*Asian Journal of Chemistry* Vol. 19, No. 1 (2007), 87–94

# Identification of Steroids in Wheat Bran

XU FENG†, DONG JIANG, YU SHAN†, TINGBO DAI, QI JING, YUNFA DONG† and WEIXING CAO*
*Key Laboratory of Crop Growth Regulation of Ministry of Agriculture*
*Nanjing Agricultural University, Nanjing 210095, P.R. China*
*Fax-Tel: (86)(25)84396575; E-mail: caow@njau.edu.cn*

To well utilize wheat product and improve its commercial value, nutritionally and medically, foundational steroids in wheat bran were identified in the present study. Steroid components in wheat bran were extracted with hexane and identified based on direct comparison with mass spectral data supplied by Finnegan. Total seven sterol 3-ols and twelve steroidal 3-ones were detected by GC-MS, which were identified for the first time in wheat bran, and eight minor steroids, *i.e.*, 24-ethyl-5α-cholestan-22-en-3-one, 24-ethyl-5α-cholestan-24(28)-en-3-one, spinasteron, 4-methyl-24-ethylcholest-4,24(28)-dien-3-one, 4-methyl-24-ethylcholest-4,22-dien-3-one, 4-methyl-αa-cholestan-3-one, 24-ethyl-5α-cholestan-24(28)-en-3β-ol, 24-ethyl-5α-cholestan-22-en-3p-ol, were first reported in wheat. Based on Takatsuto's report, biosynthetic relationships of steroids with brassinosteroids were presumed and biosynthetic pathway of steroids in wheat grain was discussed.

**Key Words: Wheat *(Triticum aestivum* L.), Bran, Steroid, GC-MS, Campestanol, Sitostanol, Spinasterone, 4-Methyl steroidal-3-one.**

## INTRODUCTION

Campestanol is a biosynthetic precursor to brassinolide, the most bioactive brassinosteroids (BRs)[1]. The application of BRs could promote grain yield of wheat *(Triticum aestivum* L.)[2–4]. Sitostanol is also expected to be a biosynthetic precursor of 29 BRs[5, 6]. Furthermore, sitostanol and its fatty acid ester could reduce cholesterol level in serum by suppressing cholesterol absorption[5, 7]. Campestanol and sitostanol have therefore attracted much interest of scientists recently.

Takatsuto *et al.*[8] demonstrated that a series of biosynthetic reactions, campesterol → (24R)-24-methylcholest-4-en-3β-ol → (24R)-24-methylcholest-4-en-3-one → (24R)-24-methyl-5α-cholestan-3-one → campestanol, take place as an early part of the brassinolide biosynthesis. Sitostanol is biosynthesized from

†The Jiangsu Provincial Key Laboratory for Medicinal Plant, Institute of Botany, Jiangsu Province and Chinese Academy of Sciences (Nanjing Botanical Garden, Mem. Sun Yat-sen), Nanjing 210014, China.

sitosterol through $C_{29}$ homolog by a pathway of sitosterol → (24R)-24-ethylcholest-4-en-3β-ol-(24R)-24-ethylcholest-4-en-3β-one → (24R)-24-ethyl-5α-choles tan-3-one → sitostanol, which is parallel to that of campestanol from campesterol.

Some steroid compositions in wheat seeds have been identified to date, including campesterol, campestanol, stigmasterol, sitosterol, sitostanol, 24-ethylcholesta-4,24(28)Z-dien-3-one, 24-methylcholest-4-en-3-one, 24-ethylcholesta-4,22E-dien-3-one, 24-ethylcholest-4-en-3-one, 24-methyl-5α-cholestan-3-one, 24-ethyl-5a-cholestan-3-one[8, 9]. However, it is not known yet whether the other steroid components exist in wheat.

As a by-product of machining flour, wheat bran is also a traditional Chinese medicine. Modern research has certified that wheat bran can relieve hematuria, diabetes, beriberi and peripheral neuritis. However, its nutritional and chemical foundations are not clear yet, especially for the healthy components such as steroids. Therefore, the primary objective of this study was to identify steroids in wheat bran on the basis of the GC-MS method. This information should help better understand medical functions of wheat bran and provide useful knowledge for improvement of wheat handling and commercial use.

## EXPERIMENTAL

Wheat (*Triticum aestivum* L.) variety Ningmai 9 were harvested in 2002 in Jiangpu Experiment Station of Nanjing Agricultural University. Bran was obtained *via* a Branbendar miller.

