Synthesis and biological activity of epoxy analogues of 3-dehydroteasterone

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Two new brassinosteroids analogues containing a 3,6-dione with a 5α-hydroxyl group and also one epoxy ring in the side chain have been synthesised from stigmasterol. Their activity as plant growth promoter has been tested using Radish (*Raphanus sativus*) tests.

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

Brassinosteroids (BS) are recognised as a new class of steroidal plant hormones that control many physiological and developmental processes. Extensive studies have been directed toward the natural occurrence, biosynthesis, metabolism, signal transduction, physiological effects and potential practical applications of these natural compounds.[1,7]

Sixty-one naturally occurring BS have now been discovered.[2,8] Among them there is only one 3-oxobrassinosteroid: the 3-dehydroteasterone **I** (Fig. 1) which was isolated for the first time from *Liliom longiflorum Thunb*.[9] This natural BS possesses 74% of the bioactivity of the 24-Epicastasterone **II** and is an intermediate in the *in vitro* enzymatic conversion of Teasterone **III** to Typhasterol **IV** in *Phaseolus vulgaris*.[5,10]

There are no natural BS with epoxy function in the side chain or with a 5α-hydroxyl group. However, many BS analogues with 22,23 epoxy ring or 5α-hydroxy-6-ketone functions have been synthesised and tested as plant growth regulators.[11-20] Here we describe in two different syntheses of two new analogues of 3-dehydroteasterone, the (22R,23R)-22,23-epoxy-5α-hydroxystigmastan-3,6-dione **6A** and the (22S,23S)-22,23-epoxy-5α-hydroxystigmastan-3,6-dione **6B.** Both steroids possess a 3,6-dione with the 5α-hydroxyl group and an epoxide in the side chain.

The biological activity of a mixture of both brassinosteroids analogues was evaluated using two different bioassays; one of them detects citokynine-like activity and the other auxin-like activity.

Results and discussion

In the first synthetic strategy (Scheme 1) the selective epoxidation of the double bond of the ring B of the stigmasterol **1** with peroxyacetic acid, generated "in situ" at 0–5°C, gave the 5αα,6αα-epoxy **2** as the major product. Hydrolysis of crude epoxide **2** using perchloric acid gave a trans-diol **3**. Simultaneous oxidation of 3β-OH and 6β-OH groups of the triol **3,** using Jones reagent in acetone at 0–5°C afforded the diketone **4**. Epoxidation of the double bond in the side chain of **4** with *m*-chloroperbenzoic acid (MCPB) at room temperature for 24 hours yielded a mixture of the 22R,23R-epoxy **6A** and its isomer 22S,23S-epoxy **6B**.

This crude mixture containing steroids **6A** and **6B** was purified by column chromatography. However, the isomers were not separated. In the final mixture the 22S,23S-epoxy **6B** predominated over the 22R,23R-epoxy **6A** by about 2:1.

In the second synthesis stigmasterol **1** was treated with peroxyformic acid generated *in situ* from hydrogen peroxide (32%) and formic acid in dichloromethane, at room temperature, to afford a 1 : 1 mixture of the diepoxides **5A** and **5B.** Their signals in the GC/EI-MS appear partially superimposed and with a difference in retention time of 0.07 minutes. The EI-MS of both peaks are practically identical. It was not possible to separate these isomers by means of column chromatography.

This mixture of **5A** and **5B** was oxidised using the Jones reagent in acetone at temperature of 0–5°C to give, after

