NOTE

Identification of Teasterone and Phytosterols in the Lipid Fraction from Seeds of *Cannabis sativa* L.

Suguru TAKATSUTO*¹, Takahiro KAWASHIMA*¹, Takahiro NOGUCHI*²,
Shozo FUJIOKA*³, and Akira SAKURAI*³

*¹ Department of Chemistry, Joetsu University of Education
(Joetsu-shi, Niigata-ken, 〒943)

*² Tama Biochemical Co. Ltd.
(Shinjuku-ku, Tokyo, 〒163)

*³ The Institute of Physical and Chemical Research (RIKEN)
(Wako-shi, Saitama-ken, 〒351-01)

Abstract : Study was made of brassinosteroids and phytosterols in unsaponifiable lipid obtained from the seeds of *Cannabis sativa* L. Bioactive substances in a rice-lamina inclination test were highly purified, derivatized and analyzed by GC-MS. The brassinosteroid, teasterone, was identified, suggesting for the first time its possible presence as a fatty acid ester in dicot plants. Phytosterols in the seeds were found to contain six phytosterols, cholesterol, campesterol, campestanol, stigmasterol, sitosterol and sitostanol, with sitosterol and campesterol present in the largest amounts. Campestanol and sitostanol were identified in the seeds for the first time. The structural relationship between brassinosteroids and phytosterols in the seeds is discussed from a biosynthetic point of view.

Key words : *Cannabis sativa* L., brassinosteroid, teasterone, campestanol, sitostanol

1 Introduction

Research on distribution of brassinosteroids (BRs), steroidal plant growth regulators, in plants has clarified that BRs occur in many higher plants and also in some lower plants and that up to date more than 40 natural BRs have been chemically characterized^{1),2)}. Screening investigation has been focused on free form of BRs, because of their high biological activity. Recently, the natural occurrence of BR fatty acid esters, teasterone 3-myristate and its 3-laurate, has been reported in the hexane-soluble fraction obtained from the lily anthers by Abe and co-workers^{3),4)}. We have also reported the possible presence of the fatty acid esters of teasterone and its 3-epimer, typhasterol, in the hexane-soluble fraction obtained from the anthers of *Erythronium japonicum* Decne⁵⁾. Very recently, Abe and co-workers have also reported the possible presence of the fatty acid esters of teasterone, typhasterol, and some BRs with lower polarity in the hexane-soluble fraction obtained from the pollen of *Typha latifolia* L⁶⁾. The above-reported three plants are monocot plants. However, with respect to the dicot plants, there is no information of the occurrence of BR esters. In our previous paper, we have reported the identification of teasterone and castasterone (**Fig. 1**) as a free form in a chloroform-soluble fraction obtained from the methanol extract of the seeds of *Cannabis sativa* L⁷⁾. Although the hexane-soluble fraction obtained from the methanol extract of the seeds was found to be inactive in the rice-lamina inclination test, which is known to be highly specific to BRs⁸⁾, the above-described recent reports on BR esters have prompted us to re-investigate the hexane fraction. Interestingly, it has been now found that an unsaponifiable lipid obtained from the hexane fraction was found to be active in the bioassay.

Corresponding author : Suguru TAKATSUTO

Fig. 1 Structures of Brassinosteroids and Phytosterols Identified in the Seeds of *Cannabis sativa* L.

Therefore, it is suggested that BRs esters should be present and that the biological activity elicited by BR esters is nullified by unknown compounds, because it has been demonstrated that teasterone fatty acid esters showed high biological activity⁹⁾. In order to have a more hint for the occurrence of BR fatty acid esters in the plant kingdom and also to get an information on what kinds of BRs are present as esters in the seeds of *C. sativa*, we have re-examined BRs contained in the hexane fraction obtained from the seeds. In this paper, we report the identification of BRs in the unsaponifiable lipid and also the composition of phytosterols in the seeds, because the C_{28} sterols are assumed to be the biosynthetic precursors of the C_{28} BRs.

2 Experimental

2.1 Authentic steroids

Cholesterol was purchased from Wako Pure Chemical Industries Co. Ltd., Osaka, Japan, and campesterol, stigmasterol, sitosterol, campestanol, and sitostanol were kindly supplied by Tama Biochemical Co. Ltd., Tokyo, Japan. Castasterone and teasterone were synthesized as described in our previous paper^{10),11)}.

2.2 Bioassay

Purification of a BR fraction was monitored by a rice-lamina inclination test, which was carried out using etiolated seedlings of rice (*Oryza sativa* L. cv. Nipponbare), according to the reported method⁸⁾.

