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STEROIDAL PLANT GROWTH REGULATORS, CASTASTERONE AND TYPHASTEROL (2-DEOXYCASTASTERONE) FROM THE SHOOTS OF SITKA SPRUCE (*PICEA SITCHENSIS*)

TAKAO YOKOTA, MASAHIRO ARIMA, NOBUTAKA TAKAHASHI and ALAN CROZIER*

Department of Agricultural Chemistry, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan; *Department of Botany, The University, Glasgow G12 8QQ, U.K.

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Key Word Index—*Picea sitchensis*; Pinaceae; steroidal growth regulators; brassinosteroids; castasterone; typhasterol; 2-deoxycastasterone.

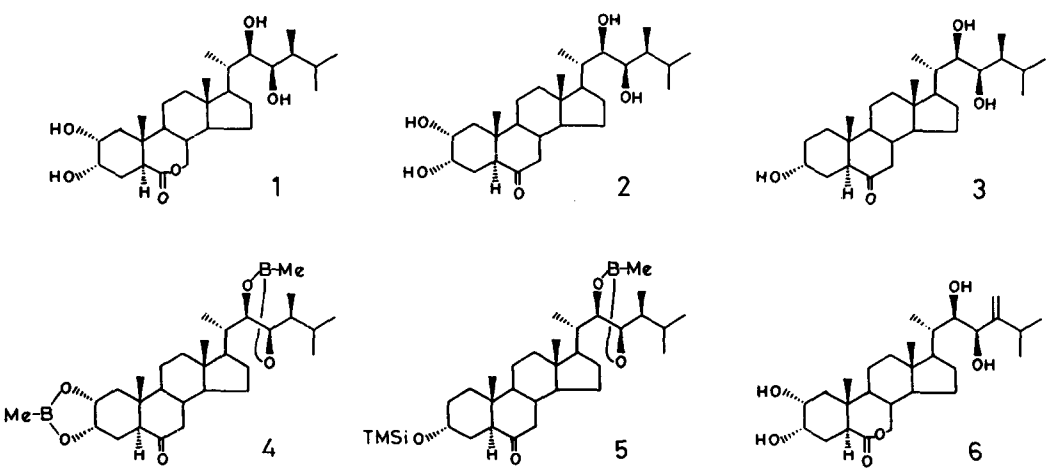
Abstract—Castasterone, [(22*R*,23*R*,24*S*)-2α,3α,22,23-tetrahydroxy-24-methyl-5α-cholestan-6-one] and typhasterol (2-deoxycastasterone) have been identified in purified extracts from the shoots of Sitka spruce (*Picea sitchensis*) by GC/MS.

INTRODUCTION

Brassinolide (**1**) is a steroidal plant growth regulator possessing unique biological activities [1]. In recent years a number of steroidal growth regulators have been found to occur in several species of angiosperm [2, 3]. Recently, we isolated castasterone (**2**) and typhasterol (2-deoxycastasterone) (**3**) from the pollen of Japanese black pine (*Pinus thunbergii*) [4]. This was the first indication of the occurrence of steroidal growth regulators in gymnosperms. Castasterone was first isolated from the insect gall of chestnut (*Castanea crenata*) [5] and was later found in *Thea sinensis* [6], *Dolichos lablab* [7], *Phaseolus vulgaris* [8], *Oryza sativa* [3], *Brassica campestris* [9] and *Distylium racemosum* [9]. Typhasterol was originally detected in an extract from pollen of *Typha latiforia* [10]. Further interest in the distribution of such compounds in gymnosperms prompted us to analyse an extract from the shoots of Sitka spruce (*Picea sitchensis* Bong Carr).

