Triterpene Alcohol and Sterol Ferulates from Rice Bran and Their Anti-inflammatory Effects

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Six novel feruloyl esters of triterpene alcohols and sterols, viz., two *trans*-ferulates, cycloeucalenol and 24-methylenecholesterol *trans*-ferulates, and four *cis*-ferulates, cycloartenol, 24-methyelenecycloartanol, 24-methylcholesterol, and sitosterol *cis*-ferulates, besides five known *trans*-ferulates, cycloartenol (CAR), 24-methylenecycloartanol (24-MCA), 24-methylcholesterol, sitosterol, and stigmastanol *trans*-ferulates, and one known *cis*-ferulate, stigmastanol *cis*-ferulate, were isolated from the methanol extract of edible rice bran. These and eight other synthetic *trans*- and *cis*-ferulates of triterpene alcohols and sterols, along with the corresponding free alcohols, were evaluated with respect to their anti-inflammatory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation (1 μg per ear) in mice. All of the ferulates showed marked inhibitory activity, and their 50% inhibitory dose (ID_{50}) was 0.1−0.8 mg per ear. On the other hand, whereas two free triterpene alcohols, CAR and 24-MCA, showed strong inhibition (ID_{50} 0.2−0.3 mg/ear), eight free sterols examined showed weaker activity (ID_{50} 0.7−2.7 mg/ear) than their corresponding ferulates.

Keywords: *Rice bran; trans- and cis-feruloyl esters; triterpene alcohol; sterol; antiedema; TPA-induced ear edema*

INTRODUCTION

In the course of our search on potential antitumor-promoters (cancer chemopreventive agents) from edible fungi and plants, and from crude herbal drugs, we have found that various triterpene alcohols and sterols and their oxygenated derivatives exhibited activity on in vivo primary screening assay for antitumor-promoters by inhibiting the inflammatory ear edema induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mice and on tumor promotion in two-stage carcinogenesis in mouse skin initiated by 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by TPA (Akihisa et al., 1997; Yasukawa et al., 1991, 1997). We were especially interested to examine rice bran and γ-oryzanol, a characteristic component of rice bran oil containing triterpene alcohol and sterol ferulic acid (4-hydroxy-3-methoxycinnamic acid) esters. γ-Oryzanol has been reported to possess diverse physiological effects including the lowering blood lipids, growth promotion, gonadotrophic action, and stimulation of the hypothalamus (Kahlon et al., 1992, 1994; Seetharamaiah and Chandrasekhara, 1988; Shinomiya et al., 1983; Sugano et al., 1996, 1997, 1999; Yoshino et al., 1989). Our recent study demonstrated that four *trans*-feruloyl esters, viz., cycloartenol (*trans*-CAR), 24-methylenecycloartanol (*trans*-24-MCA), 24-methylcholesterol (*trans*-24-MCO), and sitosterol (*trans*-SITO) *trans*-ferulates, isolated from the methanol extract of rice bran and γ-oryzanol markedly inhibited the TPA-induced inflammation in mice (Yasukawa et al., 1998). In addition, *trans*-CAR has been found to strongly inhibit the tumor-promoting activity of TPA in DMBA-initiated mice (Yasukawa et al., 1998). In continuing our study on the feruloyl ester components of rice bran, we have isolated six novel triterpene alcohol and sterol ferulates, viz., two *trans*- and four *cis*-ferulates, besides five and one known *trans*- and *cis*-ferulates, respectively. In this paper, we report the characterization of the novel ferulates as well as the inhibitory effect against TPA-induced inflammation in mice of 12 ferulates from rice bran extract and eight other synthetic ferulates along with the corresponding 10 free triterpene alcohols and sterols.

MATERIALS AND METHODS

Crystallizations were performed from acetone−MeOH (1:1, v/v). Melting points measured are uncorrected. Preparative thin-layer chromatography (TLC) on silica gel (Kieselgel 60G, Merck; 0.5 mm thick; 20 × 20 cm) was developed using benzene−$CHCl_3$ (3:1, v/v). Normal-phase high-performance liquid chromatography (HPLC) was carried out on a silica column (Silica-1301N column, 4.6 mm i.d. × 30 cm; ERC Co., Ltd., Tokyo, Japan) at 25 °C with *n*-hexanes−ethyl acetate (EtOAc) (97:3, v/v; 1.3 mL/min) as the mobile phase. Reverse-phase HPLC was performed on an ODS column (ODS-2152 column, 10 mm i.d. × 25 cm; ERC Co., Ltd.) at 25 °C with MeOH (4 mL/min) as the mobile phase. Gas−liquid chromatography (GLC) for free triterpene alcohols and sterols was performed on a Shimadzu GC-14B instrument (Shimadzu Co.,

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Kyoto, Japan) using a DB-17 fused-silica capillary column (30 m × 0.3 mm i.d.; column temperature, 275 °C) and nitrogen as a carrier gas at 60 mL/min (split ratio 60:1). For both HPLC and GLC, cholesterol (cholest-5-en-3β-ol) was the standard for the determination of relative retention times (RR_t). Ultraviolet (UV) spectra were recorded in $CHCl_3$. Electron-impact mass spectra (MS) were recorded on a Hitachi M-80B double-focusing gas chromatograph-mass spectroscopy (GC-MS) instrument (70 eV) using a direct inlet system. ^{1}H Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM LA-500 spectrometer at 500 MHz in $CDCl_3$ with tetramethylsilane (TMS) as internal standard.

