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# 6-DEOXO-28-NORCASTASTERONE AND 6-DEOXO-24-EPICASTASTERONE—TWO NEW BRASSINOSTEROIDS FROM *ORNITHOPUS SATIVUS*

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**Key Word Index**—*Ornithopus sativus*; Leguminoseae; brassinosteroids; 6-deoxo-24-epicastasterone [(22*R*, 23*R*, 24*R*)-2α, 3α, 22, 23-tetrahydroxy-24-methyl-5α-cholestane]; 6-deoxo-28-norcastasterone; phytosterols; GC-MS.

**Abstract**—Two new brassinosteroids could be detected from shoots of *Ornithopus sativus* and identified as 6-deoxo-24-epicastasterone and 6-deoxo-28-norcastasterone by GC-MS. Furthermore, the known brassinosteroids castasterone, 24-epicastasterone and 6-deoxocastasterone were found in the same plant material.

## INTRODUCTION

Brassinosteroids are a new class of naturally occurring plant growth regulators with high biological activity found in a wide variety of higher plants [1–3]. Recently, we reported the co-occurrence of the two epimeric brassinosteroids castasterone (**1**) and 24-epicastasterone (**2**) in seeds of the Leguminoseae Serradella (*Ornithopus sativus* Brot.) [4]. For comparison of the brassinosteroid pattern we have now investigated young shoots of this plant. In the course of these studies besides **1**, **2** and 6-deoxo-24-epicastasterone (**4**), the new members 6-deoxocastasterone (**3**) and 6-deoxo-28-norcastasterone (**5**) were identified.

## RESULTS AND DISCUSSION

The harvested plants were separated in roots and shoots. The shoots were dried at 40 and pulverized. The powdered material was extracted with methanol and the extracts were concentrated *in vacuo*. The aqueous residue was extracted with chloroform. The chloroform extract was partitioned between *n*-hexane and 80% methanol. Repeated silica gel column chromatography of the concentrated 80% methanol extract using several methanol–chloroform gradient systems led to two fractions eluted with 5 and 6% methanol which showed bioactivity in the rice lamina inclination test. Both fractions were concentrated and further purified on LH-20 Sephadex chromatography using methanol–chloroform (4:1) as eluents. The biological activity appeared in the eluates having 0.7–0.72 of the elution volume per total volume. The bioactive fractions from the Sephadex LH-20 chromatography were combined and further purified by DEA ion exchange chromatography. Final purification was carried out by preparative HPLC on a Eurospher 80-C18 column using an acetonitrile–water system. The biologically active fractions with $R_t$s of 22 min (fraction A), 23 min (fraction B), 24 min (fraction C), 25–26 min (fraction D), 51–52 min (fraction E) and 55–56 min (fraction F) were analysed by GC-MS after derivatization with methaneboronic acid.

Castasterone (**1**) was found in fractions A–C. The GC retention times and the full scan mass spectra were in agreement with those of authentic bismethaneboronate of **1** ($RR_t$ 1.81, $[M]^+$ at $m/z$ 512). 24-Epicastasterone (**2**) could be identified in fraction D by comparison of its GC-MS data with an authentic sample ($RR_t$ 1.86, $[M]^+$ at $m/z$ 512). In fraction E the presence of two 6-deoxobrassinosteroids (**4**, **5**) and in fraction F of a further compound **3** was indicated. The comparison of the mass spectra as well as the GC retention times of the bismethaneboronates suggested that **3** ($RR_t$ 1.48, $[M]^+$ at $m/z$ 498) is the known 6-deoxocastasterone [5, 6], whereas **4** ($RR_t$ 1.53, $[M]^+$ at $m/z$ 498) represents its 24-epimer.

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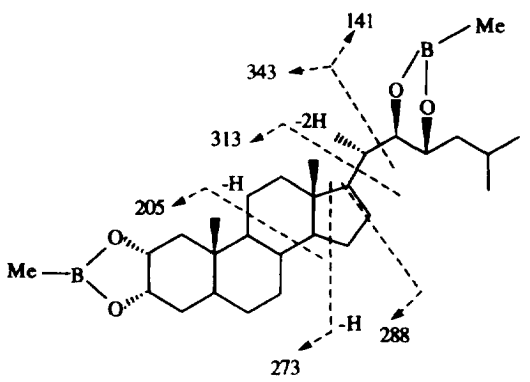
Both compounds show an identical fragmentation pattern. Also, the retention times of both brassinosteroids on the reversed-phase HPLC were in good agreement with this suggestion. The final proof of 6-deoxo-24-epicastasterone was furnished by direct GC-MS comparison with an authentic sample synthesized from 24-epicastasterone following the procedure of Anastasia *et al.* [7]. Recently, the presence of two 6-deoxocastasterone epimers in wheat grains (*Triticum aestivum*) was indicated [8], one of them probably identical with **4** ($RR_t$ 1.04 with respect to 6-deoxocastasterone).

