of the 3β-acetoxy function with potassium hydroxide (1%) in ethanol at room temperature yielded after purification by column chromatography, (22R, 23R)-3β,5-dihydroxy-22,23-epoxy-5α-stigmastan-6-one **6** as the final product.

For the purpose of evaluating the bio-activity of the (22R,23R)-3β,5-dihydroxy-22,23-epoxy-5α-stigmastan-6-one **6**, and of the mixture of the epoxides 22R,23R and 22S,23S (**6´**), we used the radish (*Raphanus sativus*)[22] cotyledon expansion test. This test detects the cytokine-like activity which stimulates plant cell division.

Compound **6** was active in this biological test at a concentration of 10^{-5} mg/ml; but the mixture **6'** of steroid **6** and its 22S,23S diasteroisomer (3:2) was even more active and it promoted a similar growth effect at a lower dose (10^{-7} mg/ml).

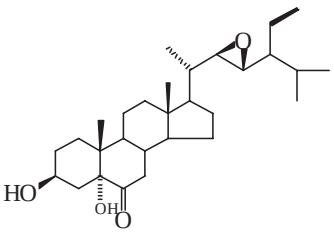
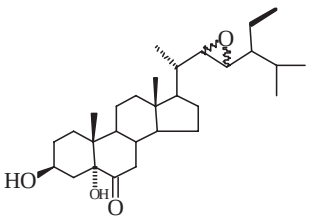
Experimental

Melting points (m.p.) were determined on a Stuart Scientific SMP3 apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker ACF 250 MHz spectrometer. Chemical shifts are given in ppm, with TMS as internal reference. Coupling constant values are given in Hz. IR spectra were obtained on a Phillips Analytical PV 9600 FTIR. Elemental analyses were performed on Carlo Erba-1106, Carlo Erba EA-1108 and Perkin Elmer CHN 2400 instruments. Unless otherwise indicated, all solvents and reagents used were of commercial grade. Reactions were monitored by thin layer chromatography (TLC) on plates pre-coated with silica gel F254 0.2 mm (Merck). Column chromatography was carried out on silica gel 60, 0.04 ± 0.063 mm (Merck). The stigmasterol acetate was synthesised as reported in the literature.[23]

(22E)-3β-acetoxy-5α-stigmast-22-en-5,6α-epoxy (**3**)**:** A solution of stigmasterol acetate **2** (3 g, 4.7 mmol), dichloromethane (18 ml) and triethyl-terbutylammonium chloride (0.09 g) was stirred under reflux (50˚C) for 1 h; the solution of magnesium monoperoxyphthalate hexahydrate (MMPP) (4, 32 g) in water (24 ml) was added in dropwise. The resultant mixture was then cooled and stirred for 3 h. The organic layer was separated from the aqueous phase, and the aqueous phase was then extracted with dichloromethane. The combined organic layer was washed sequentially with water and brine, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure yielding 2.56 g of a crude mixture (80.2%) which was purified by column chromatography on silica gel, using heptane/EtOAc (6%) as the eluent, to produce compound **3** (1.56 g, 2.45 mmol, 52%). This reaction was repeated and the resulting crude product **3'** (2.76g, 86.5%) was used as starting material for the following reaction**.** m.p. (acetone):140.8–142.5˚C, IR (KBr, cm^{-1}): 2953 and 2870 (ν_{CH}), 1735 ($\nu_{C=O}$), 1637 ($\nu_{C=C}$), 1463 and 1373 (δ_{CH}), 1248($\nu_{C-O,}$ acetate), 1036 and 968 ($\nu_{C-O,}$ epoxide). ^{1}H NMR ($CDCl_3$, δ, ppm): 5.1 (dd, H_{22}), 4.9 (dd, H_{23}), 4.9 (m, H_3), 2.8 (d, H_6β, ^3J = 4.3 Hz), 1.9 (s, CH_3-Ac), 1.0 (s, CH_3-19), 0.9 (d, CH_3-21, ^3J = 6.5 Hz), 0.8 and 0.7 (d, CH_3-26 and CH_3-27, J = 6.5 Hz), 0.7 (t, CH_3-29, J = 7.4Hz), 0.6 (s, CH_3-18). ^{13}C NMR ($CDCl_3$, δ, ppm): 170.2(C=O, acetate), 138.2 (C_{22}), 129.3 (C_{23}), 71.3 (C_3), 65.1 (C_5), 59.1 (C_6), 56.8 (C_{14}), 55,6 (C_{17}), 51.2 (C_{24}), 44.4 (C9), 42.2 (C_{13}) , 40.4 (C_{20}) , 39.2 (C_{12}) , 36.1 (C_4), 35.0 (C_{10}), 32.1 (C_1), 31.8 (C_{25}), 29, 8 (C_8), 28.7 (C_7 and C_{16}), 27.2 (C2), 25.4 (C_{28}), 24.1 (C_{15}), 22.0 and 19.0 (C_{26} and C_{27}), 21.1 (C_{21}), 21.1 (Ac), 20.5 (C_{11}), 15.8 (C_{19}), 12.2 (C_{29}), 12.0 (C_{18})., Analysis cal. C, 79.1%; H, 10.7%; found: C, 79.2%; H, 10.8%.