Chemicals and Materials. Cycloartenol (CAR), 24-methylenecycloartanol (24-MCA), cycloeucalenol (CEU), gramisterol (GRM), citrostadienol (CTR), 24-methylcholesterol (a mixture of 24*R*- and 24*S*-epimers; 24-MCO), 24-methylenecholesterol (24-MEC), sitosterol (SITO), stigmasterol (STIG), and stigmastanol (STAN) (Goad and Akihisa, 1997) were used as the reference specimens and as the starting materials for the preparation of their ferulates. Edible rice bran was purchased at a market in Tokyo (Japan). Four *trans*-ferulates, *trans*-CAR, *trans*-24-MCA, *trans*-24-MCO, and *trans*-SITO also were used as the reference compounds (Yasukawa et al., 1998). *trans*-Ferulic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) was donated by Shiratori Pharmaceutical Co., Ltd. (Chiba, Japan). TPA was purchased from ChemSyn Laboratories (Lenexa, KS). Indomethacin and hydrocortisone were obtained from Sigma Chemical Co. (St. Louis, MO). The structures of triterpene alcohols and sterols and their ferulates described in this paper are shown in Figure 1.

Extraction and Isolation. Rice bran (1 kg) was extracted three times at room temperature for 3 days each with methanol (MeOH) to give an extract (158 g). A portion (100 g) of the extract was partitioned between *n*-hexane−MeOH−H_2O (19:19:2, v/v/v). The *n*-hexane fraction (42 g) was subjected to column chromatography on silica gel (800 g) using an *n*-hexanes−EtOAc gradient of 1:0−1:1 (v/v), which yielded a mixture containing the feruloyl esters of triterpene alcohols and sterols (11.2 g). Preparative TLC of the mixture yielded purified *trans*-ferulate (R_f 0.36; 692 mg) and *cis*-ferulate (R_f 0.49; 96 mg) fractions which on reverse-phase HPLC, and when necessarily on normal-phase HPLC, eventually yielded three *trans*-ferulates, *trans*-CEU (7.0 mg), *trans*-24-MEC (5.4 mg), and *trans*-STAN (8.1 mg), and five *cis*-ferulates, *cis*-CAR (3.8 mg), *cis*-24-MCA (16.2 mg), *cis*-24-MCO (5.4 mg), *cis*-SITO (5.4 mg), and *cis*-STAN (1.9 mg), besides recently reported four *trans*-ferulates, *trans*-CAR (88 mg), *trans*-24-MCA (177 mg), *trans*-24-MCO (88 mg), and *trans*-SITO (62 mg) (Yasukawa et al., 1998). All extraction and isolation procedures for ferulates were carried out in "white" fluorescent light to prevent isomerization of the ferulates (Arín et al., 1995; Hartley and Jones, 1975; Van Boven et al., 1996).

Another portion (58 g) of the MeOH extract was alkaline hydrolyzed giving a nonsaponifiable lipid fraction (6.2 g) which on TLC yielded fractions of triterpene alcohols (R_f 0.27; 396 mg; Fr. A), 4α-methylsterols (R_f 0.22; 12 mg; Fr. B), and sterols (R_f 0.16; 558 mg; Fr. C). Fr. A contained CAR (45.6% of the fraction; RR_t 1.96 in GLC), 24-MCA (51.2%; RR_t 2.14) and other components (3.2%), Fr. B contained CEU (34.0% of the fraction; RR_t 1.73), GRM (29.8%; RR_t 1.73), CTR (15.5%; RR_t 2.28), and other components (20.7%), and Fr. C contained 24-MCO (24.8% of the fraction; RR_t 1.28), 24-MEC (1.0%; RR_t 1.35), SITO (49.9%; RR_t 1.54), STIG (18.5%; RR_t 1.38), STAN (1.4%; RR_t 1.54), and other components (4.4%). Reverse-phase HPLC of the fractions A, B, and C yielded CAR and 24-MCA; CEU, GRM and CTR; and 24-MCO, 24-MEC, SITO, STIG, and STAN; respectively. The compositions of individual fractions were determined based on the GLC and reverse-phase HPLC data.

Preparation of *trans*-Feruloyl Esters. To the solution of *trans*-ferulic acid (1 g) in dry pyridine (3 mL) in a flask equipped with a drying tube of $CaCl_2$ was added propionic anhydride (1 g), and the mixture was stirred for 48 h at room temperature. Extraction of the reaction mixture with $CHCl_3$ yielded 4-propionyl ferulate (1.3 g) (Tamura et al. 1962). Into the solution of STIG (20 mg), 4-propionyl ferulate (60 mg), and DMC (40 mg) in dry CH_2Cl_2 (10 mL) was poured dry pyridine (30 mL) dropwise under water cooling, and the solution was stirred for 2 h at room temperature. Water was added to the reaction mixture, and the CH_2Cl_2 layer was washed successively with diluted HCl, saturated $NaHCO_3$ solution, and water and then dried over Na_2SO_4. This yielded 4-propionyl ferulate of STIG. Hydrolysis of a propionyl ester moiety of the ester by 0.1 M KOH/MeOH solution at 50 °C for 20 min yielded *trans*-STIG (11 mg). Two other *trans*-ferulates, *trans*-GRM and