**Extraction of lipid fraction from bran:** 1 kg of wheat bran was exhaustively extracted with 95% ethanol. The extract was filtered and concentrated *in vacuo*, leaving an aqueous residue, which was extracted with hexane. The hexane fraction (30 g) was mixed with 100 mL 1,4-dioxane, 50 mL MeOH and 10 mL water (in which potassium hydroxide (15 g) was dissolved in advance), and then heated at 70°C for 1 h[10]. Unsaponifiable liquid (5 g) was obtained using the usual work-up (diethyl ether) and was applied to a column of silica gel (100–200 mesh). The elution was done with stepwise increase of ethyl acetate (EtOAc) in hexane and each fraction with a volume of 100 mL was collected and monitored by TLC ($F_{254}$, 0.25 mm thickness; hexane/EtOAc (5/1) (v/v)). Fractions of steroidal 3-one were eluted with hexane/EtOAc (10/0–10/1) (v/v) and pooled as Fraction I (0.6 g). Fractions of steroidal-3-one, including 4-methyl steroidal-3-one were eluted with hexane/EtOAc (10/1–8/1) (v/v) and pooled as Fraction II (0.8 g). Sterol fractions were further eluted with hexane/EtOAc (5/1–4/1) (v/v) and combined into Fraction III (1.7 g).

**GC-MS analysis:** GC-MS analysis was conducted with a gas chromatograph-mass spectrometer (GC-MS, Finnegan Trace DSQ) using a fused silica DB-1 capillary column (30 m × 0.2 5mm i.d., 0.25 μm film thickness, J&W Scientific) with the following protocol. Ionization condition was EI (70 eV) and injection port temperature was 240°C. Column oven temperature was maintained at 170°C for 1.5 min followed by temperature programming to 250°C at a rate of 32°C/min, then elevated to 270°C at a rate of 1.0°C/min and retained at 270°C for 20 min and then elevated to 290°C at a rate of 5.0°C/min, and kept isothermal for 3 min. The carried gas was He with a flow rate of 0.8 mL/min using splitless mode (1.5 min sampling time). Each sterol fraction was converted to trimethylsilyl (TMS) derivative and then analyzed by GC-MS, whereas the 3-one steroids were directly analyzed by GC-MS.

## RESULTS AND DISCUSSION

Liquid extract obtained from the wheat bran was fractionated into three fractions. The identified steroids are listed in Table-1, together with sterol compositions by GC-MS analysis of these fractions. The structures of some steroids are shown in Fig. 1–3.

TABLE-1
STEROIDS IN WHEAT BRAN

| Fraction | Yield of steroid (g) | Steroids identified | Composition (%) |
|---|---|---|---|
| Fraction I | 0.6 | 24-Methylcholest-4-en-3-one (WS1) | 23.9 |
| | | Stigmasta-4,22E-dien-3-one (WS2) | 16.4 |
| | | Stigmast-4-en-3-one (WS3) | 51.5 |
| | | 5α-Stigmast-22-en-3-one (WS4) | 6.6 |
| | | 5-Stigmast-24(28)-en-3-one(WS5) | 1.6 |
| Fraction II | 0.8 | Stigmasta-4,24(28)-dien-3-one (WS6) | 20.8 |
| | | 24-Ethyl-5α-cholestan-3-one (WS7) | 9.6 |
| | | 24-Methyl-5α-cholestan-3-one (WS8) | 17.7 |
| | | Spinasterone (WS9) | 16.4 |
| | | 4-Methylstigmasta-4,24(28 )-dien-3-one (WS10) | 3.5 |
| | | 4-Methylstigmasta-4,22-dien-3-one (WS11) | 18.6 |
| | | 4-Methyl-5α-cholestan-3-one (WS12) | 13.5 |
| Fraction III | 1.7 | Campesterol(WS13) | 11.1 |
| | | Campestanol(WS14) | 23.6 |
| | | 24-Ethylcholest-24(28)-en-3-ol (WS15) | 1.1 |
| | | 24-Ethylcholest-22-en-3-ol (WS16) | 0.6 |
| | | Stigmasterol (WS17) | 5.7 |
| | | Sitostanol (WS18) | 31.4 |
| | | Sitosterol (WS19) | 26.4 |

The retention time (Rt) (min) and mass spectral data (characteristic fragment ions) of identified steroidal 3-ones are:

1. 24-Methylcholest-4-en-3-one, Rt 34.28, MS m/z: 398 ($M^+$, 8), 344 (15), 327 (8), 282 (5), 175 (68), 161 (55), 147 (74), 143 (89), 127 (100).
2. 24-Ethylcholest-4,22E-dien-3-one, Rt 35.69, MS m/z: 410 ($M^+$, 14), 312 (10), 97 (10), 271 (28), 256 (14), 243 (24), 229 (14), 174 (40), 173 (84), 161 (52), 147 (63), 135 (74), 127 (83), 109 (94), 107 (100).

