Purification and Identification of New Acyl-conjugated Teasterone in Lily Pollen

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Typical brassinosteroid activity was found in the alkaline hydrolysate of the n-hexane fraction of lily pollen. Two acyl conjugates of teasterone were purified from the n-hexane fraction by HPLC and analyzed by GC-MS and/or LC-MS, resulting in the identification of teasterone esters with lauric acid and myristic acid. Syntheses of the teasterone esters are also reported. The acyl conjugates of typhasterol, castasterone, and brassinolide did not occur in lily pollen. This is the first time that acyl conjugates have ever been discovered among naturally occurring brassinosteroids.

Key words: acyl-conjugated teasterone; brassinolide; brassinosteroid; Lilium longiflorum; pollen

The search for brassinosteroid [BS] by using the rice-lamina joint test has demonstrated that BS is present in all plant parts. The BS content in vegetative tissue is extremely low compared with that of known plant hormones, but the BS content in plant male gametophyte (pollen) tissue is exceptionally high. In particular, it is enriched with 2-deoxy BS such as teasterone [TE] and typhasterol [TY]. A change in the levels of BS activity has been observed during the maturation of lily and green tea pollen. In the pollen of Lilium longiflorum cv. Georgia, endogenous BS has been found to change in quantity and quality during pollen maturation. Immature uninnucleate lily pollen after meiosis contained only a trace amount of TE, but the mature pollen, just before anthesis, contained large amounts of TE, TY, castasterone [CS], and brassinolide [BL]. The apparent absence of TE, a biosynthetic precursor of TY, in the immature uninnucleate pollen suggests that TE may be conjugated. An earlier paper provided the first definitive evidence for the existence of the fatty acid ester conjugate of TE. In this present paper, we report in detail the separation and purification by HPLC, and the structural identification by GC/MS or LC/MS of the ester conjugates, teasterone 3-laurate [TE-La] as a new acyl conjugate and teasterone 3-myristate [TE-My].

Materials and Methods

Extraction of conjugated BSs and purification of released BS after hydrolysis: The extraction of acyl-conjugated BSs from pollen with anthers (8440 anthers, 711.3 g fr. wt.) of lily, Lilium longiflorum cv. Georgia, and the purification and identification of released BS after hydrolysis have been described in detail in our previous report.17

Purification of TE esters. The combined n-hexane-soluble fractions (23.6 g) were twice submitted to silica gel chromatography with stepwise elution (1st column: Wakogel C-200, 140 g, 3.5 cm i.d. x 28 cm; n-hexane containing 0, 20, 40, 60, and 80% EtOAc, and 100% EtOAc; 2nd column: Wakogel C-200, 73.6 g, 2.6 cm i.d. x 27 cm; n-hexane containing 20, 25, 30, 35, 40, 45, 50, 55, and 60% EtOAc). The active substance was in the 40% and 60% EtOAc fractions from the first column (2.2 g), and in the 35% and 40% EtOAc fractions from the second column (116.8 mg). The combined fractions were further purified by Sephadex LH-20 (2.2 cm i.d. x 65 cm; 50% CH3OH in MeOH; flow rate, 0.5 ml/min, fractionated in 2-ml portions). The active substances eluted in Frs. 49-58 (33.1 mg) were purified by Devoselods ODS-5 HPLC (4.6 mm i.d. x 250 mm; MeOH: flow rate, 1 ml/min), separating into four active fractions: 0-11 min (ester-A, 9.5 mg, weak), 11-15 min (ester-B, 5.2 mg), 15-18 min (ester-C, 2.0 mg), and 18-23 min (ester-D, 3.4 mg weak).

Ester-B was dissolved in THF-CH3CN-H2O (35:50:30) and was purified in a Waters fatty acid analysis (FAA) column (3.6 mm i.d. x 150 mm; THF-CH3CN-H2O (25:50:40); flow rate, 1.0 ml/min), giving active fractions (tR 11-13 min) for GC/MS analysis.