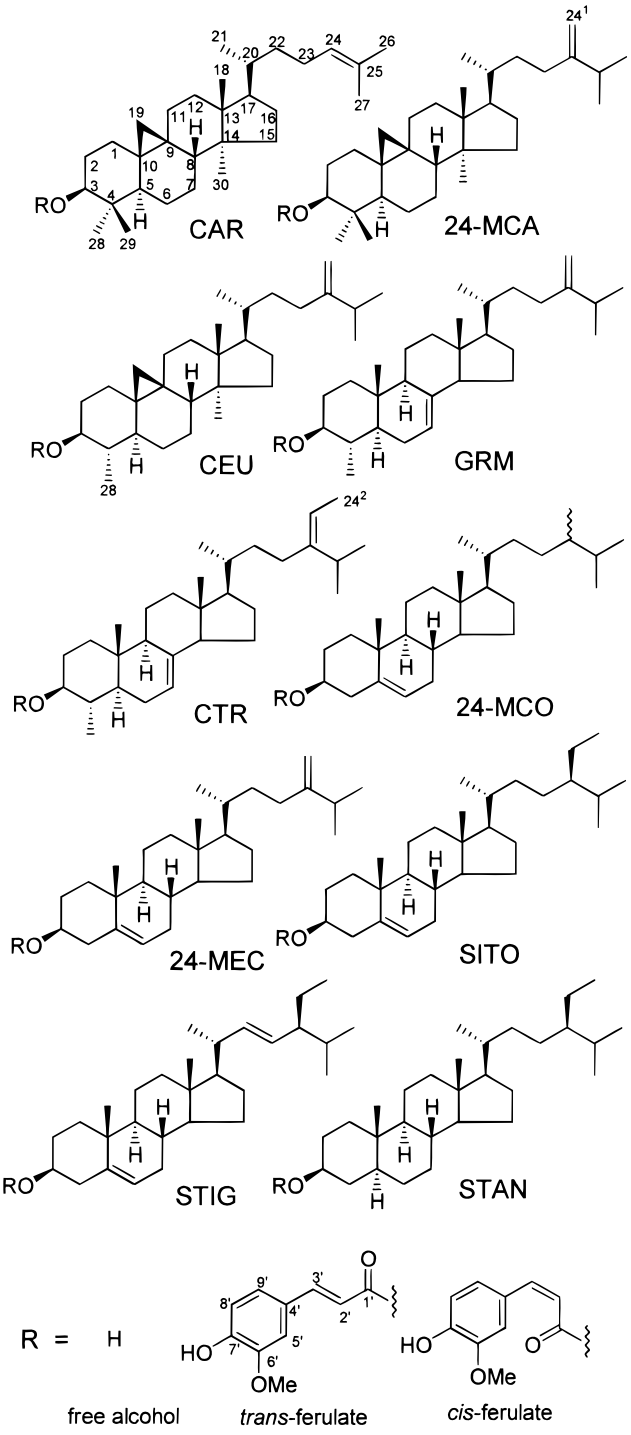

Figure 1. Structures of cycloartenol (CAR), 24-methylenecycloartanol (24-MCA), cycloeucalenol (CEU), gramisterol (GRM), citrostadienol (CTR), 24-methylcholesterol (24-MCO), 24-methylenecholesterol (24-MEC), sitosterol (SITO), stigmasterol (STIG), and stigmastanol (STAN), and their *trans*- and *cis*-ferulates.

Table 1. Relative Retention Times (RR_t)[a] in the HPLC of Triterpene Alcohols, Sterols, and Their Ferulates

compound (abbreviated and systematic names)	free alcohol		*trans*-ferulate		*cis*-ferulate	
	RR_t(I) [b]	RR_t(II) [c]	RR_t(I) [b]	RR_t(II) [c]	RR_t(I) [b]	RR_t(II) [c]
Triterpene Alcohol						
cycloartenol (CAR) (cycloart-24-en-3β-ol)	0.66	1.09	0.85	1.38	0.24	1.64
24-methylenecycloartanol (24-MCA) [24-methylcycloart-24(24^1)-en-3β-ol]	0.54	1.20	0.80	1.72	0.25	1.96
4α-Methylsterol						
cycloeucalenol (CEU) [4α,14α-dimethyl-9β,19-cycloergost-24(24^1)-en-3β-ol]	0.74	1.07	0.86	1.37	0.29	1.65
gramisterol (GRM) [4α-methylergosta-7,24(24^1)-dien-3β-ol]	0.89	1.02	0.91	1.34	0.26	1.87
citrostadienol (CTR) {[24(24^1)*Z*]−4α-methylstigmasta-7,24(24^1)-dien-3β-ol}	0.85	1.20	0.88	1.98	0.24	2.16
Sterol						
24-methylcholesterol (24-MCO) [(24*R*,*S*)-ergost-5-en-3β-ol]	0.96	1.12	0.79	1.79	0.22	1.92
24-methylenecholesterol (24-MEC) [ergosta-5,24(24^1)-dien-3β-ol]	1.02	0.84	0.78	1.38	0.24	1.47
sitosterol (SITO) [(24*R*)-stigmast-5-en-3β-ol]	0.92	1.28	0.65	2.14	0.19	2.16
stigmasterol (STIG) [(22*E*,24*S*)-stigmasta-5,22-dien-3β-ol]	0.94	1.15	0.68	1.86	0.21	1.89
stigmastanol (STAN) [(24*R*)-stigmastan-3β-ol]	0.97	1.42	0.71	2.58	0.20	2.70