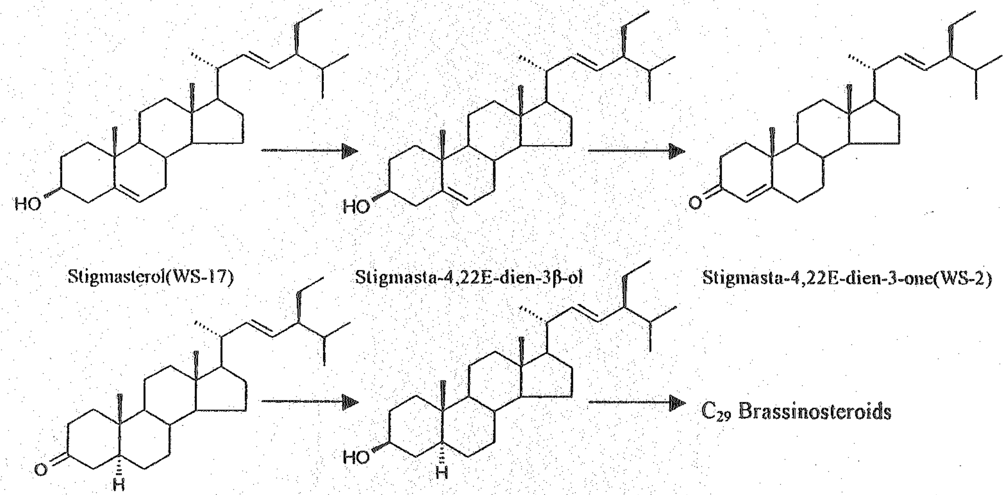


Fig. 1. Hypothetical biosynthetic pathway of 24-ethylcholest-22-en-3-ol from stigmasterol

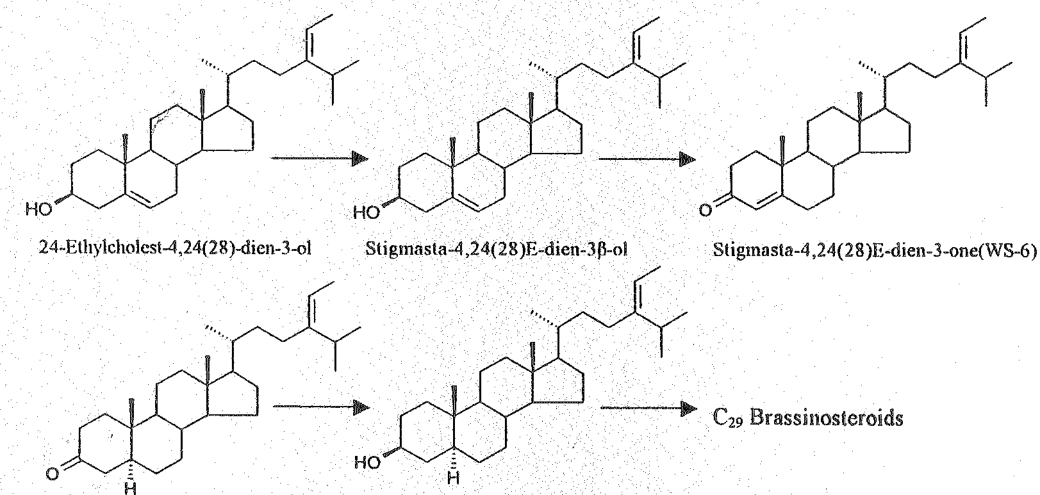


Fig. 2. Hypothetical biosynthetic pathway of 24-ethylcholest-24(28)-en-3-ol from 24-ethyl-5α-cholest-4,24 (28)-dien-3-ol

3. 24-Ethylcholest-4-en-3-one, Rt 39.32, MS m/z: 412 ($M^+$, 6), 383 (6), 356 (4), 281 (3), 271 (8), 229 (7), 187 (16), 175 (100), 174 (75), 159 (46), 133 (51), 107 (67).
4. 24-Ethyl-5α-cholestan-22-en-3-one, Rt 39.79, MS m/z: 412 ($M^+$, 8), 394 (8), 281 (4), 271 (5), 243 (5), 226 (5), 175 (70), 159 (36), 143 (100), 142 (72), 127 (52), 105 (40).
5. 24-Ethyl-5α-cholestan-24 (28)-en-3-one, Rt 42.10, MS m/z: 412 ($M^+$, 6), 316 (6), 281 (12), 258 (7), 245 (7), 229 (13), 174 (32), 149 (60), 147 (42), 121 (70), 109 (100).