(22E)-3β-acetoxy-5α-stigmast-22-en-5,6β-diol **(4):** Perchloric acid (0.86 ml) was added to a solution of the crude **3'** (2.76 g, 4.1 mmol) in acetone (120.8 ml) and water (6.9 ml). The resulting solution was stirred for 5 hours until none of the starting materials were detected using TLC. The solvent was then removed under reduced pressure until a third part of the volume remained. Then it was vacuum filtered and washed with NaOH al 1%, water and brine**.** The organic phase was dried over (Na_2SO_4) and evaporated under reduced pressure until dry. The solids yielded a total of 2.37 g (82.9 %) of the crude **4'** which was used (unpurified) as starting material for the following reaction**.** A small portion (0.283 g) of this crude was chromatographed on silica gel using n-hexane/ethyl acetate (20 %) as eluent, to give 0.144 g (0.2 mmol, 51%) of **4**. m.p. (acetone): 230.9–231.0˚C, IR (KBr,

Table 1 Biological activity

Compounds	Cothyledons	
	F-ratio of ANOVA table : 9.256	
	Conc. (mg/ml)	Harmonic mean (g^{-1})
	10^{-5}	5.29 a
	10^{-4}	4.54 b
	10^{-7}	4.43 b
	Control	4.38 b
(22R,23R)-22,23-epoxy-3β,5α-dihydroxy-estigmastan-6-one **6**	10^{-6}	4.16 b
	F-ratio of ANOVA table : 3.527	
	Conc. (mg/ml)	Harmonic mean (g^{-1})
	10^{-7}	3.645 a
	control	2.885 b
	10^{-6}	2.535 b
(22R,23R)-22,23-epoxy-3β,5α-dihydroxy-estigmastan-6-one and (22S,23S)-22,23-epoxy-3β,5α-dihydroxy-estigmastan-6-one. **6`** (ratio: 3:2).	10^{-5}	2.120 b
	10^{-4}	1.565 b

Ωcm^{-1}): 3469 (ν_{OH}), 2955 and 2868 (ν_{CH}), 1713 ($\nu_{C=O}$), 1637 ($\nu_{C=C}$), 1461 and 1364 (δ_{CH}), 1276 and 1036 (ν_{C-O}). ^{1}H NMR ($CDCl_3$, δ, ppm): 5.1 (dd, H_{22} , $^3J_1$ = 15.1 Hz, $^3J_2$ = 8.4Hz), 5.0 (dd, H_{23}, $^3J_1$ = 15.1 Hz, $^3J_2$ = 8.2 Hz), 3.5 (m, $H_{6\alpha}$), 2.2 (dd, $H_{4\beta}$), 2.0 (s, CH_3-Ac), 1.2 (s, CH_3-19), 1.0 (d, CH_3-21, ^3J = 6.6 Hz), 0.8 and 0.8 (d, CH_3-26 and CH_3-27, J = 6.5 Hz), 0.8(t, CH_3-29, J = 7.3Hz) , 0.7 (s, CH_3-18). ^{13}C NMR ($CDCl_3$, δ, ppm): 171.0 (C=O, acetate) 138.3 (C_{22}), 129.3 (C_{23}), 76.2 (C_6), 75.6 (C_5), 71.4 (C_3), 56.0 ($C_{14}$and C_{17}), 51.2 (C_{24}), 45.4 (C_9), 42.6 (C_{13}), 40.6 (C_{20}), 39.8 (C_{12}), 38.3 (C_{10}), 36.9 (C_4), 34.6 (C_7), 32.1 (C_1), 31.9 (C_{25}), 30.2 (C_8), 28.9 (C_{16}), 26.7 (C_2), 25.4 (C_{28}), 24.2 (C_{15}), 21.4 (Ac), 21.2 (C_{21}), 21.1 (C_{11} and C_{26}), 19.0 (C_{27}), 16.7 (C_{19}), 12.2 (C_{18}), 12, 13 (C_{29}).Analysis Calc.: C, 76.2%; H, 10.7%; Found: C, 76.3%; H, 10.6%.