[a] Cholesterol: $RR_t = 1.00$. [b] RR_t(I): Silica-1301N column (*n*-hexane−EtOAc = 97:3). [c] RR_t(II): ODS-2152 column (MeOH).

trans-CTR, were prepared by the same manner from the corresponding free alcohols.

Preparation of *cis*-Feruloyl Esters. *cis*-Ferulates were prepared from the corresponding *trans*-ferulates by exposing to UV light in a similar manner as described in the literature (Hartley and Jones, 1975; Van Boven et al., 1996). A solution (5 mg in 100 mL of MeOH) of *trans*-STIG was irradiated under N_2 with a 100-W mercury-vapor-discharge lamp (UM-102 lamp, Ushio Inc., Tokyo, Japan) having the radiation at 365 nm in an immersion-type silica tube unit for 1 h, and the suspected *cis*-STIG and unchanged *trans*-STIG isomers were separated by normal-phase HPLC. The same isomerization reaction yielded four other *cis*-ferulates, *cis*-CEU, *cis*-GRM, *cis*-CTR, and *cis*-24-MEC, from the corresponding *trans*-ferulates.

Identification and Characterization. Identification of four *trans*-ferulates, *trans*-CAR, *trans*-24-MCA, *trans*-24-MCO, and trans-SITO, and 10 free alcohols, from CAR to STAN (Table 1), was performed by spectroscopic (UV, MS, and ^{1}H NMR) and chromatographic (normal- and reverse-phase HPLC) comparison with reference compounds. Characterization of six other *trans*-ferulates, *trans*-CEU, *trans*-GRM, *trans*-CTR, *trans*-24-MEC, *trans*-STIG, and *trans*-STAN, and 10 *cis*-ferulates, from *cis*-CAR to *cis*-STAN (Table 1), was undertaken by spectral (UV, MS, and ^{1}H NMR) comparison with those of the above *trans*-ferulates and with the literature data for the relevant compounds (Goad and Akihisa, 1997). The melting points and the mass and UV spectral data of six *trans*-ferulates, *trans*-CEU, *trans*-GRM, *trans*-CTR, *trans*-24-MEC, *trans*-STIG, and *trans*-STAN, and 10 *cis*-ferulates, from *cis*-CAR to *cis*-STAN (Table 1), are shown below. The *cis*-ferulates exhibited the coupling constant between H′-2 and H′-3 ($J_{H'-2,3} \sim 13$ Hz) in the ^{1}H NMR spectra which unambiguously distinguished them from the *trans*-diastereoisomers ($J_{H'-2,3} \sim 16$ Hz) (Kondo et al., 1988; Otsuka et al., 1996).

Cycloeucalenol *trans*-Ferulate (*trans*-CEU). Mp 155−156 °C. MS *m*/*z* (%): 602 (M^+, 4), 408 (34), 393 (18), 302 (1), 300 (2), 283 (5), 194 (50), 177 (80), 55 (100). High-resolution (HR)-MS: *m*/*z* 602.4345 [Calcd for $C_{40}H_{58}O_4$ (M^+): 602.4332]. UV λ_{max} nm: 243 (log ϵ 4.0), 293 (4.2), 319 (4.3).

Gramisterol *trans*-Ferulate (*trans*-GRM). Mp 179−180 °C. MS *m*/*z* (%): 588 (M^+, 44), 573 (2), 504 (2), 461 (16), 394 (10), 379 (7), 310 (4), 269 (11), 267 (12), 241 (7), 227 (15), 194 (91), 177 (100). HR-MS: *m*/*z* 588.4197 [Calcd for $C_{39}H_{56}O_4$ (M^+): 588.4176]. The UV spectral data of this compound and four *trans*-ferulates described below (*trans*-CTR, *trans*-24-MEC, *trans*-STIG, and *trans*-STAN) were essentially the same with that of *trans*-CEU.

Citrostadienol *trans*-Ferulate (*trans*-CTR). Mp 118−123 °C. MS *m*/*z* (%): 602 (M^+, 23), 504 (17), 461 (28), 408 (4), 393 (4), 310 (9), 295 (6), 267 (13), 241 (7), 227 (15), 194 (74), 177 (100). HR-MS: *m*/*z* 602.4304 [Calcd for $C_{40}H_{58}O_4$ (M^+): 602.4332].

24-Methylenecholesterol *trans*-Ferulate (*trans*-24-MEC). Mp 172−173 °C. MS *m*/*z* (%): 574 (M^+, 16), 447 (4), 380 (100), 365 (10), 296 (24), 253 (15), 194 (74), 177 (71). HR-MS: *m*/*z* 574.4028 [Calcd for $C_{38}H_{54}O_4$ (M^+): 574.4019].