2.3 Hexane fraction from the seeds

As described in our previous paper⁷⁾, the hexane-soluble fraction (41.2 g) was obtained by solvent partitioning of the methanol (MeOH) extract of the seeds (2.1 kg) of *C. sativa*.

2.4 Saponification of the hexane fraction

The hexane fraction (41.2 g) was treated with 10% NaOH in 80% aqueous MeOH (300 mL) at 100°C for 2 h. Work-up (diethyl ether-toluene, 1 : 1, vol/vol) gave an unsaponifiable lipid (9.37 g).

2.5 Column chromatography of the unsaponifiable fraction

The lipid (9.37 g) was charged onto a column of silica gel (3.5 cm i.d. $\times$ 20.0 cm, Kieselgel

60, 70~230 mesh, Merck). Elution was carried out stepwise with increasing concentration of ethyl acetate (EtOAc) in toluene and, after elution with sterol fractions, then with 20% MeOH in EtOAc. Eluates with 2 and 4% EtOAc in toluene were combined to give a sterol fraction (4.34 g). An eluate with 20% MeOH in EtOAc was concentrated to give a BR fraction (1.91 g).

2-6 Purification of a BR fraction

The BR fraction (1.91 g) was chromatographed on silica gel (2.5 cm i.d. $\times$ 21.5 cm) eluting stepwise with 0, 0, 2, 2, 4, 6, 8, 10, 12, 15, 20, 30, and 100% MeOH in chloroform (CHCl_3) (each fraction 100 mL). Eluates with 4~15% MeOH in CHCl_3 were combined (0.29 g) and then applied to a column of reversed-phase silica gel (2.0 cm i.d. $\times$ 20 cm, Kieselgel 60 silanisiert, 70~230 mesh, Merck). Elution was done stepwise with 50, 50, 50, 67, 75, 80, 83, 83, 100, and 100% MeOH in H_2O (each fraction 50 mL). A strongly active eluate with 75% MeOH in H_2O was concentrated to give a fraction A-a (25 mg). Weakly active eluates with 80 and 83 (first fraction) % MeOH in H_2O were combined into a fraction A-b (113 mg).

The fraction A-a (25 mg) was purified by preparative TLC (Kieselgel 60F₂₅₄, 20 cm $\times$ 20 cm, 0.25 mm thickness, Merck) with EtOAc-ethanol (22 : 3, vol/vol). Active fractions with R_F 0.48~0.73 were concentrated to give a fraction B-1 (2.2 mg). In the same manner, the fraction A-b (113 mg) were purified by preparative TLC to give an active fraction B-2 (12.8 mg) with R_F 0.44~0.80.

The fractions B-1 and B-2 were combined (15 mg) and purified by preparative TLC (Kieselgel 60 silanisiert, 20 cm $\times$ 20 cm, 0.25 mm thickness, Merck) with MeOH- H_2O (3 : 1, vol/vol). Active fractions with R_F 0.38~0.53 were combined to give a BR fraction C (5.2 mg).

2-7 GC-MS analysis of BRs

One-third of the fraction C was methaneboronated and then trimethylsilylated, as described in a previous paper¹²⁾. GC-MS was carried out as previously described¹²⁾.

2-8 Purification of a fraction of phytosterols

The crude sterol fraction (4.34 g) was charged onto a column of silica gel (3.0 cm i.d. $\times$ 20 cm) and eluted with toluene-EtOAc (50 : 1 to 25 : 1, vol/vol). Eluates with toluene-EtOAc (25 : 1, vol/vol) containing sterols were combined and concentrated to give a sterol fraction (1.25 g). This fraction was further chromatographed on silica gel (3.0 cm i.d. $\times$ 25 cm) eluting with hexane-EtOAc (20 : 1 to 3 : 1, vol/vol). Eluates with hexane-EtOAc (3 : 1, vol/vol) containing 4-desmethylsterols were combined to provide a fraction of phytosterols (0.67 g).

2-9 Isolation of a saturated phytosterol fraction

The phytosterol fraction (100 mg) was acetylated with acetic anhydride (1 mL) and pyridine (2 mL) at room temperature overnight. Work-up (EtOAc) gave a crude sterol acetate, which in CHCl_3 (5 mL) was then treated with *m*-chloroperbenzoic acid (80 mg) at room temperature overnight. Work-up (EtOAc) gave a crude product, which was chromatographed on silica gel (2.0 cm i.d. $\times$ 20 cm) with hexane-toluene (1 : 1 to 0 : 1, vol/vol) to give the fractions of stanol acetate. These combined fractions in tetrahydrofuran (4 mL) was treated with 5% KOH in MeOH (2 mL) at room temperature overnight. Work-up (diethyl ether) followed by purification by preparative TLC (20 cm $\times$ 20 cm, 0.5 mm thickness) with toluene-EtOAc (10 : 1, vol/vol) provided a saturated phytosterol fraction (2.4 mg).