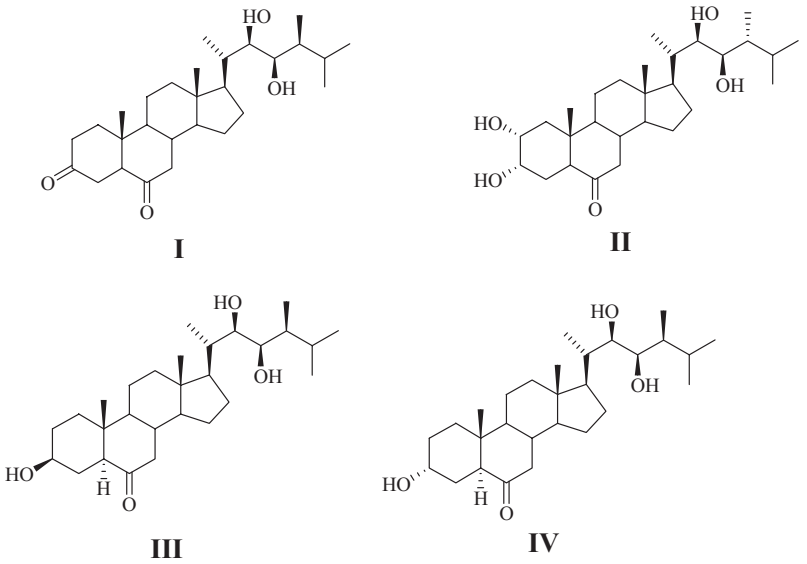

Fig. 1 Structure of 3-dehydroteasterone **I**, 24-epicasthasterone **II**, teasterone **III** and thyphasterol **IV.**

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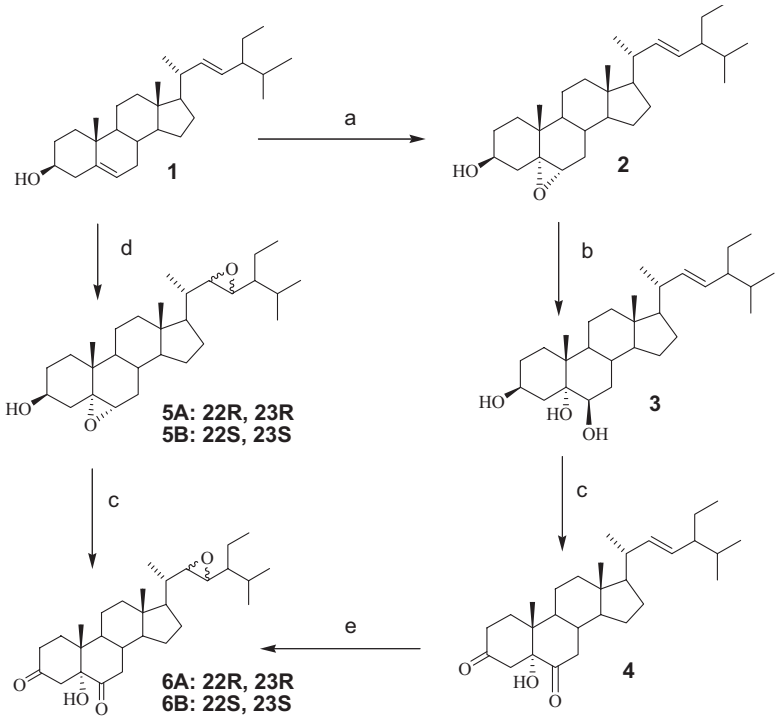


Scheme 1 Reagents and conditions **(a)** $(CH_3CO)_2O/NaAc/H_2O_2/CHCl_3$; **(b)** $HClO_4/H_2O$/acetone; **(c)** Jones R./acetone; **(d)** $HCO_2H/H_2O_2/CHCl_3$; **(e)** $MCPB/CHCl_3$.

chromatographic purification, a 2 : 1 mixture of (22R,23R)-22,23-epoxy-5α-hydroxystigmastan-3,6-dione **6A** and (22S, 23S)-22,23-epoxy-5α-hydroxystigmastan-3,6-dione **6B**. This **6A/6B** mixture was used to evaluate the biological activity in the Radish (*Raphanus sativus*) tests.

In the oxidation of compounds **5A** and **5B** it was observed that the reaction with the Jones reagent opened and oxidised the epoxy group in the ring B but did not affect the epoxide of the side chain. Then we concluded that it was not necessary to carry out the selective epoxidation of the double bond in ring B as in the first synthesis.

All synthesised compounds were examined by means of IR and NMR spectroscopy and subjected to GC/EI-MS analysis. The NMR spectral assignments for the steroids were based on previously reported data from similar steroids.[12,20] The integral of the H_{22} and H_{23} signals in the ^1H NMR spectra[12] were used to asses the proportions of diastereomeric 22,23-epoxides in the mixtures **5A/5B** and **6A/6B**.

The ^{13}C NMR spectra (Table 1), allowed us to determine the stereochemistry of the oxirane ring in the side chain.