*(22R,23R)-3*β*-acetoxy-22,23-epoxy-5*α*-stigmastan-5,6*β*-diol* **5** *and (22S,23S)-3*β*-acetoxy-22,23-epoxy-5*α*-stigmastan-5,6*β*-diol* **5A**: A solution of MCPBA (1.16 g) in benzene (19.6 ml) was added to a solution of crude **4'** (2.09 g, 3.09 mmol) in benzene (28.2 ml) at 0°C in the dark. The mixture was stirred and kept at room temperature for 24 h. The reaction mixture was washed with solutions of NaOH 1 % (3 × 100 ml) and $NaHCO_3$ 10 % (3 × 200 ml). The organic phase was dried over anhydrous Na_2SO_4 and subsequently over $CaCl_2$, and then evaporated under reduced pressure. Recrystallisation from acetone resulted in the epoxide mixture of **5** and **5A** (1.76 g, 84.4 %); 0.250 g of this crude **5'** were chromatographed on silica gel using petroleum ether /ethyl acetate (17%) as the eluent, to give 0.118g of 22R,23R-epoxy **5** and 0.058 g of 22S,23S-epoxy **5A.** Compound **5**: m.p. (acetone):188.5–190.7°C, IR (KBr, cm^{-1}): 3480 (ν_{OH}), 2957, 2937 and 2870 (ν_{CH}), 1715 ($\nu_{C=O,}$ ketone), 1450 and 1383 (δ_{CH}), 1245 and 1029 (ν_{C-O}). ^{1}H NMR ($CDCl_3$, δ, ppm): 5.2 (m, $H_{3\alpha}$), 3.5 (m, $H_{6\alpha}$), 2.7 (dd, H_{23}, $^3J_1$ = 7.08Hz, $^3J_2$ = 2.34Hz), 2.5 (dd, H_{22}, $^3J_1$ = 6.10Hz, $^3J_2$ = 2.27Hz), 2.2 (dd, $H_{4\beta}$, J_1 = 12.8Hz, J_2 = 11.4Hz), 2.0 (s, CH_3-Ac), 1.2 (s, CH_3-19), 1.0 (d, CH_3-21), 1.0 (t, CH_3-29, ^3J = 7.5Hz), 0.9 (d, CH_3-26 and CH_3-27, J = 6.9), 0.7 (s, CH_3-18). ^{13}C NMR ($CDCl_3$, δ, ppm): 171.0 (C=O, acetate), 79.1 (C_6), 75.7 (C_5), 71.3 (C_3), 62.1 (C_{22} and C_{23}), 55.4 (C_{14}), 53.5 (C_{17}), 48.3 (C_{24}), 45.49 (C_9), 43.0 (C_{13}), 39.70 (C_{12} and C_{20}), 38.4 (C_{10}), 37.0 (C_4), 34.7 (C_7), 32.1 (C_1), 30.2 (C_8), 29.1 (C_{25}), 27.9 (C_{16}), 26.7 (C_2), 24.4 (C_{15}), 21.4 (Ac), 21.0 (C_{11}), 20.9 (C_{28}), 20.2 and 19.6 (C_{26} and C_{27}), 16.7 (C_{19}), 16.2 (C_{21}), 12.5 (C_{29}), 12.10 (C_{18}). Analysis Calc: C 73.8%; H 10.4%; found: C 73.7%; H 10.3 %. *Compound* **5A:** ^{1}H NMR ($CDCl_3$, δ, ppm): 5.2(m, $H_{3\alpha}$), 2.2(dd; $H_{4\beta}$), 3.5(m; H6), 6.7(s; CH_3-18), 1.2(s; CH_3-19), 1.0(d; CH_3-21), 2.5 (dd; H_{22}), 2.5(dd; H_{23}), 0.9(d; CH_3-26 y 27), 0.9(t; CH_3-29), 2.0(s; CH_3-Ac). ^{13}C NMR ($CDCl_3$, δ, ppm): 172.3(C=O, Acetate), 21.5(CH_3-Acet), 12.4(C_{29}), 21.0(C_{28}), 19.5*(C_{27}), 19.4*(C_{26}), 29.4(C_{25}), 48.8(C_{24}), 58.6(C_{23}), 63.1(C_{22}), 16.3(C_{21}), 38.9(C_{20}), 16.7(C_{19}), 12.5(C_{18}), 56.1(C_{17}), 27.1(C_{16}), 24.4(C_{15}), 55.4(C_{14}), 43.1(C_{13}), 39.8(C_{12}), 21.1(C_{11}), 38.4(C_{10}), 45.5(C_9), 30.2(C_8), 34.7(C_7), 76.1(C_6), 75.7(C_5), 37.0(C_4), 71.3(C_3), 26.7(C_2), 32.1(C_1). Analysis Calc: C 74.8%; H 11.4%; found: C 74.7%; H 11.3 %.