Stigmasterol *trans*-Ferulate (*trans*-STIG). Mp 145−146 °C [lit. mp 147−148 °C (Tamura et al., 1962)]. HR-MS: *m*/*z* 588.4242 [Calcd for $C_{39}H_{56}O_4$ (M^+): 588.4176]. The mass fragmentation was essentially the same with that reported for the same compound (Tanaka and Kato, 1975).

Stigmastanol *trans*-Ferulate (*trans*-STAN). Mp 153−154 °C [lit. mp 156−157 °C (Tamura et al., 1958)]. HR-MS: *m*/*z* 592.4501 [Calcd for $C_{39}H_{60}O_4$ (M^+): 592.4488]. The mass fragmentation was essentially the same with that reported for the same compound (Evershed et al., 1988).

Cycloartenol *cis*-Ferulate (*cis*-CAR). Mp 143−145 °C. MS *m*/*z* (%): 602 (M^+, 3), 587 (1), 425 (4), 408 (20), 393 (6), 365 (6), 339 (7), 316 (6), 297 (4), 286 (2), 203 (8), 194 (26), 177 (100). HR-MS: *m*/*z* 602.4357 [Calcd for $C_{40}H_{58}O_4$ (M^+): 602.4332]. UV λ_{max} nm: 242 (log ϵ 4.0), 298 (4.2), 318 (4.3).

24-Methylenecycloartanol *cis*-Ferulate (*cis*-24-MCA). Mp 125−126 °C. MS *m*/*z* (%): 616 (M^+, 2), 601 (1), 439 (4), 422 (20), 407 (7), 379 (5), 316 (5), 300 (1), 297 (3), 203 (8), 194 (23), 177 (100). HR-MS: *m*/*z* 616.4483 [Calcd for $C_{41}H_{60}O_4$ (M^+): 616.4488]. The UV spectral data of this compound and eight *cis*-ferulates described below were essentially the same with that of *cis*-CAR.

Cycloeucalenol *cis*-Ferulate (*cis*-CEU). Amorphous gum. MS *m*/*z* (%): 602 (M^+, 14), 587 (2), 425 (10), 408 (100), 393 (43), 353 (7), 325 (6), 311 (6), 283 (12), 194 (27), 177 (62), 108 (35). HR-MS: *m*/*z* 602.4335 [Calcd for $C_{40}H_{58}O_4$ (M^+): 602.4332].

Gramisterol *cis*-Ferulate (*cis*-GRM). Mp 128−129 °C. MS *m*/*z* (%): 588 (M^+, 45), 573 (2), 504 (2), 461 (18), 394 (11), 379 (9), 310 (4), 269 (9), 267 (12), 241 (6), 227 (16), 194 (92), 177 (100). HR-MS: *m*/*z* 588.4163 [Calcd for $C_{39}H_{56}O_4$ (M^+): 588.4176].

Citrostadienol *cis*-Ferulate (*cis*-CTR). Mp 92−93 °C. MS *m*/*z* (%): 602 (M^+, 20), 504 (16), 461 (35), 408 (5), 393 (6), 310

Table 2. ^{1}H NMR Data (δ/ppm, 500 MHz, $CDCl_3$) of Six *trans*-Feruloyl Esters of Sterols[a]