The 22R,23R epoxy steroids **5A** and **6A** differ from 22S,23S epoxy compounds **5B** and **6B** by the chemical shifts of C17, C22 and C23, and the values correspond with the previous report.[12,20-22] In the R,R-epoxy, the C22 and C23 show a very similar chemical shift ($\Delta\delta \approx 0.1$–0.2 ppm); whereas in the S,S-epoxy those carbons are clearly distinguishable ($\Delta\delta \approx 4.3$–4.6 ppm) .[21]

In the radish (*Raphanus sativus*) cotyledon expansion test[23], which is used to detect citokynine-like activity, solutions of the mixture of **6A** and **6B** showed plant growth promoting activity in all concentrations (10^{-4}–10^{-7} mg/ml). The effect consisted of an increased weight of the treated cotyledons compared to those of the un-treated germinated seeds (Table 2).

However, in the second test, where the length of the radish's hypocotyls is measured to evaluate the presence of auxin type activity, differences between the treated and the non-treated hypocotyls were not detected at any of the studied concentrations.

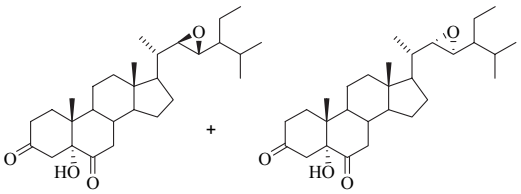
Table 1 ^{13}C NMR chemical shifts (δ in ppm) of all synthesised compounds

C	Compounds						
	2	3	4	5A	5B	6A	6B
1	32.4	31.3	31.8	32.4	32.4	31.7	31.8
2	31.0	31.0	37.5	31.1	31.1	37.4	37.4
3	68.7	65.7	210.8	68.7	68.7	211.1	211.1
4	39.8	39.6	44.8	39.9	39.9	44.7	44.7
5	65.7	74.3	82.8	65.7	65.7	82.7	82.7
6	59.3	74.1	210.8	59.2	59.3	210.9	210.9
7	28. 8	34.4	41.9	28.8	28.8	41.8	41.8
8	29.8	30.0	37.3	29.9	29.9	37.3	37.3
9	42.5	44.6	44.7	42.7	42.7	44.6	44.6
10	34.8	37.8	43.2	34.9	34.9	43.2	43.2
11	20.6	21.1	21.6	20.6	20.6	21.0	21.0
12	39.3	40.0	39.4	39.2	39.3	39.3	39.4
13	42.2	42.1	43.0	42.6	42.6	43.4	43.4
14	56.9	55.9	56.4	56.5	56.5	55.9	55.9
15	24.1	23.8	24.1	24.3	24.3	24.2	24.2
16	28.7	28.6	28.7	27.8	26.9	27.8	26.9
17	55.6	55.4	55.9	53.1	55.8	53.4	56.0
18	12.0	12.1	12.3	11.9	12.0	12.0	12.1
19	15.9	16.2	13.9	15.9	15.9	13.8	13.8
20	40.5	40.9	40.4	38.7	38.9	38.5	38.8
21	21.1	20.9	21.2	16.2	16.3	16.0	16.3
22	138.2	138.1	137.9	62.2	63.1	62.0	63.0
23	129.2	128.7	129.7	62.1	58.6	62.0	58.8
24	51.2	50.6	51.2	48.3	48.8	48.3	48.8
25	31.8	32.0	31.9	29.1	29.3	29.1	29.3
26	21.1	20.7	21.1	20.2	19.4	20.2	19.4
27	18.9	18.8	19.0	19.5	19.4	19.6	19.4
28	25.4	24.8	25.4	20.9	20.9	21.0	21.0
29	12.2	12.1	12.2	12.5	12.4	12.4	12.4

Experimental

Melting points (m.p.) were determined on a Reichert-Jung apparatus and are uncorrected. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 MHz spectrometer. Chemical shifts are given with TMS as internal reference. IR spectra were obtained on a Phillips Analytical PV 9600 FTIR. Elemental analyses were performed on CHNS analyser-932LECO instruments. Mass spectrometry by gas

Table 2 Biological activity of **6A/6B** mixture in the radish cotyledon expansion test

Compounds	Increased weight of treated cothyledons	
	Conc./mg/ml	Harmonic mean*/g^{-1}
	Control	12 a
	1.00E-07	15.9 d
	1.00E-06	18.3 e
	1.00E-05	14.5 c
	1.00E-04	13.6 b

6A: 22R, 23R **6B: 22S, 23S**

(ratio: 2:1)

*Different letters denote statistically significant difference at the 95% confidence level.

chromatography/electron ionisation was performed with a Voyager GCMS/Thermoquest model spectrometer at 70 eV. 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). "Usual work up" refers to drying organic phase over (Na_2SO_4) and evaporated under reduced pressure until dry. The ^{13}C NMR data of all compounds were reported in Table 1**.**

