Identification of Sterols and Steroidal 3-Ones in the Seeds of *Echinochloa frumentacea*

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Abstract : Sterols and steroidal 3-ones were detected in the seeds of *Echinochloa frumentacea* (Roxb.) Link. Twenty sterols were identified by direct GC-MS comparison with authentic samples and the compositions of both free and esterified sterol fractions were determined. Eight steroidal 3-ones were found present in the seeds.

Key words : *Echinochloa frumentacea* (Roxb.) Link, seed, sterol, steroidal 3-one

1 Introduction

Recently, much attention has been focused on stanols, not only because sitostanol and its fatty acid esters lower the serum cholesterol level by suppressing cholesterol absorption^{1,2)}, but because campestanol is a precursor of a phytohormone brassinolide³⁾. As an early part of brassinolide biosynthesis, it has recently been demonstrated that a series of biosynthetic reactions, campesterol $\rightarrow$ (24*R*)-24-methylcholest-4-en-3 β -ol $\rightarrow$ (24*R*)-24-methylcholest-4-en-3-one $\rightarrow$ (24*R*)-24-methyl-5 α -cholestan-3-one $\rightarrow$ campestanol, takes place in the shoots of *Arabidopsis thaliana* and the cultured cells of *Catharanthus roseus*⁴⁾. It has analogously been proposed that sitostanol is also biosynthesized from sitosterol through C₂₉ homologs by a pathway parallel to that of campestanol from campesterol in plants, based on the co-occurrence of the corresponding intermediates in the seeds of *Cannabis sativa*^{5,6)}, wheat⁷⁾, and foxtail millet⁷⁾. We have recently reported a high content of sitostanol in the milled grains and seeds of foxtail millet^{8,9)}.

Continuing our research program of sterols in cereal grains, we have now examined trace sterols and steroidal 3-ones in the seeds of *Echinochloa frumentacea* (Roxb.) Link, because of our preliminary results that cholesterol, 24-methylenecholesterol, and isofucosterol are present along with campesterol, campestanol, stigmaterol, sitosterol, and sitostanol. The seeds seems to be ideal to obtain basic information about the biosynthetic pathways of stanols, because of the co-occurrence of the sterols with six types of side chain. In this paper, we wish to report our identification results of sterols and steroidal 3-ones in *E. frumentacea* seeds.

2 Experimental

2.1 Authentic Steroids

The following authentic steroids used in this study (listed in **Table 1** and **2**) are available : sterol (1) was obtained from Wako, and sterols (4, 5, 7-11, 13-16, and 18-20) are available as previously described^{7,10,11)}, and steroidal 3-ones (23-28) are available as previously described^{7,12)}. Sterols (2, 3, 6, 12, and 17) and steroids 21 and 22 were synthesized¹³⁾.

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2·2 Extraction of a Lipid Fraction from the *E. frumentacea* Seeds

According to the reported method⁷⁾, the homogenized seeds of *E. frumentacea* (3.0 kg, harvested in China and the seeds were identified by Dr. Y. Iokawa of Joetsu University of Education) were extracted with a 1 : 1 mixture of chloroform and methanol and the extract was subjected to solvent partitioning to give a hexane-soluble lipid fraction (155.84 g).

2·3 Separation of Fractions of Sterols from Steryl Esters, Steroidal 4-En-3-ones, 5 α -Steroidal 3-Ones, and Free Sterols

According to the reported method⁷⁾, a sterol fraction A (1.27 g) derived from a steryl ester fraction, a 5 α -steroidal 3-one fraction B (3.5 mg), a steroidal 4-en-3-one fraction C (16.2 mg), and a free sterol fraction D (1.29 g) were obtained from the lipid fraction.

2·4 Fractionation of Sterol Fractions A and D

The sterol fraction A (300 mg) was acetylated in the usual manner and the resulting acetate was fractionated by 10% AgNO₃-silica gel column (2.0 cm i.d. $\times$ 32 cm). Each fraction was collected in the amount of 10 mL. The fractions 14~19 with 1% diethyl ether (Et₂O)/dichloromethane (CH₂Cl₂) were collected as fraction A-1 (21.1 mg), the fractions 20~23 with 1% Et₂O/CH₂Cl₂ as fraction A-2 (128.4 mg), the fractions 24~31 with 1% Et₂O/CH₂Cl₂ as fraction A-3 (46.4 mg), the fractions 32~39 with 1% Et₂O/CH₂Cl₂ as fraction A-4 (11.4 mg), the fractions 40~50 with 3% Et₂O/CH₂Cl₂ as fraction A-5 (4.8 mg), the fractions 51~60 with ethyl acetate as fraction A-6 (3.7 mg) and the fractions 61~70 with ethyl acetate as fraction A-7 (76.8 mg).