2-10 GC-MS analysis of phytosterols

A Shimadzu GC-MS QP-5000 equipped with a fused silica ULBON HR-1 capillary column (50 m $\times$ 0.25 mm i.d., 0.25 μm film thickness, Shimadzu) was used under the following conditions : ionization, electron impact mode (70 eV) ; mass range, 100~550 ; scan interval, 0.5 s. ; injection temp., 300°C ; interface temp., 250°C ; carrier gas, helium ; carrier gas pressure, 170 kPa ; total flow rate, 50 mL/min, split ratio 61 : 1, flow rate, 0.7 mL/min ; column temp., 300°C.

A small portion (0.5 mg) of each sterol fraction was derivatized as a trimethylsilyl (TMS) ether by treatment with *N*-trimethylsilylimidazole (100 μ L) and pyridine (50 μ L) at 60°C for 30 min.

Relative retention times (R_{tR}) to cholesterol TMS ether (13.741 min) and mass spectral data (characteristic fragment ions) of the identified phytosterols as TMS ethers were as follows. Cholesterol TMS ether, R_{tR} , 1.000, MS m/z (rel. int.) : 458 (M^+ , 19), 443 (17), 368 (20), 353 (22), 329 (23), 247 (15), 129 (100). Campesterol TMS ether, R_{tR} , 1.179, MS m/z : 472 (M^+ , 13), 457 (4), 382 (29), 367 (12), 343 (30), 255 (10), 129 (100). Campestanol TMS ether, R_{tR} , 1.202, MS m/z : 474 (M^+ , 34), 459 (42), 417 (12), 384 (33), 369 (23), 306 (35), 230 (19), 215 (100), 201 (16), 147 (57), 121 (29), 107 (75). Stigmasterol TMS ether, R_{tR} , 1.231, MS m/z : 484 (M^+ , 16), 394 (19), 379 (8), 355 (25), 351 (7), 342 (8), 282 (9), 255 (36), 129 (64). Sitosterol TMS ether, R_{tR} , 1.360, MS m/z : 486 (M^+ , 10), 471 (3), 396 (23), 381 (10), 357 (23), 329 (2), 275 (5), 255 (8), 129 (100). Sitostanol TMS ether, R_{tR} , 1.379, MS m/z : 488 (M^+ , 31), 473 (42), 431 (13), 398 (20), 383 (30), 359 (5), 306 (33), 290 (9), 257 (12), 230 (23), 215 (100), 201 (20), 147 (36), 121 (47), 107 (95).

3 Results and Discussion

The hexane-soluble fraction, obtained from the MeOH extract of the seeds of *C. sativa*⁷⁾, was saponified and the unsaponifiable lipid was chromatographed on silica gel to give a sterol fraction and a BR fraction. At first, the BR fraction was further purified, as described in **Experimental**, to give an active fraction C with R_F 0.38~0.53. The R_F value of the fraction corresponds to those of authentic teasterone and castasterone, suggesting the occurrence of these BRs.

The active fraction C was methaneboronated and analyzed by GC-MS. However, castasterone could not be detected. Thus, the derivatized fraction C was further trimethylsilylated and analyzed by GC-MS, affording an incomplete mass spectrum of a teasterone methaneboronate-TMS derivative, because of the abundant intensity of background ions and also its inadequate purity. In the GC-selected ion monitoring (SIM) analysis (**Fig. 2**), however, characteristic fragment ions at m/z 544 (M^+), 529 (M^+-15), and 515 (M^+-29) of the teasterone

Fig. 2 GC-SIM of Characteristic Ions at m/z 544 [M^+ , (A)], 529 [M^+-15 , (B)], and 515 [M^+-29 , (C)] of a Teasterone Methaneboronate-TMS Derivative and Total Ion Chromatogram (D), Obtained from the Bioactive Fraction C.