Ester-C was dissolved in THF-CH3CN-H2O (35:50:30), and the soluble substances were loaded into an aluminum oxide column (Merck aluminum oxide, 90, grade II-III, 10 g; 18 mm i.d. x 25 m). Eluting with 80% EtOAc in n-hexane and 100% EtOAc, and with 20, 40, 60, and 80% EtOH in EtOAc (15 ml each) gave strong activity in the eluate with 20% EtOH in EtOAc (0.8 mg). This eluate was further purified by HPLC with a Sclshupak DIOL-1251-R column (4.6 mm i.d. x 250 mm; 33% CH3OH, n-hexane (0.8 min), 30-100% CH3OH, n-hexane (8.18 min), 100% CH3OH, 18.30 min; flow rate, 1 ml/min), yielding active fractions (tR 20-21 min). Further HPLC with Devoselods ODS-5 (4.6 mm i.d. x 250 mm; MeOH: flow rate, 1 ml/min) yielded active fractions (tR 17.19 min), which were finally purified with an FAA column as already described. The active fractions (tR 12-15 min) were then analyzed by GC-MS and LC-MS.

Biassay. During the foregoing chromatographic separations, the rice-lamina inclination bioassay was used for detecting biological activity. A sample of each fraction was hydrolyzed with 1% KOH in 80% EtOH, the hydrolysate being extracted with Et2O and the extract was assayed.

GC-MS and LC-MS analyses. For the analysis of TE-esters, a JEOL JMS SX-102A-MS system coupled to an HP 5890 GC system with a capillary column (J & W DB-1 HT, 0.25 mm i.d. x 15 m; 0.1 µm film thickness) was used. The column temperature was programmed to be held at 200 °C for 1 min, and then to rise to 320 °C at the rate of 32 °C/min. The analysis was performed for 20 min, the substances being ionized by EI (70 eV; ion source temperature, 250-270 °C). Methaneboronate derivatives were prepared according to the procedure already described, and the LC-MS analysis for TE-My being described in detail in our previous report.17

Syntheses of TE esters. All melting point (mp) data were determined with Yawaza hot-stage microscope apparatus and are uncorrected. NMR spectra were recorded at 270 MHz with tetramethylsilane as the internal standard by a JEOL JNM-EX270 spectrometer. EI-MS were measured.

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Abbreviations: BS, brassinosteroid; TE, teasterone; TY, typhasterol; CS, castasterone; BL, brassinolide; TE-La, teasterone 3-laurate; TE-My, teasterone 3-myristate; TE-Pa, teasterone 3-palmitate; TE-St, teasterone 3-stearate; TE-OI, teasterone 3-octate; TE-Lo, teasterone 3-linoleate; Waters FAA, Waters fatty acid analysis.
with a JEOL JMS SX-102A mass spectrometer.

TE 22,23-acetone was prepared from TE (25 mg) according to the method of Aburatani et al.\(^\text{33}\) (24.4 mg, 90.0% yield). To the acetone (12.2 mg, 25 μmol) dissolved in pyridine (250 μl) and dry n-hexane (2 ml), 4-dimethylamino)pyridine (1 mg) and myristyl chloride (65 mg, 263 μmol) were added and reacted for 1 h at room temperature. The reaction mixture was then diluted with distilled water, extracted with EtOAc, and purified with Sephadex LH-20 (CHCl₃, MeOH = 1:1) and Lobar® columns (Merck LiChroprep Si60, size: A, B, EtOAc = 50:1, yielding TE-My-acetone (12.6 mg, 72.2% yield). After refluxing in 80% acq. acetic acid for 2 h and then purifying with a silica gel column (CHCl₃, EtOAc = 6:1), TE-My was obtained (10.4 mg, 87.3% yield), mp 173-177 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 736 [M⁺, 6%], 455 (100), 439 (32), 436 (10), 246 (7), 155 (55).

Teastone 3-laurate (TE-La), palmitate (TE-Pa), stearate (TE-St), oleate (TE-OI), linoleate (TE-Lo), and linolenate (TE-Ln) were synthesized from lauroyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, linoleoyl chloride, and linolenoyl chloride, respectively, by using the same procedure. The physicochemical data for TE-La and TE-Ln could not be obtained because of insufficient amounts of the synthetic compounds.

TE-La: mp 179 182 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 654 [M⁺, 6%], 454 (100), 439 (31), 436 (8), 426 (7), 155 (55).

TE-Pa: mp 168-171 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 710 [M⁺, 6%], 454 (100), 439 (31), 436 (8), 426 (7), 155 (55).

TE-St: mp 160 164 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 670 [M⁺, 6%], 454 (100), 439 (31), 436 (8), 426 (7), 155 (55).

Teastone 3-laurate (TE-La), palmitate (TE-Pa), stearate (TE-St), oleate (TE-OI), linoleate (TE-Lo), and linolenate (TE-Ln) were synthesized from lauroyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, linoleoyl chloride, and linolenoyl chloride, respectively, by using the same procedure. The physicochemical data for TE-La and TE-Ln could not be obtained because of insufficient amounts of the synthetic compounds.

