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24-*Epi*-secasterone and 24-*epi*-castasterone from *Lychnis viscaria* seeds ☆

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Abstract

The brassinosteroids 24-*epi*-castasterone and the hitherto unknown (22*R*, 23*R*, 24*R*)-22,23-dihydroxy-2β,3β-epoxy-24-methyl-5α-cholestan-6-one (24-*epi*-secasterone) could be identified from seeds of *Lychnis viscaria* (Caryophyllaceae). In the phytosterol fraction of the same plant material spinasterol was found as the main component. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Lychnis viscaria*; Caryophyllaceae; Seeds; Brassinosteroids; 24-*Epi*-secasterone; 24-*Epi*-castasterone; Phytosterols

1. Introduction

Brassinosteroids are an important group of phytohormones with a high growth-promoting activity. These plant growth regulators have been found in a wide variety of higher plants and occur in trace amounts in a range of ng to μg per kg plant material. These natural products show profitable effects in field experiments such as stimulation of germination, promotion of plant growth and increase in crop yield. In addition, a higher tolerance of plants to abiotic and biotic stress have been observed after application (Clouse & Sasse, 1998; Cutler, Yokota & Adam, 1991; Fujioka & Sakurai, 1997; Marquardt & Adam, 1991; Takatsuto, 1994; Yokota, 1997).

Plant promoting effects of natural agents produced on the basis of different plant extracts of Caryophyllaceae have been described for practical application in agriculture (Gajic, 1988). Further investigation of plant promoting effects resulted in the production of effective agents, including extracts of *Lychnis viscaria* seeds as a main component.

In this paper we report the identification of 24-*epi*-castasterone and 24-*epi*-secasterone in seeds of *L. viscaria*.

2. Results and discussion

Powdered seeds of *L. viscaria* were extracted with methanol and ethyl acetate over a time of two weeks. After concentration of extracts *in vacuo* the residue was partitioned between water and ethyl acetate. The ethyl acetate extract was then partitioned between *n*-hexane and 90% methanol. After concentration the aqueous methanol fraction was subjected to a silica gel chromatography eluted stepwise with increasing concentrations of methanol in chloroform. The biological activity of one hundredth of each eluted fraction was assayed with the rice lamina inclination test. The fraction eluted with 5 % methanol in chloroform displayed biological activity and was further purified by preparative reverse-phase HPLC on a RP-18 column with a water–methanol gradient system. The biological ac-

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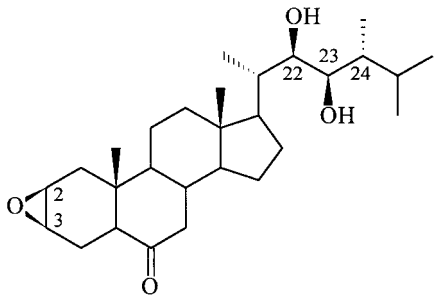
tivity in rice inclination assay was found for the fractions with R_t of 25.5–29.5 min. This biologically active fraction was analysed by GC–mass spectrometry (MS) after derivatization with methylboronic acid.



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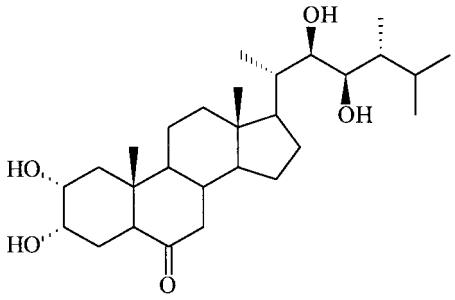

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The EI mass spectrum of the methylboronated compound **1** showing a molecular ion M^+ at m/z 470 is very similar to those obtained for 2,3-epoxybrassinosteroids (Adam, Porzel, Schmidt, Schneider & Voigt, 1996; Schmidt et al., 1995b) and 3-dehydroteasterone (Abe et al., 1994; Yokota, Nakayama, Wakisaka, Schmidt & Adam, 1994). The fragmentation is mainly characterized by cleavages in the side chain leading to the key ions at m/z 399 ($[M\text{-}71]^+$), 357 ($[M\text{-}113]^+$), 316 (bond scission C-20/C-22), 287/286/285 (splitting between C-17/C-20) and 245 (ring D-cleavage, C-13/C-17 and C-14/C-15). While the base peak ion at m/z 155 is typical for a methylboronated side chain with a methylation at C-24, the ion at m/z 137 comprises the ring A (Schmidt et al., 1995b). Both the mass spectral data and the relative retention time RR_t of **1** (0.945) with respect to 24-*epi*-castasterone in the GC were in good agreement with those of synthesized 24-*epi*-secasterone [0.947, (22*R*, 23*R*, 24*R*)-22,23-dihydroxy-2β,3β-epoxy-24-methyl-5α-cholestan-6-one] as shown by comparison with an authentic sample (Schmidt et al., 1995b; Voigt, Takatsuto, Yokota & Adam, 1995). This is the first report of the isolation of 24-*epi*-secasterone from a natural source, representing besides secasterone from seeds of *Secale cereale* (Schmidt et al., 1995b) the second brassinosteroid with a 2β,3β-epoxy function. In regard to their biosynthesis it is remarkable that both epoxides do not fit into the hitherto known pathways (Fujioka & Sakurai, 1997; Yokota, 1997) reflecting a greater diversity of biosynthetic sequences than assumed until now.