Each fraction A-1~7 was saponified in the usual manner to provide, after the extractive work-up (Et₂O), fraction A-1a (19.3 mg), A-2a (116.7 mg), A-3a (36.1 mg), A-4a (5.1 mg), A-5a (2.5 mg), A-6a (2.6 mg), and A-7a (62.6 mg), respectively.

In the same manner, the sterol fraction D (300 mg) was acetylated, fractionated by AgNO₃-silica gel column and saponified to give fraction D-1a (15.1 mg), D-2a (163 mg), D-3a (65 mg), D-4a (3 mg), D-5a (1.1 mg), D-6a (27.7 mg), and D-7a (2.8 mg).

2·5 GC-MS Analysis

GC-MS analysis was performed with a gas

chromatograph-mass spectrometer (GC-MS QP-5000, Shimadzu Corporation) equipped with a fused silica DB-1 capillary column (30 m $\times$ 0.25 mm i.d., 0.25 μ m film thickness, J & W Scientific), under the same condition as previously described¹²⁾. Each sterol fraction was converted to trimethylsilyl (TMS) derivative and analyzed, whereas the steroidal 3-one and 4-en-3-one fractions were directly analyzed.

The relative retention times (Rt_R) of each sterol TMS ether to cholesterol TMS ether were summarized in Table 1. Mass spectral data of the identified sterols were as follows. Cholestanol (2) TMS ether, *m/z* (rel. int., %) : 460 (M⁺, 19), 445 (35), 403 (13), 370 (24), 367 (15), 355 (37), 306 (27), 262 (14), 257 (10), 237 (8), 230 (18), 215 (100), 201 (20). Lathosterol (3) TMS ether, *m/z* : 458 (M⁺, 93), 443 (36), 368 (10), 353 (35), 345 (14), 303 (11), 255 (100), 229 (34), 213 (58), 201 (13). 24-Methylenecholesterol (4) TMS ether, *m/z* : 470 (M⁺, 7), 455 (5), 386 (25), 380 (14), 371 (8), 365 (10), 343 (15), 341 (15), 296 (20), 281 (14), 257 (13), 255 (8), 253 (17), 243 (5), 213 (8), 129 (100). 24-Methyl-5 α -cholest-24(28)-en-3 β -ol (6) TMS ether, *m/z* : 388 (M⁺-84, 100), 373 (34), 345 (54), 283 (26), 255 (49), 229 (23), 215 (36). Episterol (10) TMS ether, *m/z* : 470 (M⁺, 7), 455 (15), 386 (21), 380 (5), 372 (8), 365 (12), 343 (100), 318 (4), 303 (4), 281 (9), 255 (16), 253 (22), 227 (21), 213 (28). 24-Ethylcholesta-5,25-dien-3 β -ol (12) TMS ether, *m/z* : 484 (M⁺, 20), 469 (6), 394 (20), 386 (5), 379 (10), 371 (6), 355 (15), 343 (5), 296 (5), 281 (7), 255 (8), 253 (9), 227 (4), 213 (12), 129 (100). 24-Ethyl-5 α -cholest-24(28)Z-en-3 β -ol (17) TMS ether, *m/z* : 388 (M⁺-98, 100), 374 (13), 345 (14), 305 (19), 283 (25), 255 (16), 229 (18), 215 (39), 201 (10). The mass spectral data of respective TMS ether of cholesterol (1), campesterol (5), campestanol (7), stigmasterol (8), Δ^2 -stigmastenol (9), 24-methylathosterol (11), fucosterol (13), sitosterol (14), isofucosterol (15), sitostanol (16), 24-ethyldestmosterol (18), 24-ethylathosterol (19), and avenasterol (20) were essentially the same as previously reported^{7),11),12),14)}.

The Rt_{RS} of each steroidal 4-en-3-one to cholesterol TMS ether were summarized in Table 2. Mass spectral data of the identified steroidal 3-ones were as follows. Cholest-4-en-3-one (21), *m/z* (rel. int., %) : 384 (M⁺, 29), 342 (14), 261 (27), 229 (33), 211 (9), 187 (14), 175 (9), 149 (33), 135 (19),

124 (100). 24-Methylcholesta-4,24(28)-dien-3-one (22), m/z : 396 (M^+ , 15), 381 (14), 313 (78), 312 (100), 297 (93), 281 (6), 269 (86), 256 (23), 245 (23), 243 (31), 231 (41), 229 (36), 227 (67), 209 (18), 187 (24), 173 (33), 161 (28), 149 (50), 147 (52), 135 (45), 133 (58), 124 (95). The mass spectral data of 24-methylcholest-4-en-3-one (23), 24-ethylcholesta-4,22 E -dien-3-one (24), 24-ethylcholest-4-en-3-one (25), 24-ethylcholesta-4,24(28) Z -dien-3-one (26), 24-methyl-5 α -cholestan-3-one (27), and 24-ethyl-5 α -cholestan-3-one (28) were essentially the same as reported previously^{7),12)}.