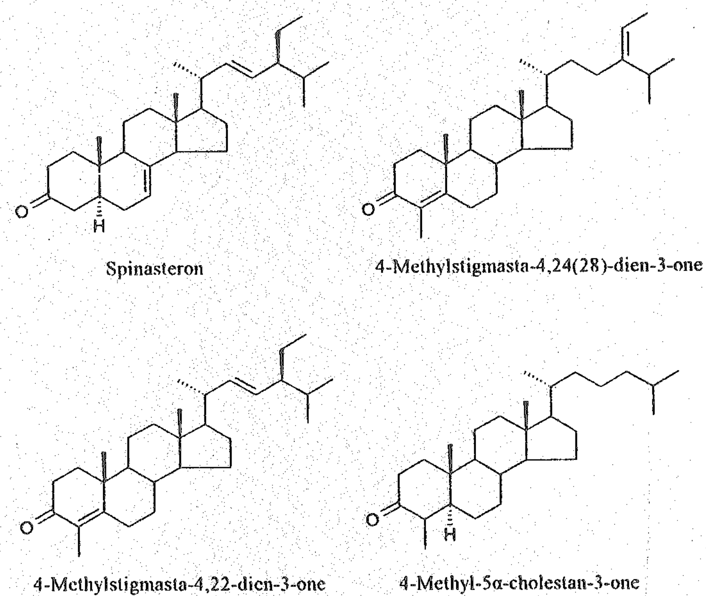


Fig. 3 Structures of spinasterone and 4-methyl-steroidal-3-ones found in wheat bran

6. 24-Ethylcholest-4,24(28)-dien-3-one, Rt 38.24, MS m/z: 410 ($M^+$, 3), 395 (3), 311 (2), 284 (3), 229 (3), 218 (100), 149 (14), 147 (32), 135 (40), 105 (60).
7. 24-Ethyl-5α-cholestan-3-one, Rt 39.87, MS m/z: 414 ($M^+$, 5), 387 (2), 259 (3), 220 (20), 218 (100), 191 (36), 189 (51), 175 (16), 149 (16), 147 (34), 133 (64).
8. 24-Methyl-5α-cholestan-3-one, Rt 40.85, MS m/z: 400 ($M^+$, 1), 386 (2), 357 (2), 300 (3), 241 (3), 219 (18), 189 (20), 177 (13), 159 (20), 147 (53), 121 (94), 107 (100).
9. Spinasterone, Rt 41.87, MS m/z: 410 ($M^+$, 3), 366 (3), 297 (3), 270 (3), 227 (6), 218 (10), 175 (15), 161 (17), 149 (30), 147 (40), 135 (55), 121 (63), 109 (96), 107 (100).
10. 4-Methyl-24-ethylcholest-4,24(28)-dien-3-one, Rt 44.33, MS m/z: 424 ($M^+$, 5), 409 (5), 316 (4), 281 (6), 227 (10), 174 (16), 161 (20), 147 (39), 133 (75), 107 (100).
11. 4-Methyl-24-ethylcholest-4,22-dien-3-one, Rt 46.16, MS m/z: 424 ($M^+$, 6), 380 (4), 297 (2), 282 (3), 256 (2), 175 (10), 159 (21), 145 (26), 135 (43), 133 (71), 121 (67), 109 (93), 107 (100).
12. 4-Methyl-5α-cholestan-3-one, Rt 49.04, MS m/z: 400 ($M^+$, 10), 358 (10), 281 (6), 242 (7), 217 (15), 161 (21), 159 (40), 147 (39), 135 (56), 133 (82), 119 (84), 107 (100).

The retention time (Rt) (min) to TMS ether and mass spectral data (characteristic fragment ions) of identified sterols are:

13. Campesterol TMS ether, Rt 33.23, MS m/z: 472 ($M^+$, 2), 382 (4), 343 (5), 255 (10), 129 (100), 107 (70).
14. Campestanol TMS ether, Rt 33.79, MS m/z: 474 ($M^+$, 2), 459 (2), 417 (4), 384 (4), 368 (3), 306 (3), 230 (2), 215 (5), 147 (33), 107 (100).