RESULTS AND DISCUSSION

A neutral benzene soluble extract from 1 kg of shoots of *P. sitchensis* was partitioned between hexane and 80% methanol. When tested in the rice-lamina inclination bioassay [11], which is very sensitive to brassinolide and related compounds, biological activity equivalent to 13 μg of brassinolide was located exclusively in the 80% methanol fraction. The 80% methanol fraction was purified on a silica gel column eluted step-wise with increasing amounts of methanol in chloroform. Biological activity was detected in the 5%, 20% and 100% methanol fractions. The latter two fractions were combined and purified by Sephadex LH-20 chromatography using a methanol–chloroform mobile phase. However, only minor biological activity was observed in the eluates from this column. The analysis of these fractions was, therefore, not pursued further. When the 5% methanol fraction was similarly purified on Sephadex LH-20, a clear zone of



biological activity was detected. This was purified on a Sephadex LH-20 column eluted with 70% aqueous ethanol. Once again a peak of biological activity was detected and this was further purified by reversed-phase high performance liquid chromatography (HPLC). Distribution of biological activity following HPLC is shown in Fig. 1. Major activity was present in fractions 9 and 14.

Fraction 9 was analysed by GC/MS after methaneboronation [12]. The predominant peak observed in the reconstructed ion current trace had the same R_t (6.35 min) as authentic castasterone bismethaneboronate (4) and gave a mass spectrum identical with that of 4. Major fragment ions were observed at *m/z* (rel. int.): 512 $[M]^+$ (44), 497 (2), 441 (10), 358 (20), 287 (21), 228 (7) and 155 (100). Fraction 14 was subjected to methaneboronation followed by trimethylsilylation. The major constituent in the reconstructed ion current trace had the same R_t (5.55 min) as the authentic 3-*O*-trimethylsilyl ether of typhasterol methaneboronate (5) and yielded a mass spectrum identical with that of 5. Major fragment ions were observed at *m/z* (rel. int.): 544 $[M]^+$ (100), 529 (56), 526 (28), 515 (81), 454 (71), 439 (20), 300 (18), 229 (32) and 155 (44). It can, therefore, be concluded that the steroidal plant growth regulators castasterone (2) and typhasterol (3) are endogenous constituents of the shoots of *P. sitchensis*.

It might be of taxonomic significance that both castasterone (2) and typhasterol (3) have now been found in two species belonging to the Pinaceae, *Pinus Thunbergii* [4] and *Picea sitchensis*. Although chemotaxonomic aspects of the growth-promoting steroids have not been studied in any detail, it is evident that the main constituents do appear to vary between taxonomically different plants. For example, brassinolide (1) is the major active constituent in rape (*Brassica napus*) [1], while castasterone (2) is the predominant component in chestnut (*Castanea crenata* [2, 5] and dolicholide (6) in *Dolichos lablab* [13].

The amounts of castasterone and typhasterol in the purified extract from 1 kg of *Picea* shoots were estimated by GC-selected ion monitoring (SIM) to be *ca* 5 and 7 μg, respectively. These are obviously underestimates as internal standards were not used to assess losses encountered during sample purification. It is evident that the levels of castasterone (2) and typhasterol (3) present in *Picea* shoots could be sufficient to affect growth since submicrogram quantities of such compounds can elicit responses in various bioassay systems [14–21]. However, as yet the biological activity of steroidal growth regulators has not been investigated in test systems utilizing plant material from a member of the Pinaceae.

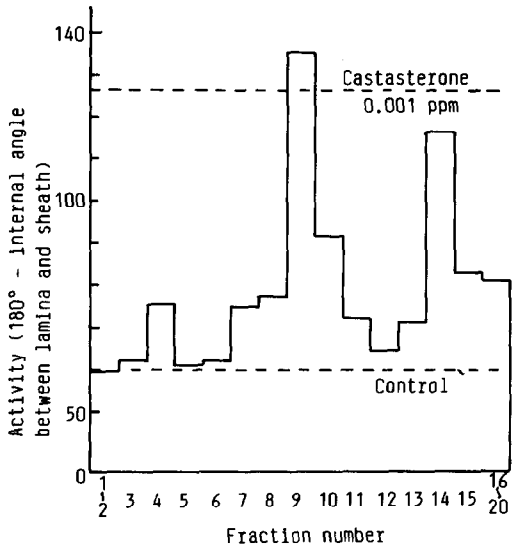


Fig. 1. Distribution of biological activity determined by the rice-lamina inclination test after reversed-phase HPLC of a partially purified extract from 1 kg *P. sitchensis* shoots. Fractions assayed at a 50-fold dilution.