TE-La: mp 179 182 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 654 [M⁺, 6%], 454 (100), 439 (31), 436 (8), 426 (7), 155 (55).

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Teastone 3-laurate (TE-La), palmitate (TE-Pa), stearate (TE-St), oleate (TE-OI), linoleate (TE-Lo), and linolenate (TE-Ln) were synthesized from lauroyl chloride, palmitoyl chloride, stearoyl chloride, oleoyl chloride, linoleoyl chloride, and linolenoyl chloride, respectively, by using the same procedure. The physicochemical data for TE-La and TE-Ln could not be obtained because of insufficient amounts of the synthetic compounds.

TE-La: mp 179 182 C (recrystallized from CHCl₃, MeOH H:O); 1H-NMR (CDCl₃, J = 5.0 Hz, H-12), 5.36 (1H, d, J = 8.0 Hz, H-22), 3.72 (1H, dd, J = 8.0, 2.0 Hz, H-23), 4.68 (1H, m, H-3). EI-MS of the methaneboronate m/z: (rel. int., %): 654 [M⁺, 6%], 454 (100), 439 (31), 436 (8), 426 (7), 155 (55).

Results and Discussion

Presence of the n-hexane-soluble conjugate and released BS after the hydrolysis

BS conjugate was found in the n-hexane-soluble fraction. Ether extracts obtained from the alkaline hydrolysate of the conjugate fraction were purified in silica gel, aluminum oxide and C18-Bond Elut columns, and finally by ODS-HPLC, giving active fractions at tR 20-22 min (Fig. 2), which agree with that of authentic TE. The active principle was identified by GC-MS analysis after conversion to the methaneboronate-trimethylsilyl derivative as previously described.\(^\text{21}\) The mature pollen contains TY, CS, and BL in addition to TE,\(^\text{13}\) but these could not be detected in the hydrolysate. Esterification only occurred with TE: this is suggested to have possibly resulted from the substrate specificity of the enzyme-catalyzing system. In this case, the β-configuration of the hydroxyl group at C-3 could be required for substrate specificity, such substrate specificity having been known in the esterification of phytosterols.\(^\text{15}\)

 establishment of a purification method by HPLC for acyl-conjugated TE

Seven presumed TE esters were synthesized, which included TE-LA, TE-MY, TE-Pa, TE-St, TE-OI, TE-Lo, and TE-Ln. These TE esters were subjected to HPLC by using columns of DIOL, ODS, and FAA, providing very useful information about the eluting solvent system and tR values. With the DIOL column, all esters were eluted at 20-21 min by using a linear gradient mode of CHCl₃ in n-hexane. The synthetic TE esters were detected on a thin-layer chromatogram as a purple spot under ultraviolet light upon heating with sulfuric acid containing 0.5% vanillin. DIOL-HPLC was very useful for removing large quantities of impurities. The fatty acid esters of estradiol have been purified by using the same HPLC method and eluting with an isocetane-CH₂Cl₂ solvent system in an isocratic mode.\(^\text{7}\) However, the TE esters could not be...
purified by eluting with an isoctane-CH₂Cl₂ solvent system (neither isocratic nor gradient) because of elution broadening. With the ODS column and a MeOH solvent, the TE esters were detected by UV (210 nm), enabling all the esters except TE-Pa and TE-OI to be distinguished according to the chain length of fatty acids as shown in the Table. With the ODS column, TE-LO and TE-Ln eluted at $t_R$ 18–22 min and 13–16 min, respectively, using crude TE-LO and TE-Ln. Therefore, this column made it possible to distinguish between saturated fatty acid esters or between unsaturated ones. However, this column could not distinguish between TE-Pa and TE-OI, TE-My and TE-Lo, and TE-La and TE-Ln. FAAA-HPLC, using a THF-CH₃CN-H₂O (25:50:40) solvent system, divided both into two distinguishable peaks, when monitoring by UV detection at 210 nm (Table). The water concentration was important in this solvent system, because TE-Pa and TE-OI came out at 13.9 and 15.0 min, respectively, with a 25:50:35 THF-CH₃CN-H₂O solvent, and at 9.2 and 9.8 min, respectively, with a 25:50:30 THF-CH₃CN-H₂O solvent.