15. 24-Ethyl-5α-cholesten-24(28)-en-3β-ol TMS ether, Rt 34.78, MS m/z: 486 ($M^+$, 2), 381 (4), 356 (3), 284 (4), 269 (6), 216 (8), 161 (15), 147 (40), 133 (61), 130 (54), 107 (100).
16. 24-Ethyl-5α-cholestan-22-en-3β-ol IMS ether, Rt 35.35, MS m/z: 486 ($M^+$, 3), 375 (7), 267 (3), 158 (13), 217 (11), 147 (48), 133 (34), 107 (100).
17. Stigmasterol IMS ether, Rt 36.04, MS m/z: 484 ($M^+$, 1), 458 (5), 326 (11), 305 (2), 281 (8), 269 (11), 256 (14), 230 (3), 159 (45), 129 (60), 107 (100).
18. Sitostanol IMS ether, Rt 38.60, MS m/z: 488 ($M^+$, 1), 487 (9), 397 (10), 303 (6), 273 (4), 229 (8), 159 (26), 147 (30), 130 (50), 129 (92), 107 (71), 105 (100).
19. Sitosterol IMS ether, Rt 39.18, MS m/z: 486 ($M^+$, 3), 459 (10), 385 (14), 358 (14), 300 (11), 257 (15), 230 (9), 217 (33), 176 (32), 145 (60), 129 (80), 107 (100).

In this paper, configurations at the C-24 positions of 24-methyl- and 24-ethylsteroids are not given, because 24-stereoisomers of these steroids could not be separated under GC conditions in the present study. The trivial steroid names used in this paper do not specify the C-24 stereochemistry. However, in higher plants, $C_{28}$ sterols such as campesterol are generally a mixture of (24R)-isomer (major) and (24S)-isomer (minor), while $C_{29}$ sterols such as sitosterol and stigmasterol comprise a single (24R)-isomer[5].

GC-MS analysis of Fraction I from wheat bran based on direct comparison with mass spectra data supplied by Finnegan resulted in the identification of 24-methylcholest-4-en-3-one (WS-1), 24-ethylcholest-4,22E-dien-3-one (WS-2), 24-ethylcholest-4-en-3-one (WS-3), 24-ethyl-5α-cholestan-22-en-3-one (WS-4), 24-ethyl-5α-cholestan-24(28)-en-3-one (WS-5). From Fraction II, 24-ethylcholest-4,24(28)-dien-3-one (WS-6), 24-ethyl-5α-cholestan-3-one (WS-7), 24-methyl-5α-cholestan-3-one (WS-8), spinasterone (WS-9), 4-methyl-24-ethylcholest-4,24(28)-dien-3-one (WS-10), 4-methyl-24-ethylcholest-4,22-dien-3-one (WS-11), 4-methyl-5α-cholestan-3-one (WS-12).

Comparing with data from Takatsuto *et al.*'s report[8], wheat bran contains all steroidal 3-one fractions from wheat seeds. Furthermore, six other steroidal 3-ones, WS-4, WS-5, WS-9, WS-10, WS-11, WS-12, in the present paper were first reported from wheat, including 4-methyl-steroidal 3-ones, WS-10, WS-11, WS-12.

From Fraction III, nine sterol components were first identified from wheat bran, *i.e.*, campesterol (WS-13), campestanol (WS-14), 24-ethyl-5α-cholestan-24(28)-en-3β-ol (WS-15), 24-ethyl-5α-cholestan-22-en-3β-ol (WS-16), stigmasterol (WS-17), sitostanol (WS-18), sitosterol (WS-19). Five of these sterol compositions, WS-13, WS-14, WS-17, WS-18, WS-19, were ever reported from wheat seed.

Takatsuto *et al.*[8] has demonstrated a series of biosynthetic reactions from campesterol to campestanol and from sitosterol to sitostanol in wheat seeds, but the expected sterols, 24-methylcholest-4-en-3β-ol and 24-ethylcholest-4-en-3βol, the putative metabolites derived from campesterol and sitosterol could not be detected. This paper reports a similar result, the two sterols could neither be detected from wheat bran.

The pathway of campestanol seems to occur in a wide range of plants[11]. The intermediate, 24-methylcholest-4-en-3β-ol, was not detected in the present study, and the levels of other intermediates in the conversion of campesterol to campestanol were very low in wheat bran. Thus, it is assumed that these intermediates, especially for 24-methylcholest-4-en-3β-ol, should have very short life time and be rapidly metabolized.