Furthermore, a new brassinosteroid **5** ($RR_t$ 1.39, $[M]^+$ at *m/z* 484) was found in fraction E. Its bismethaneboronate showed besides a molecular ion at *m/z* 484 significant key ions at *m/z* 469, 343, 313, 288, 273, 205 and 141. The prominent ions at *m/z* 288, 273 (base peak) and 205 are characteristic of 6-deoxobrassinosteroids and give a hint for a 2,3-diol moiety in ring A [5]. The ion at *m/z* 141 indicates that **5** has hydroxyls at C-22 and C-23, and no methyl at C-24 in the side chain. Both the molecular ion and other key ions in **5** showed a mass shift of 14 amu compared with **3** and **4** (Scheme 1). Therefore, **5** is proposed to be 6-deoxo-28-norcastasterone.

GC-MS co-injection of aliquot amounts of the methaneboronated fractions A–D showed that the ratio of castasterone (**1**)/24-epicastasterone (**2**) is approx. 9:1. As estimated from the GC-MS co-injection experiment of the methaneboronated fractions E and F the ratio of 6-deoxocastasterone (**3**)/6-deoxo-24-epicastasterone (**4**) is in agreement to this value.

A part of the residue of the *n*-hexane phase was repeatedly chromatographed on a silica gel column using a *n*-hexane–ethylacetate gradient system and chloroform as eluents. The fractions collected were monitored by TLC. Six phytosterols could be identified by GC-MS in comparison with authentic samples (Table 1), among them cholesterol having the same basic side chain structure as 6-deoxo-28-norcastasterone (**5**).

The co-occurrence of 6-deoxocastasterone (**3**) and 6-deoxo-24-epicastasterone (**4**) along with castasterone (**1**) and 24-epicastasterone (**2**) suggests that **3** and **4** are the biosynthetic precursors of their corresponding 6-ketobrassinosteroids **1** and **2**, respectively. Yokota *et al.* found that immature seeds of *Phaseolus vulgaris* contain castasterone (**1**), dolichosterone as well as their corresponding 6-deoxocompounds 6-deoxocastasterone (**3**) and 6-deoxodolichosterone [6]. 6-Deoxo-28-norcastasterone is assumed to be synthesized in a parallel pathway.




Scheme 1. Mass spectral fragmentation of **5** as bismethaneboronate.

Table 1. Phytosterol composition in shoots of *Ornithopus sativus* Brot.

| Phytosterol | Composition (%) |
|---|---|
| Cholesterol | 1.6 |
| Campesterol | 9.7 |
| Campestanol | trace |
| Stigmasterol | 14.0 |
| Sitosterol | 72.9 |
| Stigmastanol | 1.8 |

The roots of the young plants of *Ornithopus sativus* were also analysed with respect to brassinosteroids, but no fraction with biological activity was found after silica gel chromatography.

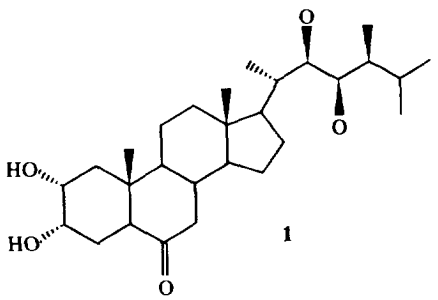
## EXPERIMENTAL

*Plant material.* The seeds of *Ornithopus sativus* Brot. were obtained from "Saat und Pflanzen" Wittenberg, Germany. They were sowed in a mixture of earth and sand. After 3 weeks the plants (grown in a greenhouse, *ca.* 23°) were harvested and sepd in roots and shoots. The roots were washed with distilled $H_2O$. Roots and shoots were dried at 40° and pulverized.

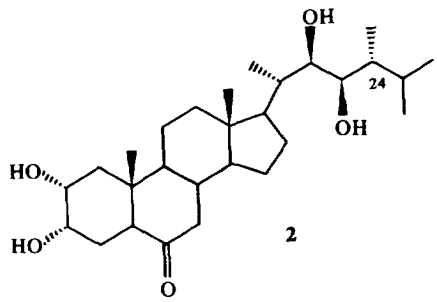
*Bioassay.* The rice lamina inclination test was carried out using the cultivar Koshihikari as described in ref. [9].

*Extraction of brassinosteroids.* The powdered shoots (246 g) were extracted 3× with MeOH. The combined MeOH extracts were evapd to dryness *in vacuo*. The residue was partitioned 3× between $H_2O$ and $CHCl_3$. The residue after evapn of the $CHCl_3$ phase (12.7 g) was partitioned between *n*-hexane (500 ml) and 80% MeOH (500 ml). The *n*-hexane phase was partitioned a second time with 80% MeOH, and the combined 80% MeOH frs were concd (8.5 g).