Table 1
Phytosterols from *Lychnis viscaria* seeds

Phytosterol[a]	Relative composition (%)[b]
Cholesterol	0.7
24-Methylcholesta-5,22-dien-3β-ol	0.6
24-Methylcholesta-7,22-dien-3β-ol	1.4
Campesterol	1.1
Campestanol	0.2
Stigmasterol	0.6
Stigmast-22-en-3β-ol	0.3
24-Methylcholest-7-en-3β-ol	2.7
Spinasterol	88.2
Sitosterol	traces
Sitostanol	1.2
Unidentified Δ^7-sterol	0.2
Dihydrospinasterol	2.8

[a] Determined as acetates.
[b] The relative composition of the sterols was calculated from their peak areas in the GC total ion current chromatogram.

Furthermore, the already known 24-*epi*-castasterone (**2**) could be identified by comparing with an authentic sample. Compound **2** was firstly found in the green alga *Hydrodictyon reticulatum* (Yokota et al., 1987). Occurrences in higher plants are described for *Ornithopus sativus* (Schmidt, Spengler, Yokota & Adam, 1993; Spengler, Schmidt, Voigt & Adam, 1995), *Beta vulgaris* (Schmidt, Kuhnt & Adam, 1994), *Rheum rhabarbarum* (Schmidt, Himmelreich & Adam, 1995a) and *Daucus carota* (Schmidt, Porzel & Adam, 1998).

A further brassinosteroid-like compound eluting later than 24-*epi*-castasterone in the GC was found in amounts being comparable with both other brassinosteroids. Its EIMS also display the typical side chain fragment m/z 155 as well as some other brassinosteroid-type ions. The relative retention time RR_t of 1.36 suggests an unpolar brassinosteroid conjugate, the structural determination of which needs further investigations.

The Silenoideae subfamily of Caryophyllaceae is known as phytoecdysteroid rich plant source (Bathori, Mathe, Solymosi & Szendrei, 1987). The identification of 24-*epi*-secasterone and 24-*epi*-castasterone in *L. viscaria* is the first report of brassinosteroids in the *Lychnis* genus of the Caryophyllaceae family and represents another example, that compounds of the 24-*epi* (24*R*) series can occur as the only brassinosteroids in plants. It is interesting to note, that also in the earlier described occurrence of a brassinosteroid in a

Caryophyllaceae with 24-*epi*-brassinolide from *Gypsophila perfoliata* a member of the 24-*epi*-series was identified (Schmidt, Böhme & Adam, 1996).

The sterol fraction mainly contains Δ^7-sterols (ca. 95%). In this fraction spinasterol could be identified as the main component (88%) by the GC–MS analysis of the phytosteryl acetates (Table 1).

3. Experimental

3.1. Plant material

The seeds of *L. viscaria* were collected from naturally grown plants in Italy in summer of 1996 and were obtained from Polus & Partner (Lindenfels, Germany).

3.2. Bioassay

The rice inclination bioassay was carried out using the cultivar "Koshihikari" according to the method of Arima, Yokota and Takahashi (1984).

3.3. Extraction and purification of brassinosteroids

Powdered seeds (25 g) of *L. viscaria* were extracted three times with 200 ml MeOH over a time of 1 week and three times with 200 ml of a MeOH–EtOAc (1:1 v/v) solvent over a time of 1 week. The combined extracts were concentrated in vacuo. The residue was partioned three times between 150 ml H_2O and 150 ml EtOAc. The residue of the combined EtOAc phases was further partitioned three times between *n*-hexane and 90% MeOH. The combined aqueous MeOH phases were concentrated and partitioned between 150 ml EtOAc and 150 ml saturated sodium hydrogencarbonate solution.