3 Results and Discussion

As described in **Experimental**, a hexane-soluble lipid was obtained from the homogenized seeds of *E. frumentacea*. From this lipid, a sterol fraction A of a steryl ester fraction, a steroidal 4-en-3-one fraction B, a 5 α -steroidal 3-one fraction C, and a free sterol fraction D were obtained.

The acetate of the sterol fraction A was fractionated by 10% AgNO₃-silica gel column. After converting to the TMS ether, each subfraction was analyzed by GC-MS. In the same manner,

Table 1 Sterol Composition of the Seeds of *Echinochloa frumentacea*.

Sterol (as a TMS ether)	R _{tR} ^{a)}	Composition (%)	
		Free sterol (430 mg/kg)	Esterified sterol (423 mg/kg)
Cholesterol (1)	1.000	1.21	4.64
Cholestanol (2)	1.012	0.02	0.14
Unidentified ^{b)}	1.029	n.d. ^{c)}	0.49
Lathosterol (3)	1.078	n.d. ^{c)}	0.92
Unidentified ^{b)}	1.116	0.52	trace ^{d)}
24-Methylencholesterol (4)	1.140	2.00	4.30
Campesterol (5)	1.156	16.35	15.62
24-Methyl-5 α cholest-24(28)-en-3 β -ol (6)	1.158	0.05	n.d. ^{c)}
Campestanol (7)	1.172	7.22	5.28
Stigmasterol (8)	1.199	7.58	2.34
Δ^{22} -Stigmasterol (9)	1.219	0.80	0.26
Unidentified ^{b)}	1.224	trace ^{d)}	0.03
Episterol (10)	1.225	0.44	1.52
Unidentified ^{b)}	1.228	0.01	n.d. ^{c)}
24-Methyl-lathosterol (11)	1.242	0.07	0.38
Unidentified ^{b)}	1.246	trace ^{d)}	0.08
24-Ethylcholesta-5,25-dien-3 β -ol (12)	1.263	0.71	0.47
Fucoesterol (13)	1.289	0.47	0.28
Sitosterol (14)	1.294	51.02	40.36
Unidentified ^{b)}	1.300	n.d. ^{c)}	0.01
Isocuposterol (15)	1.315	4.75	16.87
Sitostanol (16)	1.317	5.19	3.32
Unidentified ^{b)}	1.323	n.d. ^{c)}	trace ^{d)}
24-Ethyl-5 α -cholest-24(28) Z -en-3 β -ol (17)	1.333	0.01	0.02
24-Ethyl-desmosterol (18)	1.354	0.65	0.57
Unidentified ^{b)}	1.380	0.03	n.d. ^{c)}
24-Ethyl-lathosterol (19)	1.384	0.07	0.36
Avenasterol (20)	1.406	0.20	1.42
Unidentified ^{b)}	1.451	trace ^{d)}	trace ^{d)}

a) Relative retention time (R_{tR}) to the cholesterol TMS ether (10.645 min).

b) The structure was not determined because of the lack of authentic samples.

c) n.d., not detected.

d) trace, less than 0.01%.

Table 2 Steroidal 4-En-3-one Composition of the Seeds of *Echinochloa frumentacea*.

Steroidal 3-one	R _{tR} ^{a)}	Composition (%)
Steroidal 4-en-3-one fraction (5.4 mg/kg)		
Cholest-4-en-3-one (21)	1.092	0.21
24-Methylcholesta-4,24(28)-dien-3-one (22)	1.245	5.39
24-Methylcholest-4-en-3-one (23)	1.260	21.78
24-Ethylcholesta-4,22E-dien-3-one (24)	1.310	14.19
24-Ethylcholest-4-en-3-one (25)	1.415	46.97
24-Ethylcholesta-4,24(28)Z-dien-3-one (26)	1.428	0.70
Unidentified ^{b)}	1.457	5.80

a) Relative retention time (R_{tR}) to the cholesterol TMS ether (10.545 min).

b) The structure was not determined because of the lack of authentic sample.

the free sterol fraction D was fractionated and analyzed. Sterol compositions were calculated from the peak area of total ion chromatogram and the amount of each analyzed sterol fraction. Based on direct GC-MS comparison with authentic samples, sterols listed in **Table 1** were identified, in which the compositions of both free and esterified sterol fractions were included. Although the C-24 epimers of 24-methyl- and 24-ethylsterols could not be separated under our GC conditions, we tentatively use trivial names throughout this text.