*(22R,23R)-3*β*,5*α*-dihydroxy-22,23-epoxy-stigmastan-6-one* (**6**): Jones reagent (3.78 ml) was added in drops to a solution of a mixture of both diols **5** and **5A** (1.51g, 2.19 mmol) in acetone (21.2 ml). After 1 h of isopropyl alcohol (19.1 ml) was added to the stirred reaction and the resulting mixture was concentrated to half volume, then poured into cold water (100 ml) and filtered. Re-crystallisation from EtOH afforded 1.36 g of a crude product, which was dissolved in a solution of KOH 1 % in EtOH (151ml). The reaction was stirred for 1 h at room temperature and then neutralised with diluted HOAc. Cold water was added and the solid was filtered, washed with cold water and dried. It afforded 1.27g (93.8%) of a crude product which was then purified by column chromatography on silica gel, using petroleum ether (b.p. 60–90°C)/AcOEt (40%) as eluent to produce 1.17 g (92.3%) of **6**. m.p. (acetone): 214.2–214.8°C, IR (KBr, cm^{-1}): 3415 (ν_{OH}), 2940 and 2871 (ν_{CH}), 1708 ($\nu_{C=O,}$ C=O), 1465 and 1385 (δ_{CH}), 978 (ν_{C-O}, epoxide). ^{1}H NMR ($CDCl_3$, δ, ppm): 3.9 (m, $H_{3\alpha}$), 2.8 (dd, $H_{7\alpha}$), 2.7 (dd, H_{23}, $^3J_1$ = 7.14Hz, 2.30Hz), 2.5 (dd, H_{22}), 1.0 (d, CH_3-21), 1.0 (t, CH_3-29, ^3J = 7.5Hz), 0.9 (d, CH_3-26 and CH_3-27, ^3J = 6.9 Hz), 0.8 (s, CH_3-19), 0.6 (s, CH_3-18). ^{13}C NMR ($CDCl_3$, δ, ppm): 214.0 (C = 0), 80.3 (C_5), 67.2 (C_3), 62.0 (C_{22} and C_{23}), 55.9 (C_{14}), 53.4 (C_{17}), 48.3 (C_{24}), 44.2 (C_9), 43.4 (C_{13}), 42.5 (C_{10}), 41.8 (C_7), 39.4 (C_{12}), 38.5 (C_{20}), 37.4 (C_8), 35.8 (C_4), 29.8 and 29.7 (C_1 and C_2), 29.1 (C_{25}), 27.8 (C_{16}), 24.2 (C_{15}), 21.37 (C_{11}), 20.9 (C_{28}), 20.1 and 19.6 (C_{26} and C_{27}), 16.0 (C_{21}), 13.9 (C_{19}), 12.4 (C_{29}), 11.9 (C_{18}). Analysis calc.: C 76.1% ; H 11.0%; found: C 76.2 %; H 11.15%.

Biological activity: Plant material: radish seeds. Growth conditions: The seeds were germinated for three days in the dark. The cotyledons were excised and the rest of the plant was discarded. The cuttings were placed on Petri dishes containing the brassinosteroid analog at different concentrations (10^{-4} to 10^{-7} mg/ml). The plates were then left under controlled conditions such as constant illumination, 25°C temperature and 85% relative humidity. The weight of the cotyledons (gr.) was measured after three days.

Data analysis: The results were statistically analysed using Statgraphics Version 5.1. Software.

A simple ANOVA and the Duncan's Multiple Range Test comparison procedures were carried out in order to compare the different treatments used. Different letters denote statistically significant difference at the 95% confidence level.

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