The present study demonstrates that wheat bran contained campesterol (WS-13), 24-methylcholest-4-en-3-one (WS-1), 24-methyl-5α-cholestan-3-one (WS-8) and campestanol (WS-14). It seems that biosynthetic reactions from sitosterol to sitostanol take place in parallel to those from campesterol to campestanol in wheat as postulated. Likewise, no evidence was found since the absence of putative 24-ethylcholest-4-en-3β-ol. Wheat contains $C_{28}$ BRs, but does not contain $C_{29}$ BRs at the detectable levels[12]. Takatsuto *et al.*[8] presumed that deficiency of $C_{29}$ BRs in wheat is not ascribed to impaired synthesis of sitostanol, but to blockage in the further downstream reactions. The present study confirmed this postulation, since levels of sitosterol were much higher than those of campesterol in wheat bran.

It was found that stigmasterol (WS-17) co-occurred with 24-ethylcholest-4,22E-dien-3-one (WS-2), 24-ethyl-5α-cholestan-22-en-3-one (WS-4) and 24-ethyl-5α-cholestan-22-en-3β-ol (WS-16) in wheat bran. This implied that stigmasterol may also be converted to 24-ethyl-5α-cholestan-22-en-3β-ol *via* WS-2 and WS-6 (Fig. 1) in a similar pathway as indicated[8]. Furthermore, it seems that biosynthesisreactions from 24-ethylcholest-4,22-dien-3β-ol to 24-ethyl-5α-cholestan-24(28)-en-3β-ol (WS-15) *via* 24-ethylcholest-4,24(28)-dien-3-one (WS-6) and 24-ethyl-5α-cholestan-24(28)-en-3-one (WS-5) also take place in parallel to those from stigmasterol to 24-ethyl-5α-cholestan-22-en-3β-ol as shown in Fig. 2. However, 24-ethylcholest-4,24(28)-dien-3β-ol could not be obtained, and 24-ethylcholest-4,22E-dien-3β-ol and 24-ethylcholest-4,24(28)E-dien-3β-ol were not at the detectable levels. It seems that 4-methyl-steroid 3-one fractions, 4-methyl-24-ethylcholest-4,24(28)-dien-3-one (WS-10), 4-methyl-24-ethylcholest-4,22-dien-3-one (WS-11), 4-methyl-5α-cholestan-3-one (WS-12), may be the branch pathway in the biosynthesis reactions.

## ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (30030090, 30571093), the SRFPD (20050307006) and the Innovation Foundation of Jiangsu Province (BK2005419). The authors are thankful to Mr. Han Hangru, State Key Laboratory of Crop Genetics and Germplasm Enhancement for assistance in GC-MS analysis of this work.

## REFERENCES

1. S. Fujioka and A. Sakurai, *Nat. Prod. Rep.*, **14**, 1 (1997).
2. V. Marquardt, *G. Adam. Chem. Plant Prot.*, **7**, 103 (1991).
3. S. Takatsuto and F. Futatsuya, *Yukagaku*, **39**, 227 (1990).
4. N. Ikckawa and Y.J. Zhao, in: G. Cutler, T. Yokota and G. Adam (Eds.), Brassinosteroids, Chemistry, Bioactivity and Applications (ACS Symposium Series 474), Am. Chem. Soc., Washington DC, p. 280 (1991).
5. H. Hibino, *J. Jpn. Oil Chem. Soc.*, **46**, 1127 (1997).
6. T. Yokota, *Trends Plant Sci.*, **2**, 137 (1997).
7. M. Sugano, *Nippon Nogeikagaku Kaishi*, **71**, 769 (1997).
8. S. Takatsuto and N. Kosuga, *J. Plant Res.*, **112**, 27 (1999).
9. P. C. Dutta and L.-A. Appelqvist, *J. Sci. Food Agric.*, **71**, 383 (1996).
10. Y Narumi and S. Takatsuto, *J. Jpn. Oil Chem. Soc.*, **49**, 719 (2000).
11. T. Akihisa, W.C.M.C. Kokke and T. Tamura, in: G. W. Patterson and W.D. Nes (Eds.), Physiology and Biochemistry of Sterols, American Oil Chemists' Society, Champaign, Illinois, p. 172 (1991).
12. T. Yokota, M. Nakayama, T. Walkisaka, J. Schmidt and G. Adam, *Biosci. Biotech. Biochem.*, **58**, 1183 (1994).

(*Received*: 8 April 2005; *Accepted*: 16 May 2006) AJC-4911