(22E)-3β-Hydroxyi-5α-22-stigmasten-5,6α-epoxy (**2**): A mixture of stigmasterol **1** (4 g, 8.0 mol), chloroform (5 ml) and sodium acetate (40 mg, 0.5 mol), was cooled to a temperature between 0 and 5°C. Acetic anhydride (0.1 ml, 0.86 mol) and hydrogen peroxide (0.2 ml, 4.5 mol) were added in a slow dropwise fashion. The reaction was stirred to room temperature for 7 h. Then the organic phase was washed with a solution of sodium carbonate and brine. On usual "work up" it yielded 3.2 g of **2**. A small portion of this crude product was purified to afford **2**. M.p. (acetone) = 144–148.5°C. IR (KBr, cm^{-1}): 3305 (OH). ^1H NMR ($CDCl_3$) (δ, ppm): 0.78 (s, 3H-18) 0.98 (s, H-21), 2. 78 (d, J = 4.4 Hz H-6β), 3.89 (m, H-3), 5.01 (dd, *J* = 15.1/8.4 Hz, H-23), 5.15 (dd, J = 15.1/8.4 Hz, H-22). EIMS (*m/z*, relative peak height): 428 (M^+, 11), 367 (14), 349 (9), 286 (17), 253.1 (25). EA: Calcd for $C_{29}H_{48}O_2$ (428,37), C 81.2; H 11.3; Found: C 81.4; H 11.2%.

(22E)-5α-22-stigmasten-3β, 5α, 6β-trihydroxy (**3**): Perchloric acid (1.1 ml) was added to a solution of the impure epoxide **2** (3 g, 7 mmol) in acetone (147 ml) and water (8 ml). The resulting solution was stirred for 2–4 h 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 (1%), water and brine. Usual "work-up" yielded 2.7 g (86%) of the crude of **3**. A small portion of this crude (0.7 g) was purified by flash chromatography on silica gel using *n*-hexane/ethyl acetate (10%) as eluent, resulting in 0.073 g (10.4%) of the pure steroid **3**. M.p. (acetone): 222–226°C. IR (NaCl, cm^{-1}): 3469 (OH), 1637 (C=C), and 1364 (CH), 1276 and 1036 (C-O). ^1H NMR (DMSO-d_6) (δ, ppm): 0.65 (s H-18), 0.77 (d, *J* = 6.0 Hz, H-26-27), 0.775 (t, *J* = 7.2 Hz, H-29), 0.82 (d, *J* = 6.2 Hz, H-26-27), 0.98 (d, *J* = 6.4 Hz, H-21) 1.02 (3H-19), 3.62 (s, OH-5), 4.14, 4.37 (d, OH-3, OH-6). EIMS (*m/z*, relative peak heigth): 428 (M^+, 21), 367 (51), 271 (83), 253 (57), 229 (26), 158 (33), 134 (35), 122 (41), 109 (50), 97 (62), 83 (94), 81(100). EA: Calcd for $C_{29}H_{50}O_3$ (446,38), C 77.9; H 11.3; Found: C 77.8; H 11.3%.

(22R,23R)-5α-hydroxi-22,23-stigmasten-3,6-dione (**4**): Jones reagent (1.6 ml) was added in drops to a solution of a crude **3** (1.5 g) in acetone (17 ml). When the starting material was consumed ethanol (30 ml) was added to the stirred reaction. A white solid precipitated and was vacuum filtered. This produced 1.4 g (91%) of the crude **4**. This crude was chromatographed using *n*-hexane/ethyl acetate (10%) as eluent, to give 1 g (2.3 mmol, 70%) of **4**. M.p. (acetone): 242–245°C. IR (KBr, cm^{-1}): 3358 (OH), 1712 (C=O), (CH). EIMS (*m/z*, relative peak height): 442 (M^+, 2), 289 (9), 109 (100). EA: Calcd for $C_{29}H_{46}O_3$ (442,34), C 78.7; H 10.5; Found: C 78.9; H 10.4%.