Among these sterols detected, eight sterols (1, 4, 5, 7, 8, 14, 15, and 16) were previously found present in the seeds of *Echinochloa crus-galli*¹⁴⁾. In the current study, cholesterol (1) was present in much higher content in the esterified sterol fraction than the free sterol one. Lathosterol (3) was detected only in the esterified sterol fraction, and episterol (10), 24-methylathosterol (11), 24-ethylathosterol (19), and avenasterol (20) were found mainly as esterified forms. 24-Methylenecholesterol (4) and isofucoesterol (15) were more abundantly detected in the esterified sterol fraction than the free sterol one. Campestanol (7) and sitostanol (16) were accumulated in both free and sterol ester fractions. It is noteworthy that the content of campestanol (7) was higher than that of sitostanol (16) in both sterol fractions, although sitosterol (14) was present in much higher amount than campesterol (5).

Since no report has been published on the steroidal 3-ones in *Echinochloa* plants, both the steroidal 4-en-3-one fraction B and the 5 α -steroidal 3-one fraction C from *E. frumentacea* seeds were examined. Six steroidal 4-en-3-ones listed in the

Table 2 were identified from the fraction B by direct GC-MS comparison with authentic samples. Similarly, the full-scan mass spectral data of 24-methyl-5 α -cholestan-3-one (27, R_{tR} 1.137) and 24-ethyl-5 α -cholestan-3-one (28, R_{tR} 1.278) were obtained from the crude fraction C. Although these steroidal 3-ones were reported to occur in many plants^{4),6),7),15),16)}, the current study is the first report of the co-occurrence of six types of steroidal 4-en-3-ones (21-26) in plants.

The co-occurrence of C₂₈ steroids (10, 4, 5, 23, 27, and 7) suggests that *E. frumentacea* may have the same early biosynthetic pathway of episterol (10) $\rightarrow$ 4 $\rightarrow$ 5 $\rightarrow$ 23 $\rightarrow$ 27 $\rightarrow$ campestanol (7) as *A. thaliana*^{4),16),17)}. Based on the present identification data, we propose that all six types of Δ^5 -sterols, i.e., cholesterol (1), 24-methylenecholesterol (4), isofucoesterol (15), campesterol (5), sitosterol (14), and stigmasterol (8), may be converted via the steroidal 4-en-3-ones (21, 22, 26, 23, 25, and 24) into the corresponding C-5 saturated sterols, i.e., cholestanol (2), 24-methyl-5 α -cholest-24(28)-en-3 β -ol (6), 24-ethyl-5 α -cholest-24(28)Z-en-3 β -ol (17), campestanol (7), sitostanol (16), and Δ^{22} -stigmasterol (9), respectively, by the same series of enzymatic reactions. Metabolic study using deuterio-labeled steroids is necessary to verify our proposals.

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[ノート] ヒエ種子中に含まれるステロールおよび ステロイド系 3- オンの同定

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ヒエ (*Echinochloa frumentacea* (Roxb.) Link) 種子中に含まれるステロールおよびステロイド系 3- オンの同定を行った。20 種類のステロールを標品との GC-MS による直接比較により同定し、遊離型およびエステル型ステロール分画のステロール組成を解明した。さらに、ステロイド系 3- オンとして 8 種の化合物を同定した。

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[速報] フォスファチジルコリン／セラミド 3 混合単分子膜 および混合二分子膜中でのドメイン形成と相分離

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L- α -ジパルミトイルフォスファチジルコリン (DPPC) と 2*S*,3*S*,4*R*-2-ステアロイルアミド -1,3,4-オクタデカントリオール (Ceramide 3) から成る混合単分子膜中および混合二分子膜中における相互作用を表面圧測定、原子間力顕微鏡法 (AFM) および示差走査熱量測定 (DSC) により検討した。

表面圧 10mN/m でマイカ上に一層に累積した DPPC/Ceramide 3 混合単分子膜 (LB 膜) の AFM 画像には楕円形のドメインが示された。DPPC リポソーム溶液を用いて測定した DSC 曲線からは、DPPC 二分子膜の相転移温度が Ceramide 3 の添加によって高温側にシフトすること、および DPPC 単独系で見られたシャープなピークは DPPC/Ceramide 3 混合系ではブロードなピークに変化し、更には Ceramide 3 のモル分率が高いものでは DSC ピークが分裂することが観察された。結論として、DPPC/Ceramide 3 混合単分子膜中および混合二分子膜中において相分離が認められた。Ceramide 3 は液体膨張膜の状態にある DPPC と相互作用を起こすことによって、ドメイン形成を伴う相分離を起こすことが示唆された。

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