proton	*trans*-CEU	*trans*-GRM	*trans*-CTR	*trans*-24-MEC	*trans*-STIG	*trans*-STAN
H-3	4.65 (dt, 4.9, 10.7)	4.55 (dt, 4.6, 10.9)	4.55 (dt, 4.5, 10.9)	4.74 (tt, 5.5, 11.0)	4.75 (m)	4.82 (tt, 5.1, 11.4)
H-6				5.41 (br d, 4.1)	5.40 (m)	
H-7		5.19 (m)	5.19 (m)			
H-18	0.98 (s)	0.55 (s)	0.55 (s)	0.69 (s)	0.71 (s)	0.66 (s)
H-19	0.18 (1H, d, 3.9) 0.43 (1H, d, 4.0)	0.87 (s)	0.86 (s)	1.05 (s)	1.05 (s)	0.85 (s)
H-21	0.90 (d, 6.1)	0.90 (d, 6.6)	0.90 (d, 6.4)	0.96 (d, 6.8)	1.02 (d, 6.5)	0.91 (d, 6.6)
H-22					5.16 (dd, 8.5, 14.9)	
H-23					5.02 (dd, 8.8, 15.2)	
H-25	2.24 (sept, 6.7)	2.23 (sept, 6.8)	2.83 (sept, 6.7)	2.23 (sept, 6.7)		
H-26	1.03 (d, 7.0)	1.03 (d, 6.9)	0.98 (d, 7.0)	1.03 (d, 7.0)	0.80 (d, 6.7)	0.81 (d, 7.0)
H-27	1.03 (d, 7.0)	1.03 (d, 6.9)	0.98 (d, 7.0)	1.03 (d, 7.0)	0.85 (d, 6.4)	0.84 (d, 7.0)
H-24^1	4.67 (1H, br s) 4.72 (1H, br s)	4.66 (1H, br s) 4.72 (1H, br s)	5.11 (q, 6.7)	4.66 (1H, br s) 4.72 (1H, br s)		
H-24^2			1.59 (d, 6.7)		0.81 (d, 7.3)	0.85 (d, 7.0)
H-28	0.89 (d, 6.1)	0.96 (d, 6.3)	0.96 (d, 7.0)			
H-30	0.91 (s)					
H-2′	6.30 (d, 15.9)	6.30 (d, 15.9)	6.30 (d, 15.9)	6.28 (d, 15.9)	6.28 (d, 16.0)	6.27 (d, 15.8)
H-3′	7.61 (d, 15.9)	7.61 (d, 15.9)	7.61 (d, 15.9)	7.60 (d, 15.9)	7.60 (d, 15.9)	7.59 (d, 16.1)
H-5′	7.04 (d, 1.8)	7.04 (d, 1.9)	7.04 (d, 1.9)	7.03 (d, 1.8)	7.03 (d, 1.8)	7.03 (d, 1.8)
O*Me*-6′	3.93 (s)	3.93 (s)	3.93 (s)	3.93 (s)	3.92 (s)	3.92 (s)
O*H*-7′	5.82 (s)	5.84 (s)	5.83 (s)	5.82 (s)	5.83 (s)	5.83 (s)
H-8′	6.92 (d, 8.2)	6.92 (d, 8.2)	6.92 (d, 8.2)	6.91 (d, 8.2)	6.91 (d, 8.2)	6.91 (d, 8.1)
H-9′	7.08 (dd, 1.8, 8.2)	7.08 (dd, 2.0, 8.8)	7.08 (dd, 1.9, 8.4)	7.07 (dd, 2.1, 8.1)	7.07 (dd, 2.1, 8.5)	7.07 (dd, 1.8, 8.1)

[a] Figures in parentheses denote *J* values (Hz).

(7), 295 (7), 267 (15), 241 (8), 227 (16), 194 (75), 177 (100). HR-MS: *m*/*z* 602.4312 [Calcd for $C_{40}H_{58}O_4$ (M^+): 602.4332].

24-Methylcholesterol *cis*-Ferulate (*cis*-24-MCO). Mp 103−105 °C. MS *m*/*z* (%): 576 (M^+, 1), 382 (100), 367 (10), 274 (8), 261 (8), 255 (10), 228 (2), 213 (9), 194 (86), 177 (57). HR-MS: *m*/*z* 576.4217 [Calcd for $C_{38}H_{56}O_4$ (M^+): 576.4166].

24-Methylenecholesterol *cis*-Ferulate (*cis*-24-MEC). Mp 126−127 °C. MS *m*/*z* (%): 574 (M^+, 4), 380 (100), 365 (24), 296 (8), 255 (9), 253 (14), 227 (6), 213 (10), 194 (74), 177 (64). HR-MS: *m*/*z* 574.4060 [Calcd for $C_{38}H_{54}O_4$ (M^+): 574.4019].

Sitosterol *cis*-Ferulate (*cis*-SITO). Mp 102−103 °C. MS *m*/*z* (%): 590 (M^+, 4), 396 (58), 381 (7), 288 (5), 275 (5), 255 (7), 213 (5), 194 (100), 177 (46). HR-MS: *m*/*z* 590.4347 [Calcd for $C_{39}H_{58}O_4$ (M^+): 590.4332].

Stigmasterol *cis*-Ferulate (*cis*-STIG). Mp 150−151 °C. MS *m*/*z* (%): 588 (M^+, 1), 394 (86), 379 (4), 351 (7), 282 (5), 255 (23), 213 (8), 194 (64), 177 (31), 55 (100). HR-MS: *m*/*z* 588.4177 [Calcd for $C_{39}H_{56}O_4$ (M^+): 588.4176].

Stigmastanol *cis*-Ferulate (*cis*-STAN). Mp 120−121 °C. MS *m*/*z* (%): 592 (M^+, 32), 398 (2), 383 (4), 257 (4), 215 (7), 194 (100), 177 (14). HR-MS: *m*/*z* 592.4382 [Calcd for $C_{39}H_{60}O_4$ (M^+): 592.4488].

Animals. Female ICR mice were obtained from Japan SLC (Shizuoka, Japan). The animals were housed in an air-conditioned specific pathogen free room (22−23 °C) lit from 08:00 to 20:00. Food and water were available ad libitum.

Assay of TPA-Induced Inflammation Ear Edema. TPA (1 μg) dissolved in acetone (20 μL) was applied to the right ear of ICR mice by means of a micropipet. A volume of 10 μL was delivered to both the inner and outer surfaces of the ear. The samples or their vehicles, MeOH−$CHCl_3$−H_2O (2:1:1, v/v/v; 20 μL), as control, were applied topically about 30 min before TPA treatment. For ear thickness determinations, a pocket thickness gauge with a range of 0−9 mm, graduated at 0.01 mm intervals and modified so that the contact surface area was increased to reduce the tension, was applied to the tip of the ear. The ear thickness was measured before treatment (*a*), and 6 h after TPA treatment (*b* = TPA alone; *b*′ = TPA plus sample). The following values were then calculated:

$$\text{Edema A is induced by TPA alone } (b - a)$$

$$\text{Edema B is induced by TPA plus sample } (b' - a)$$

$$\text{Inhibitory ratio (\%)} = [(\text{Edema A} - \text{Edema B})/\text{Edema A}] \times 100$$

Each value was the mean of individual determinations from five mice. The 50% inhibitory dose (ID_{50}) values were determined by the method of probit-graphic interpolation for four dose levels.