The residue resulting from the EtOAc phase (145 mg) was chromatographed on a silica gel column (20 cm × 1 cm, Merck Kieselgel, 35–70 mesh). The column was successively eluted with chloroform (50 ml) and then methanol in chloroform (2, 5, 10, 15, 20, 100 vol%; each 50 ml). The eluate was collected in 25 ml frs.. Biological activity in the rice lamina inclination was found for the fraction eluted with 5 % MeOH in chloroform (4.8 mg).

The biologically active oil was further purified by preparative HPLC. HPLC separations were performed on a Beckman model 126 chromatograph equipped with a 4.6 × 250 mm ultrasphere ODS RP 18 column. Chromatographic conditions consisted of a mobile phase of A, H_2O (0.1 vol% TFA) and B (methanol) run with a flow rate of 1 ml/min. The column was eluted using the following solvent gradient profile: 0–1 min: 100% A, 1–10 min: 0–40% B (linear gradient), 10–15 min: 40% B, 15–27 min: 40–100% B (linear gradient), 27–35 min: 100% B, 35–37 min: 0–100% A (linear gradient). The fractions with activity (retention times 25.5–29.5 min) were concentrated, derivatized with methylboronic acid and examined by GC–MS.

3.4. Gas chromatography–mass spectrometry (GC–MS)

MD-800 (Fisons Instruments, EI (70 eV); source temp. 200°C; column DB-5MS (J&W, 15 m × 0.32 mm, 0.25 μm film thickness), inj. temp 260°C, interface temp. 300°C, carrier gas He, flow rate 1 ml/min, splitless injection, column temp. program: 170°C for 1 min, then raised to 290°C at a rate of 30°C/min and held on this temperature for 20 min. The relative retention times (RR_t) of the derivatized brassinosteroids were calculated with respect to 24-*epi*-castasterone ($R_t = 9.90$ min). The methylboronation of the brassinosteroids was carried out by treatment of the sample with pyridine containing methylboronic acid (Takatsuto, Ying, Morisaki & Ikekawa, 1982).

3.4.1. 24-Epi-secasterone (**1**, natural source)

$RR_t = 0.945$, EI-MS (*m*/*z*, rel. int.): 470(M^+, 49), 455 (3), 399 (6), 357 (5), 339 (6), 316 (21), 287 (7), 286 (5), 285 (11), 259 (5), 245 (11), 155 (100), 137 (7), 85 (65), 71 (76).

3.4.2. 24-Epi-secasterone (authentic sample)

$RR_t = 0.947$, EI-MS (*m*/*z*, rel. int.): 470(M^+, 37), 455 (2), 399 (3), 357 (9), 339 (2), 316 (13), 287 (7), 286 (6), 285 (5), 259 (8), 245 (30), 155 (100), 137 (12), 85 (93), 71 (57).

3.4.3. 24-Epi-castasterone (**2**)

$RR_t = 1.00$, EI-MS identical with the previously described one Spengler et al., 1995.

3.4.4. Unknown brassinosteroid

$RR_t = 1.36$, EI-MS (*m*/*z*, rel. int.): 512 (49), 470 (12), 452 (10), 437 (88), 434 (28), 408 (6), 339 (4), 316 (7), 297 (8), 269 (4), 225 (7), 155 (40), 85 (76), 71 (100).

3.5. Extraction and purification of phytosterols

Fifteen g of the same plant material were extracted three times with 150 ml MeOH over a time of three days and three times with 150 ml of a MeOH–EtOAc (1:1 v/v) solvent over a time of three days. The combined extracts were concentrated in vacuo. The residue was partioned three times between 200 ml H_2O and 200 ml EtOAc. The residue of the combined EtOAc phases was further partitioned three times between *n*-hexane and 80% MeOH. The concentrated *n*-hexane fraction (0.6 g) was subdivided into two portions (each 0.3 g) which were chromatographed on two identical

silica gel columns (20 × 1 cm, Merck Kieselgel, 35–70 mesh). The columns were stepwise eluted with *n*-hexane (50 ml) and then EtOAc in *n*-hexane (10, 30, 50, 70, 90 vol%; each 50 ml). The eluate was collected in 6 ml frs.. The frs were monitored by TLC using a $CHCl_3$–MeOH solvent (95:5 v/v) as developing system. Phytosterols were detected with 5% H_2SO_4 in acetic anhydride as spray reagent and heating the TLC-plates at 150°C. Fractions containing phytosterols were combined (49 mg) and further purified by a silica gel column (8 g silicagel) using CH_2Cl_2/*n*–heptane (1:1 v/v) as eluent system with 8 ml frs.. The sterol containing fr (2 mg) was acetylated and examined by GC–MS under the same conditions as described previously (Schmidt et al., 1996).

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