EXPERIMENTAL

Plant material. Shoots of *Picea sitchensis* Bong Carr were collected from the upper crown of four 49-year-old trees growing in the Glentress Forest, Peebleshire, U.K. on 28 September 1981. The shoots were frozen in liquid N_2 and stored at −20° prior to extraction.

Bioassay. The rice-lamina inclination test was carried out using the cultivar Koshihikari as described by Arima *et al.* [2].

Extraction. Shoots (1 kg) were homogenized and extracted twice with an excess of cold MeOH. The combined MeOH extract was reduced to the aq. phase *in vacuo*, an equal vol. of pH 8.0, 0.5 M Pi buffer was added and the extract was adjusted to pH 8.0 prior to partitioning × 3 against one-third vols of C_6H_6. The C_6H_6 extracts were combined and reduced to dryness *in vacuo*.

Purification. The C_6H_6 fraction (17.3 g) was dissolved in hexane (500 ml) and partitioned twice against 80% MeOH (500 ml). The combined 80% MeOH fraction (6.4 g) was purified on a column packed with 65 g silica gel. Elution was carried out step-wise with $CHCl_3$ (300 ml) and MeOH–$CHCl_3$ (2.5:97.5, 500 ml; 5:95, 300 ml; 10:90, 500 ml; 20:80, 500 ml; 100:0, 500 ml). The 5% MeOH eluate (0.9 g), which contained biological activity, was chromatographed on a Sephadex LH-20 column (900 × 26.6 mm i.d.) using a mixture of $CHCl_3$–MeOH (1:4) at a flow rate of 30 ml/hr. Successive 10 ml fractions were collected and biological activity detected in fractions 31–38 which, when combined and reduced to dryness, weighed 0.2 g. The sample was dissolved in 70% EtOH and centrifuged to remove insoluble materials before being chromatographed on a Sephadex LH-20 column (900 × 26.6 mm i.d.) with a mobile phase of 70% aq. EtOH. Biological activity eluted between 350 and 390 ml and the wt of the extract was reduced to 34 mg.

High performance liquid chromatography. A JASCO (Japan) model Tri Rotar SR-2 liquid chromatograph was used for HPLC and samples were introduced off-column via a Rheodyne 7125 injector (Berkeley, U.S.A.) fitting with a 5 ml loop. Samples were analysed by reversed-phase HPLC on a 250 × 20 mm i.d. column packed with 20 μm Sensyu Pak LRP-1 and eluted at a flow rate of 10 ml/min with a MeCN–H_2O mobile phase (containing 1% HOAc) comprising 0–20 min, 50% MeCN; 20–40 min, 50–70% MeCN; 40–60 min, 70% MeCN; 60–80 min, 100% MeCN. Fractions were collected every 4 min and those containing biological activity were analysed by GC/MS.

GC/MS and selected ion monitoring. GC/MS was carried out on a JEOL (Japan) DX-300 (ionization voltage, 70 eV). GC conditions were as follows: column, 2% OV-1 (1 m × 2.6 mm i.d.); column temp., 280°; flow rate of He carrier gas, 40 ml/min. SIM was carried out with a Hitachi 80A (ionization voltage, 22 eV) under the same GC conditions as described above except that a 1 m × 3 mm i.d. column was used. Quantitative data were calculated from $[M]^+$ SIM peak areas. Standard calibration curves were obtained using known amounts of authentic compounds.

Derivatization. An aliquot of each sample was dissolved in 25 μl pyridine containing 2 mg/ml methaneboronic acid and heated at 65° for 30 min in order to produce the bismethaneboronate derivative. The reaction mixture was used directly for analysis. Bis(trimethylsilyl)acetamide (10 μl) and trimethylchlorosilane (3 μl) were added to the above reaction mixture and heated at 65° for 5 min to obtain the methaneboronate–trimethylsilyl ether. The reaction mixture was used directly for analysis.

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