Statistical Analysis. Statistical analysis was carried out by Student's *t*-test.

RESULTS AND DISCUSSION

We have isolated and characterized six novel feruloyl esters of triterpene alcohols and sterols, viz., two *trans*-ferulates, *trans*-CEU and *trans*-24-MEC, and four *cis*-ferulates, *cis*-CAR, *cis*-24-MCA, *cis*-24-MCO (The ratio of the 24*R*:24*S* stereoisomers was estimated to be 2:3 based on the ^{1}H NMR data), and *cis*-SITO, as minor feruloyl ester constituents from the MeOH extract of edible rice bran in this study along with five known *trans*-ferulates, *trans*-CAR, *trans*-24-MCA, *trans*-24-MCO, *trans*-SITO, and *trans*-STAN (Diack and Saska, 1994; Evershed et al., 1988; Kondo et al., 1988; Norton, 1995; Rogers et al., 1993; Seitz, 1989; Tamura et al., 1958, 1962; Tanaka, 1971; Yasukawa et al., 1998), and one known *cis*-ferulate, *cis*-STAN (Kondo et al., 1988). In addition, 10 triterpene alcohols and sterols, from CAR to STAN (Table 1), were isolated from the nonsaponifiable lipid fraction of the MeOH extract.

This is the first instance for the characterization of naturally occurring ferulate of 4α-methylsterol, viz., *trans*-CEU. Among the five *cis*-ferulates characterized from rice bran extract in this study, *cis*-STAN has previously been isolated accompanied with its 24-methyl homologue and their *trans*-ferulate isomers (Kondo et al., 1988). Daylight and long-wavelength UV radiation induce *cis*−*trans* isomerization of feruloyl esters (Arín et al., 1995; Hartley and Jones, 1975; Van Boven et al., 1996). Even though all extraction and isolation procedures were undertaken with devoid of daylight, there is a high possibility as artifacts (Van Boven et al., 1996) for the five *cis*-ferulates characterized from rice bran extract in this study which remains to be clarified in the future. Natural occurrence of several *cis*-cinnamic acid derivative esters, viz., the *cis*-ferulate of loganic acid, an iridoid glucoside acyl ester, in *Alangium pla-*

Table 3. ^{1}H NMR Data (δ/ppm, 500 MHz, $CDCl_3$) of Ten *cis*-Feruloyl Esters of Triterpene Alcohols and Sterols[a]

