24-Epibrassinolide from *Gypsophila perfoliata*

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The scarce 24-epibrassinolide could be identified from seeds of *Gypsophila perfoliata* L. by GC/MS as the only brassinosteroid present. Furthermore, the Δ^7-phytosterols ergost-7-en-3β-ol, spinasterol and 22-dihydrospinasterol were found as main sterols in the same plant material.

Introduction

Brassinosteroids are a class of naturally occurring phytohormones with high biological activity found in a wide variety of higher plants (Adam and Petzold, 1994; Adam *et al.*, 1996; Cutler *et al.*, 1991; Sakurai and Fujioka, 1993). Brassinosteroids occur only in trace amount (ng- to µg/kg plant material). Therefore, their purification has to be monitored by a sensitive bioassay (Wada *et al.*, 1984) and microanalytical techniques must be used for the brassinosteroid analysis (Ikekawa *et al.*, 1984).

In continuation of our work on the distribution of brassinosteroids we have investigated seeds of *Gypsophila perfoliata* belonging to the family Caryophyllaceae. To our knowledge, up to now no occurrence of brassinosteroids has been described for plant species of the Caryophyllaceae family. *Gypsophila perfoliata* stems originally from Middle-Asia and was not investigated with respect to natural compounds before. The present paper deals with the isolation and identification of brassinosteroids and phytosterols from seeds of this species.

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Materials and Methods

Plant material

The seeds of *Gypsophila perfoliata* L. were obtained from the Institut für Landwirtschaftliche Forschung und Untersuchung e. V., Halle/Saale, Germany.

Bioassay

The rice lamina inclination bioassay was carried out using the cultivar "Koshihikari" as described previously (Arima *et al.*, 1984).

Extraction and purification of brassinosteroids

The powdered seeds (301 g) were extracted three times with 600 ml MeOH. The residue of the MeOH extracts was partitioned three times between 150 ml H_2O and 300 ml $CHCl_3$. The combined $CHCl_3$ phases were dried with Na_2SO_4 and the residue of the $CHCl_3$ layer (5.05 g) was partitioned between 200 ml *n*-hexane and 250 ml 80% MeOH. The *n*-hexane phase was partitioned a second time with 250 ml 80% MeOH, and the combined 80% MeOH fractions were concentrated (901 mg).

The residue resulting from the 80% MeOH fraction was chromatographed on a silica gel column. Elution was carried out stepwise with 11 fractions (100 ml) of MeOH in $CHCl_3$ (0, 2, 3, 4, 5, 7, 10, 15, 20, 50, 100 v/v%). The fraction eluted with 4% MeOH (199 mg) was biologically active and further purified using LH-20 Sephadex (PHARMACIA) chromatography with MeOH-$CHCl_3$ (4:1 v/v) as eluent. After running the combined active fractions (49 mg) on a diethylaminopropyl (DEA) ion exchange column (1 g, Analytichem Bondesil, Preparative Grade) the residue (29 mg) was subjected to HPLC using the conditions described previously (Spengler *et. al.*, 1995). The fractions with activity (retention times 17–20 min) were concentrated, derivatized with methylboronic acid and examined by GC-MS.

Purification and identification of phytosterols

A portion (2 g) of the residue of the *n*-hexane phase obtained from the partition procedure with 80% MeOH (see above) was saponified with 5%

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KOH in 80% EtOH (13 ml) for 3 hours. The unsaponifiable lipid was extracted three times with 50 ml $CHCl_3$. After drying the $CHCl_3$ layer with Na_2SO_4 the residue (1.61 g) was chromatographed on a silica gel column (81 g). Elution was carried out stepwise with an *n*-hexane/EtOAc gradient system with increasing content of EtOAc (9:1, 7:3, 5:5, 3:7, 1:9 v/v) using seventeen 25 ml-fractions of each gradient (425 ml). The fractions were monitored by TLC using $CHCl_3$-MeOH (95:5) as developing system. The phytosterol containing fractions 15–17 (45 mg) eluted with *n*-hexane-EtOAc (7:3 v/v) were further chromatographed on a silica gel column (2 g) which was eluted with $CHCl_3$. The phytosterol mixture (10 mg) was recrystallized from MeOH (boiling temperature) and acetylated with acetanhydride/pyridine at room temperature for 12 hours. The steryl acetates were examined by GC-MS (Table I).