These characteristic ions were observed at 12.15 min.

derivative were observed, and their relative intensity and retention time (12.15 min) were identical with those of the authentic sample. The amount of teasterone in the bioactive fraction C was roughly estimated by GC-SIM to be *ca.* 2.3 $\mu\text{g/kg}$ fresh weight of the seeds. Therefore, BR contained in the unsaponifiable lipid of the hexane-soluble fraction obtained from the mature seeds of *C. sativa* was rigorously identified to be teasterone. It is suggested that teasterone is originally present as an ester form. Thus, the present result coupled with our previous data⁷⁾ strongly suggests that in the seeds of *C. sativa*, teasterone occurs as both free and ester forms and castasterone occurs as a free form. Although what kind of fatty acids are esterified with teasterone remains for further investigation, the present data suggests for the first time the possibility that BRs occur as an ester form not only in the monocot plants^{3)~6)}, but also in dicot plants.

Next, we investigated the composition of phytosterols in the seeds of *C. sativa*, because of their importance as biosynthetic precursors to BRs. The sterol fraction, obtained by silica gel column of the unsaponifiable lipid, was purified, as described in **Experimental**, to obtain a phytosterol fraction. This was analyzed by GC-MS after trimethylsilylation. The respective sterol TMS ether was identified by direct comparison with authentic samples. The phytosterols identified and the composition (indicated in parenthesis), which was obtained by total ion chromatogram, were as follows: cholesterol (0.53%), campesterol (16.01), campestanol (0.45), stigmastanol (5.00), sitosterol (70.12), and sitostanol (6.82). Two unidentified sterols accounted for 1.06%. Because of the difficulty of separation of stereoisomers at the C-24 position of the 24-alkylsterols under our analytical condition, it is unknown whether or not the 24-alkylsterols are mixed with each C-24 stereoisomer. In this paper, we tentatively used the common names.

Since the mass spectrum of campestanol was incomplete and contaminated with unknown background ions, we have further investigated the very minor campestanol in the seeds. From the isolated phytosterol fraction, sterols with carbon-carbon double bond (s) were removed, as described in **Experimental**. The resulting saturated phytosterol fraction was analyzed by GC-MS after trimethylsilylation, affording clear sharp peaks of the TMS derivatives of campestanol and sitostanol. By direct comparison with authentic samples, campestanol and sitostanol were rigorously identified in the seeds of *C. sativa* by the full-scan mass spectral data.

The present identification is roughly consistent with the reported data of the seeds, in which campesterol, stigmastanol, and sitosterol were identified as major sterols^{13)~17)}. It was also reported that campesterol, stigmastanol, sitosterol, 5 α -ergosterol-3-one, 5 α -stigmastanol-3-one, 5 α -stigmast-22-en-3-one, and 5,24(28)-unsaturated sterol were identified from the callus of *C. sativa*¹⁸⁾. However, to the best of our knowledge, the present study is the first report of the identification of campestanol and sitostanol in *C. sativa*.

With respect to the biosynthesis of BRs, among the phytosterols identified, campesterol and campestanol have the same carbon skeleton as those of teasterone and castasterone. Recently, Fujioka and co-workers have reported that in cultured cells of *Catharanthus roseus*, campesterol and campestanol are the most plausible biosynthetic precursors of the C₂₈ BRs^{19),20)}. Furthermore, they have very recently demonstrated that the *det 2* mutant of *Arabidopsis thaliana* is the BR-deficient mutant and the step of the conversion of campesterol into campestanol is blocked in the BR biosynthesis^{21),22)}. Coupled with these reported data and our previous identification of the C₂₈ BRs in the seeds of *C. sativa*⁷⁾, the present identification of campesterol and campestanol suggests that campesterol and campestanol are involved in the BR biosynthesis and these two sterols selectively serve as biosynthetic precursors to the C₂₈ BRs.

Acknowledgement

The authors thank Mr. Katsuya Shimada and Miss Kana Makiuchi of Joetsu University of Education for their technical assistance.

(Received Apr. 17, 1997 ; Accepted Sept. 26, 1997)