*Purification of brassinosteroids.* The residue resulting from the 80% MeOH fr. was chromatographed on a silica gel column (53 g). Elution was carried out with $CHCl_3$ (300 ml), $CHCl_3$–MeOH (8:2, 300 ml) and MeOH (300 ml). The eluate with 20% MeOH (2.4 g) was subjected to a second silica gel column (25 g). Elution was carried out stepwise with 10 frs (100 ml) of MeOH in $CHCl_3$ (0, 3, 5, 7, 10, 15, 20, 30, 50, 100%). The frs eluted with 7–15% MeOH were biologically active. The 3 frs were combined and evapd. The residue (1 g) was charged on to a silica gel column (15 g). Elution was performed stepwise with 12 frs (50 ml) of MeOH in $CHCl_3$ (2, 4, 5, 6, 7, 8, 10, 12, 14, 17, 20, 50%). The frs eluted with 5 and 6% MeOH displayed biological activity. These frs were combined and evapd (148 mg). Further purification was carried out using LH-20 Sephadex chromatography




1

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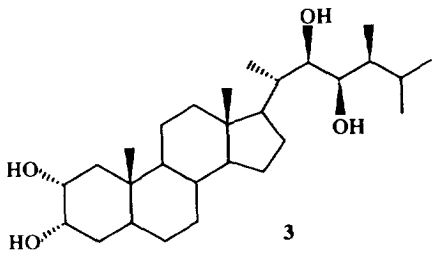


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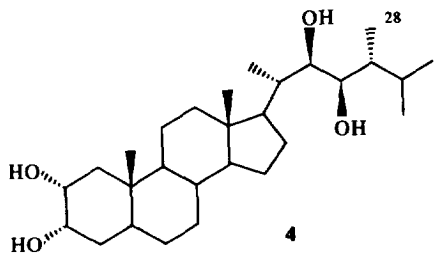


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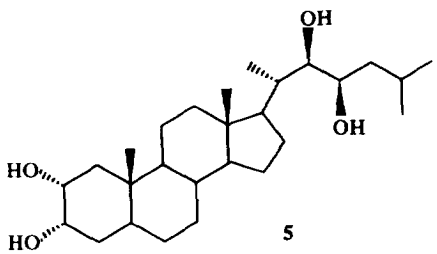


5

(bed vol. 500 ml) with MeOH–$CHCl_3$ (4:1) as eluent. The eluates were collected in 6 ml frs. Frs 35 and 36 (elution vol./total column vol. 0.70–0.72) with biological activity were combined and evapd.

The residue (30 mg) was dissolved in MeOH and runs on a DEA ion exchange cartridge (500 mg, Bond Elut). The residue resulting from the DEA chromatography (9.6 mg) was subjected to HPLC (Eurospher 80-C18, column 8 × 250 mm); flow rate, 2 ml $min^{-1}$, mobile phase, MeCN–$H_2O$ (45% MeCN for 40 min, then raised to 80% MeCN within 5 min and hold on 80% MeCN for 25 min, one run), 70 2 ml frs. The frs with activity were pooled and concd and examined by GC-MS.

*GC-MS*. MD-800 (Fisons Instruments); EI (70 eV); source temp. 200°, column DB-5MS (J&W, 15 m × 0.32 mm, 0.25 μm film thickness), inj. temp. 260°, column temp. program: 170° for 1 min, then raised to 290° at a rate of 30 grd $min^{-1}$ and held on this temp. for 20 min, interface temp. 300°, carrier gas He, flow rate 1 ml $min^{-1}$, splitless injection. The $RR_t$ values were calcd with respect to 5α-cholestane ($R_t$ = 5.45 min). The methaneboronation of the brassionosteroids was carried out with pyridine containing methaneboronic acid at 70° for 30 min [10].

*6-Deoxo-28-norcastasterone* (**5**). $RR_t$ = 1.387, EI-MS *m/z* (rel. int.): 484[M]$^+$ (34), 469 (14), 343 (3), 313 (2), 288 (13), 273 (100), 213 (15), 205 (18), 141 (13).

*6-Deoxocastasterone* (**3**). $RR_t$ = 1.480, EI-MS *m/z* (rel. int.): 498[M]$^+$ (14), 483 (8), 427 (2), 343 (4), 313 (3), 288 (12), 273 (100), 213 (25), 205 (31), 155 (45).

*6-Deoxo-24-epicastasterone* (**4**). $RR_t$ = 1.534, EI-MS *m/z* (rel. int.): 498[M]$^+$ (23), 483 (6), 427 (2), 343 (4), 313 (6), 288 (12), 273 (100), 213 (13), 205 (20), 155 (31).