Gas chromatography – mass spectrometry

The bismethylboronation of the brassinosteroids was carried out by treatment of the samples with pyridine containing methylboronic acid (2 mg/ml) at 70° for 30 min (Takatsuto *et al.*, 1982). Brassinolide and 24-epibrassinolide (each 1 μg) as reference compounds were derivatized according to the above described procedure by adding 20 μl of the methylboronic acid-pyridine solution, 1 μl (50 ng brassinosteroid) was submitted to the GC-MS analysis. The same procedure was used for the evaporated HPLC-fractions. The GC-MS measurements of the bismethylboronates of the brassinosteroids as well as of the phytosteryl acetates were performed from a MD-800 instrument (Fisons Instruments) using the conditions described previously (Spengler *et al.*, 1995). The relative retention times (RRT) were calculated with respect to 5α-cholestane.

Cholesteryl acetate, RRT=1.31, EI-MS (*m/z*, rel. int., %) : 368 ([M-HOAc]$^+$, 100), 260 (40), 255 (35), 247 (32), 213 (29).

Ergosta-7,22-dien-3β-yl acetate, RRT=1.41, EI-MS (*m/z*, rel. int., %) : 440 (M$^+$, 4), 342 (3), 313 (45), 255 (19), 229 (9), 213 (13), 81 (100).

Ergost-7-en-3β-yl acetate, RRT=1.50, EI-MS (*m/z*, rel. int., %) : 442 (M$^+$, 37), 427 (10), 367 (9), 315 (5), 273 (7), 255 (66), 229 (23), 213 (54), 81 (100).

Spinasteryl acetate, RRT=1.55, EI-MS (*m/z*, rel. int., %) : 454 (M$^+$, 7), 439 (3), 411 (4), 342 (5), 313 (49), 288 (6), 255 (32), 229 (11), 213 (16), 81 (100).

22-Dihydrospinasteryl acetate, RRT=1.63, EI-MS (*m/z*, rel. int., %) : 456 (M$^+$, 51), 441 (14), 381 (10), 315 (7), 288 (6), 273 (9), 255 (90), 229 (29), 213 (64), 81 (100).

Results and Discussion

The biologically active fractions resulting from the preparative HPLC with retention times of 17, 18 and 19–20 min were analyzed by GC-MS after derivatization with methylboronic acid. 24-Epibrassinolide (**1**) was identified as the only one brassinosteroid in each of these fractions. Its relative retention time RRT with respect to 5α-cholestane (RT = 5.63 min) in the GC and the full scan mass spectrum of its bismethylboronate were in agreement with those of authentic 24-epibrassinolide [(RRT = 2.22; EIMS (*m/z*, rel. int., %): 528 (M$^+$, 2), 457 (2), 415 (3), 374 (11), 345 (9), 332 (10), 177 (43), 155 (84), 85 (100)]. Brassinolide showing the same mass spectrum elutes earlier in the GC (RRT = 2.13). A natural occurrence of 24-epibrassinolide was only detected before in pollen of *Vicia faba* (Ikekawa *et al.*, 1988), whereas its precursor 24-epicastasterone has been found by us already in a broad spectrum of plant species (Adam *et al.*, 1996). These findings indicate that 24-epibrassinosteroids are more widespread as hitherto assumed in higher plants and can play their independent role as phytohormones in special cases. Furthermore, to our knowledge this is the first detection of a brassinosteroid in a plant of the Caryophyllaceae family.

In the sterol fraction besides traces of cholesterol only Δ^7-sterols occur. Ergost-7-en-3β-ol, spinasterol and 22-dihydrospinasterol could be identified by GC-MS as main components in

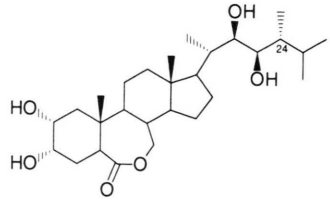

Table I. Phytosterol composition in seeds of *Gypsophila perfoliata* L.

Phytosterol	Composition (%)*
Cholesterol	0.2
Ergosta-7,22-dien-3β-ol	0.9
Ergost-7-en-3β-ol	5.6
Spinasterol	79.2
22-Dihydrospinasterol	12.4
Unidentified Δ^7-sterols	1.7

* The relative composition of the phytosterols was calculated from their peak areas in the GC total ion current (TIC) chromatogram.

comparison with authentic samples or reference data from the literature (Goad, 1991; Itoh *et al.*, 1984) (Table I). It is interesting to note, that the Δ^5-sterols ergost-5-en-3β-ol (22-dihydrobrassicasterol) or ergosta-5,22-dien-3β-ol (brassicasterol) being the potential precursors of 24-epibrassinolide could not be found suggesting that the corresponding Δ^7-sterols could be involved in the biosynthesis of 24-epibrassinolide in *Gypsophila perfoliata*.

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