References

- 1) S. Fujioka, A. Sakurai, *Nat. Prod. Rep.*, **14**, 1. (1997).
- 2) S. Takatsuto, *J. Chromatogr. A*, **658**, 3 (1994).
- 3) S. Asakawa, H. Abe, Y. Kyokawa, S. Nakamura, M. Natsume, *Biosci. Biotech. Biochem.*, **58**, 219 (1994).
- 4) S. Asakawa, H. Abe, N. Nishikawa, M. Natsume, M. Koshioka, *Biosci. Biotech. Biochem.*, **60**, 1416 (1996).
- 5) E. Yasuta, T. Terahata, M. Nakayama, H. Abe, S. Takatsuto, T. Yokota, *Biosci. Biotech. Biochem.*, **59**, 2156 (1995).
- 6) K. Mukai, H. Abe, M. Natsume, *Abstract of the 31st Annual Meeting of the Japanese Society for Chemical Regulation of Plants (in Japanese)*, (Kyoto), p. 53 (1996).
- 7) S. Takatsuto, H. Abe, T. Yokota, K. Shimada, K. Gamoh, *J. Jpn. Oil Chem. Soc.*, **45**, 871 (1996).
- 8) M. Arima, T. Yokota, N. Takahashi, *Phytochemistry*, **23**, 1587 (1984).
- 9) H. Abe, M. Aburatani, *Jpn. Pat.*, 6-25281 (1994).
- 10) S. Takatsuto, *J. Chem. Soc., Perkin Trans. I*, **1986**, 1833.
- 11) S. Takatsuto, N. Yazawa, M. Ishiguro, N. Morisaki, N. Ikekawa, *J. Chem. Soc., Perkin Trans., I*, **1984**, 139.
- 12) H. Suzuki, S. Fujioka, S. Takatsuto, T. Yokota, N. Murofushi, A. Sakurai, *J. Plant Growth Regul.*, **13**, 21 (1994).
- 13) C. Fenselau, G. Hermann, *J. Forensic Sci.*, **17**, 309 (1972).
- 14) T. Itoh, T. Tamura, T. Matsumoto, *J. Am. Oil Chem. Soc.*, **50**, 122 (1973).
- 15) R. S. Foote, L. A. Jones, *J. Agric. Food Chem.*, **22**, 534 (1974).
- 16) M. L. Mole Jr., C. E. Turner, *J. Pharm. Sci.*, **63**, 154 (1974).
- 17) T. M. Jeong, T. Itoh, T. Tamura, T. Matsumoto, *Lipids*, **9**, 921 (1974).
- 18) H. Itokawa, K. Takeya, M. Akasu, *Shoyakugaku Zasshi*, **29**, 106 (1975).
- 19) S. Fujioka, A. Sakurai, *Chem. Regul. Plants* (Shokubutsu no Kagaku Chousetsu, review in Japanese), **30**, 137 (1995).
- 20) H. Suzuki, T. Inoue, S. Fujioka, T. Saito, S. Takatsuto, T. Yokota, N. Murofushi, T. Yanagisawa, A. Sakurai, *Phytochemistry*, **40**, 1391 (1995).
- 21) S. Fujioka, Y.-H. Choi, S. Takatsuto, T. Yokota, J. Li, J. Chory, A. Sakurai, *Abstract of the 31st Annual Meeting of the Japanese Society for Chemical Regulation of Plants (in Japanese)*, (Kyoto), p. 41 (1996).
- 22) J. Li, P. Nagpal, V. Vitart, A. Chao, S. Fujioka, Y.-H. Choi, M. Guha-Biswas, T. C. McMorris, S. Takatsuto, T. Yokota, D. W. Russell, A. Sakurai, J. Chory, *Proc. Plant Growth Regul. Soc. Am.*, **23**, 11 (1996).

[ノート] アサ種子中より得られた脂質中に含まれる ティーステロン及び植物ステロールの同定

高津戸 秀^{*1}・川島 崇弘^{*1}・野口 貴弘^{*2}・藤岡 昭三^{*3}・桜井 成^{*3}

^{*1} 上越教育大学自然系化学教室 (〒943 新潟県上越市山屋敷町 1)

^{*2} タマ生化学 (〒163 東京都新宿区西新宿 2-7-1)

^{*3} 理化学研究所植物生活環境制御研究室 (〒351-01 埼玉県和光市広沢 2-1)

アサ種子中から得られた不けん化脂質中に含まれるブラシノステロイドと植物ステロールについて調べた。イネ葉身屈曲試験において活性な物質を精製し、誘導体化後 GC-MS 分析を行った。ブラシノステロイドの一種であるティーステロンを同定したことから、このものが双子葉植物中に脂肪酸エステルとして存在する可能性が初めて示唆された。また、種子中に含まれる植物ステロールについて調べ、6 種のステロール (コレステロール、カンペステロール、カンペスタノール、スティグマステロール、シトステロール及びシトスタノール) を同定したところ、カンペステロールとシトステロールが主成分であった。カンペスタノールとシトスタノールはアサ種子中から初めて同定された。アサ種子中に含まれるブラシノステロイドと植物ステロールとの関係について、生合成的観点から論じた。

(連絡者: 高津戸 秀) Vol. 46, No. 12, 1499 (1997)