*Castasterone* (**1**). $RR_t$ = 1.808, EI-MS *m/z* (rel. int.): 512 [M]$^+$ (14), 441 (4), 399 (7), 358 (9), 329 (8), 287 (25), 155 (100).

*24-Epicastasterone* (**2**). $RR_t$ = 1.864, EI-MS *m/z* (rel. int.): 512[M]$^+$ (20), 441 (2), 399 (10), 358 (9), 329 (18), 287 (23), 155 (100).

*Synthesis of 6-deoxo-24-epicastasterone* [(22*R*, 23*R*, 24*R*)-2α,3α,22,23-*tetrahydroxy*-24-*methyl*-5α-*cholestane*, **4**]. Treatment of 24-epicastasterone (**2**, 64 mg) in dry benzene (3 ml) with ethanedithiol (0.1 ml) and boron trifluoride etherate (0.1 ml) under argon and stirring for 30 min afforded the corresponding thioketal (50 mg, glass) which was dissolved in dry benzene (5 ml) and reacted with tris-*n*-butyltin hydride (0.5 ml) in the presence of 2, 2′-azobis-2-methylpropionitrile (3 mg) for 3 hr

at 80°. After dilution with EtOAc and removing of solvents the residue was purified by flash chromatography on silica gel. Elution with *n*-hexane–EtOAc (85:15) provided the 6-deoxotetraacetate (30 mg, amorphous, MS *m/z* 617 $[M-1]^+$). The 6-deoxotetra-acetate (25 mg) was treated with 5% methanolic KOH (5 ml) at room temp. for 24 hr. After removing of the solvent the residue was acidified with 2 N HCl and extracted with EtOAc, worked up and purified by flash chromatography. Elution with *n*-hexane-EtOAc (2:8) gave 6-deoxo-24-epicastasterone [(22*R*, 23*R*, 24*R*)-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestane, 10 mg, 56%], mp. 216–217° (from EtOAc–*n*-hexane); $[\alpha]_D^{25} - 6.6°$ (MeOH; *c* 3.47); IR $\nu_{max}$ $cm^{-1}$: 3406 (br OH). $^1H$ NMR (at 499.84 MHz in $CDCl_3$ with $Me_4Si$ as int. stand.): δ0.66 (3H, *s*, $H_3$-18), 0.80 (3H, *s*, $H_3$-19), 0.84 (3H, *d*, *J* = 7.02 Hz, $H_3$-28), 0.87 (3H, *d*, *J* = 6.72 Hz, $H_3$-26), 0.92 (3H, *d*, *J* = 6.72 Hz, $H_3$-27), 0.92, (3H, *d*, *J* = 6.41 Hz, $H_3$-21), 3.40 (1H, *m*, H-23), 3.70 (1H, *m*, H-22), 3.76 (1H, *m*, H-2), 3.96 (1H, br,*s*, H-3). ESI-MS *m/z*: 473 ($[M + Na]^+$(100%)). 70 eV-EI-MS (probe): *m/z* (rel. int.): 450 ($[M]^+$, 0.7), 432 (1.1), 413 (2.6), 379 (3.8), 350 (100), 331 (82), 313 (86), 295 (30), 273 (34), 255 (24), 249 (25), 231 (37). GC-MS of the bis-methaneboronate: $RR_t$ = 1.528, EI-MS *m/z* (rel. int.): 498 $[M]^+$ (18), 483 (9), 427 (2), 343 (4), 313 (4), 288 (12), 273 (100), 213 (20), 205 (24), 155 (45).

*Identification of the phytosterols.* A portion (1.2 g) of the residue of the *n*-hexane phase was chromatographed on a silica gel column (20 g). Elutin was carried out stepwise with *n*-hexane (100 ml); *n*-hexane–EtOAc 9:1 (2 × 100 ml), 7:3 (6 × 100 ml), 5:5 (100 ml), 3:7 (100 ml) and EtOAc (2 × 100 ml). The frs were monitored by TLC using $CHCl_3$–MeOH (95:5) as developing system. Fr. 4 (539 mg) eluted with *n*-hexane–EtOAc (7:3) was further chromatographed on a silica gel column (31 g) which was eluted with $CHCl_3$ in 1.5 ml frs The combined frs 45–46 containing the phytosterols (18 mg) were further purified by prep. TLC($CHCl_3$–MeOH, 95:5). A portion of the sterol fr. (7.6 mg) was acetylated and the steryl acetate mixt. was examined by GC-MS (Table 1).

*GC-MS of the phytosterols.* Temp. program (column): 170° for 1 min then elevated to 270° within 25 grd $min^{-1}$ then raised to 290° at a rate of 2 grd $min^{-1}$.

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