(22R,23R)-22,23,5α,6-diepoxi-3β-hydroxy-5α-stigmastane (**5A**) *and (22S,23S)-22,23,5α,6-diepoxi-3β-hydroxy-5α-stigmastane* (**5B**): A solution of stigmasterol **1** (2 g, 4.8 mmol), chloroform (13 ml), 90% formic acid (1.8 ml), 30% hydrogen peroxide (1.8 ml) was stirred at room temperature for 2 h. When the reaction was completed, methanol and a solution of NaOH (concentrated) were added. This mixture was neutralised with HCl (10%), extracted with chloroform and washed with a solution of $NaHCO_3$. Usual "work-up" produced a crude (1.9 g, 90%); which was then purified by flash chromatography yielding 1.7 g (3.9 mmol, 89%) of a mixture of isomers **5A** and **5B**. M.p. (acetone): 223–225°C. IR (KBr, cm^{-1}): 3305 (OH). ^1H NMR ($CDCl_3$), (δ, ppm): 2.05 (dd, *J* = 12.9/11.4 Hz, H-4β), 2.44–2.55 (m, H-22, H-23 **5B** and H-22, **5A**), 2.71 (dd, *J* = 10.0/2.3 Hz, H-23 **5A**), 2.87 (d, *J* = 4.3 Hz, H-6) 3.88 (m, H-3). EIMS (*m/z*, relative peak height): 444 (M^+, 8,1), 426(3,6), 359 (11,5), 297 (11.4), 253(14.8), 187 (14.0), 161 (23.2), 147 (28.2), 109 (40.5), 93 (50.0), 81(55.8, 69 (65.2), 55 (100). EA: Calcd for $C_{29}H_{48}O_3$ (444,36), C 78.3; H 10.9; Found: C 78.4; H 11.0%.

(22R,23R)-22,23-epoxy-5α-hydroxi-stigmastan-3,6-dione (**6A**) *and (22S,23S)-22,23-epoxy-5α-hydroxi-stigmastan-3,6-dione* (**6B**): *Method I:* A solution of MCPBA (0.75 g) in chloroform (3.8 ml) was added to a solution of crude of **4** (1 g) in chloroform (7.5 ml), at room temperature and in the dark. The mixture was then stirred and kept at room temperature for 24 h. The reaction mixture was washed with solutions of NaOH (1%), $NaHCO_3$ (saturated) and brine. The usual "workup" resulted in the mixture of **6A** and **6B** (0.690 g, 69%). This crude mixture was purified by chromatography on silica gel using *n*-hexane/ethyl acetate (20%) as eluent, resulting in 0.305 g (74%) of the mixture of steroids **6A** and **6B**. *Method II:* Jones reagent (1.84 ml) was added in drops to a solution of a mixture of **5A** and **5B** (1.7 g, 3.9 mmol) in 20 ml of acetone. When the reaction finished, ethanol (4 ml) was added to the stirred reaction and the white solid precipitate was then vacuum filtered to afford 1.6 g (96%) of crude **6A** and **6B**. This crude mixture was purified by flash chromatography yielding 1.01 g (2.14 mmol, 63%) of *a mixture* **6A** and **6B** and then compared with authentic samples by TLC. This revealed identical patterns. M.p. (acetone): 251–253°C. IR (KBr, cm^{-1}): 3474 (OH), 1713 (C=O), 1461 (CH), 1240 (C-O). ^1H NMR ($CDCl_3$), (δ, ppm): 2.27 (d, *J* = 15.8 Hz, H-4α), 2.44–2.55 (m, H-22, H-23 **6B** and H-22, **6A**), 2.72 (dd, H-23 **6A**), 2.74 (t, *J* = 12.6 Hz, H-7α) 2.90 (d, *J* = 15.8 Hz, H-4β). EIMS (*m/z*, relative peak height): 458 (M^+, 3), 442 (3), 415 (7), 373 (23), 355 (29), 327 (26), 311 (48), 283 (21), 257 (15), 136 (46), 68 (100), EA: Calcd for $C_{29}H_{46}O_4$ (458,34), C 75.9; H 10.1; Found: C 75.8; H 10.2%.

Biological activity

Radish seeds were germinated over wet cotton in the dark at 25°C for 72 h. Hypocotyls cut to 1 cm long and cotyledons, were placed in separate plates, then 5 ml of the solution containing a mixture of **6A** and **6B** in a ratio of 3:1 (concentrations 10^{-4} to 10^{-4} mg/ml) was applied over the filter paper. For control experiments, cotyledons and hypocotyls were treated with 5 ml of distilled water. After 72 h, the lengths of the hypocotyls were measured, and the cotyledons were weighed. Each experiment for each concentration consisted of three different plates containing 10 hypocotyls or cotyledons per plate. Results refer to the corresponding control experiments.

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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