Based on the results of retention times by the three kinds of HPLC columns, the natural sample was successfully purified in the order of DIOL, ODS, and FAAA-HPLC before the GC-MS and LC-MS analyses.

**Purification of acyl-conjugated TE**

The n-hexane-soluble fraction was chromatographically purified by using HPLC columns of silica gel, Sephadex LH-20 and Develosil ODS-5 as shown in Fig. 3, yielding four fractions: ester-A, ester-B, ester-C, and ester-D. Highly active ester-B and ester-C were further purified by the three kinds of HPLC columns already mentioned. The retention time in the ODS column indicated that ester-B was TE-La or TE-Lo and that ester-C was TE-My or TE-Ln. Ester-B gave two active peaks at 11–13 min (ester-B-1) and 18–20 min (ester-B-2) by FAAA-HPLC (Fig. 4). The retention time of ester B-1 is in good agreement with that of synthetic TE-La. The retention times of ester-C were 20–21 min in the DIOL column, 17–19 min in the ODS column, and 12–15 min in the FAAA column (Fig. 5), being closest to that of synthetic TE-My.

**Identification of acyl-conjugated TE by GC-MS and LC-MS**

Acyl-conjugated TE of ester B-1 was analyzed by GC-MS after its conversion to a methanoboronic derivative. As shown in Fig. 6, a molecular ion at m/z 654 (7%), a fragment ion at m/z 454 resulting from the elimination of a fatty acid

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<th>Table: Retention Times for Authentic Teasterone Esters in Develosil ODS and Waters FAAA Columns</th>
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<td>HPLC column</td>
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<td>Waters FAAA</td>
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![Diagram](Fig. 3. Purification of the Teasterone Esters from Lily Anthers.)

**Diagram:** Distribution of Biological Activity Determined by the Rice Lamina Inclination Test after Waters FAAA HPLC of Ester B.

![Diagram](Fig. 4. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test after Waters FAAA HPLC of Ester B.)
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Fig. 5. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test after HPLC of Ester C.
HPLC columns: (a) Senshu Pak DIONEX-125; (b) Develop ODS-5; (c) Waters F-A A.

(R-COOH), and ions at m/z 439 and m/z 426 appeared. In addition, a characteristic ion appeared at m/z 155 arising from the C20–C22 bond cleavage at a side chain of the methaneboronate derivative of TE. These observations indicate that the fatty acid was attached to the 3β-hydroxy group of TE and that this fatty acid was lauric acid (C_{11}H_{23}CO_{2}H). The retention time and mass spectrum agree well with the values for the authentic TE-La methaneboronate derivative. Based on these results, the TE-ester in ester B-1 was determined to be TE-La. After purification, the TE-ester in ester-C was identified to be TE-My by means of GC-MS and also by LC-MS methods as previously reported.

Many steps were required for purification of the TE esters. Although the purification of free BSs from plants has been achieved in three steps, purification of the TE esters consisted of five steps for the GC-MS analysis and eight steps for the LC-MS analysis as already described. GC-MS has been widely used in the analysis of free brassinosteroids in plants. This technique is most successful, but had the disadvantage of partial decomposition and longer retention times, even at the high column temperature used during the analysis of the TE esters. In view of this, LC-MS, using the column-switching technique, which provides a molecular ion and a fatty acid fragment ion without conversion, is very useful for analyzing such TE esters.

Our findings are the first evidence for the presence of fatty acid esterified BS in plants. Interestingly, esterification occurred only with teasterone in lily pollen which had a hydroxy group at C-3 with a β-configuration. More recently, Adam’s group has reported the acyl conjugates of 3,24-bisepibrassinolide and 3,24-bisepicastasterone as metabolites when 24-epibrassinolide and 24-epicastasterone, respectively, were exogenously applied. Both were esterified at the 3β-position. From these facts, the β-configuration

Fig. 6. GC-EIMS of the Methaneboronate Derivative of the Acyl-conjugated TE of Ester B-1 (Teasterone Laurate).
of the hydroxyl group at C-3 might be necessary for BS esterification, as has been observed during the action of acyltransferase catalyzing phytosterol esterification.\(^6\)

As mentioned in the Introduction, endogenous BS was found to change in quantity and quality during pollen maturation. TE esters exist in the uninucleate microspore in spite of the absence of TE. The quantity of TE esters decreased during the stage of binucleate pollen after mitosis. In contrast, TE appeared at this stage (this result will be published elsewhere). These facts suggest that TE esters are the storage form of TE.

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