						cis-24-MCO					
proton	*cis*-CAR	*cis*-24-MCA	*cis*-CEU	*cis*-GRM	*cis*-CTR	24*R*	24*S*	*cis*-24-MEC	*cis*-SITO	*cis*-STIG	*cis*-STAN
H-3	4.64 (dd, 6.3, 10.4)	4.64 (dd, 6.3, 10.4)	4.59 (dt, 6.1, 10.7)	4.48 (dt, 4.4, 10.9)	4.48 (dt, 4.6, 10.9)	4.68 (m)		4.67 (m)	4.67 (m)	4.67 (tt, 5.6, 11.3)	4.75 (m)
H-6						5.38 (br d, 6.8)		5.38 (br d, 5.0)	5.38 (br d, 6.4)	5.38 (br d, 6.4)	
H-7				5.19 (m)	5.18 (m)						
H-18	0.97 (s)	0.97 (s)	0.97 (s)	0.54 (s)	0.54 (s)	0.68 (s)		0.68 (s)	0.68 (s)	0.70 (s)	0.66 (s)
H-19	0.35 (1H, d, 4.4)	0.35 (1H, d, 4.4)	0.16 (1H, d, 4.0)	0.84 (s)	0.85 (s)	1.02 (s)		1.05 (s)	1.02 (s)	1.02 (s)	0.84 (s)
	0.60 (1H, d, 4.0)	0.58 (1H, d, 3.8)	0.41 (1H, d, 3.7)								
H-21	0.89 (d, 6.6)	0.90 (d, 6.6)	0.90 (d, 6.1)	0.90 (d, 6.3)	0.90 (d, 6.4)	0.91 (d, 6.8)	0.92 (d, 6.8)	0.95 (d, 6.7)	0.92 (d, 6.6)	1.02 (d, 6.7)	0.91 (d, 6.8)
H-22										5.16 (dd, 8.5, 14.9)	
H-23										5.02 (dd, 8.5, 14.9)	
H-24	5.11 (br t, 7.0)										
H-25		2.24 (sept, 6.6)	2.24 (sept, 6.7)	2.23 (sept, 6.6)	2.83 (sept, 6.7)			2.23 (sept, 6.7)			
H-26	1.69 (s)	1.03 (d, 6.9)	1.03 (d, 7.0)	1.03 (d, 6.9)	0.98 (d, 6.8)	0.85 (d, 6.9)	0.86 (d, 6.9)	1.03 (d, 7.0)	0.82 (d, 6.9)	0.80 (d, 7.0)	0.81 (d, 6.8)
H-27	1.61 (s)	1.03 (d, 6.9)	1.03 (d, 7.0)	1.03 (d, 6.9)	0.98 (d, 6.8)	0.80 (d, 7.1)	0.79 (d, 7.1)	1.03 (d, 7.0)	0.84 (d, 6.9)	0.85 (d, 6.0)	0.83 (d, 6.8)
H-24^1		4.67 (1H, br s)	4.67 (1H, br s)	4.66 (1H, br s)	5.11 (q, 6.7)	0.78 (d, 6.9)	0.78 (d, 6.9)	4.66 (1H, br s)			
		4.72 (1H, br s)	4.72 (1H, br s)	4.72 (1H, br s)				4.72 (1H, br s)			
H-24^2					1.59 (d, 7.1)				0.85 (t, 7.1)	0.81 (t, 7.3)	0.85 (t, 6.8)
H-28	0.86 (s)[b]	0.86 (s)[b]	0.86 (d, 6.1)	0.96 (d, 6.6)	0.95 (d, 6.4)						
H-29	0.87 (s)[b]	0.87 (s)[b]									
H-30	0.91 (s)	0.91 (s)	0.91 (s)								
H-2′	5.83 (d, 12.6)	5.84 (d, 12.9)	5.82 (d, 12.5)	5.82 (d, 12.9)	5.82 (d, 12.8)	5.80 (d, 12.9)		5.80 (d, 12.8)	5.80 (d, 12.9)	5.80 (d, 13.2)	5.80 (d, 12.9)
H-3′	6.78 (d, 12.6)	6.78 (d, 12.9)	6.78 (d, 12.8)	6.79 (d, 12.9)	6.78 (d, 13.2)	6.78 (d, 12.9)		6.78 (d, 12.8)	6.78 (d, 12.9)	6.78 (d, 12.8)	6.78 (d, 12.9)
H-5′	7.74 (d, 1.9)	7.74 (d, 1.9)	7.77 (d, 1.8)	7.77 (d, 1.9)	7.77 (d, 1.8)	7.73 (d, 1.9)		7.73 (d, 1.8)	7.73 (d, 1.9)	7.73 (d, 2.0)	7.73 (d, 1.9)
O*Me*-6′	3.93 (s)	3.93 (s)	3.93 (s)	3.93 (s)	3.93 (s)	3.93 (s)		3.93 (s)	3.93 (s)	3.92 (s)	3.93 (s)
O*H*-7′	5.81 (s)	5.81 (s)	5.82 (s)	5.84 (s)	5.81 (s)	5.80 (s)		5.81 (s)	5.81 (s)	5.81 (s)	5.81 (s)
H-8′	6.88 (d, 8.2)	6.88 (d, 8.29	6.88 (d, 8.2)	6.88 (d, 8.2)	6.88 (d, 8.2)	6.88 (d, 8.2)		6.88 (d, 8.2)	6.88 (d, 8.2)	6.88 (d, 8.2)	6.88 (d, 8.2)
H-9′	7.13 (dd, 1.8, 8.2)	7.13 (dd, 1.9, 8.2)	7.14 (dd, 1.6, 8.3)	7.14 (dd, 1.9, 8.2)	7.13 (dd, 1.9, 8.2)	7.12 (dd, 1.9, 8.2)		7.12 (dd, 1.8, 8.2)	7.12 (dd, 1.9, 8.2)	7.12 (dd, 1.8, 8.2)	7.12 (dd, 1.9, 8.2)

[a] Figures in parentheses denote *J* values (Hz). [b] Values bearing same superscript in each column are interchangeable.

Table 4. Inhibitory Effect of Triterpene Alcohols and Sterols, Their Ferulates, and Reference Compounds on TPA-Induced Inflammation in Mice[a]

	free alcohol		*trans*-ferulate		*cis*-ferulate	
compound	ID_{50} (mg/ear)	I.R. (%)	ID_{50} (mg/ear)	I.R. (%)	ID_{50} (mg/ear)	I.R. (%)
Triterpene Alcohol						
CAR	0.3	92[b]	0.2[c]	79[c]	0.8	47
24-MCA	0.2	85	0.2[c]	88[c]	0.5	62
4a-Methylsterol						
CEU	1.9	38	0.2	86	0.4	68
GRM	0.7	66	0.5	72	0.5	55
CTR	0.7	60	0.5	64	0.5	72
Sterol						
24-MCO	1.7	44	0.3[c]	85[c]	0.3	84
24-MEC	2.7	28	0.4	77	0.5	66
SITO	1.8	43	0.2[c]	93[c]	0.3	74
STIG	1.9	44	0.2	93	0.3	87
STAN	1.0	53	0.1	94	0.1	94
Reference Compound						
quercetin	1.6	40				
indomethacin	0.3	96				

[a] ID_{50}: 50% inhibitory dose. I.R.: inhibitory ratio. Unless otherwise stated, the I.R. was at 1 mg/ear, and $p < 0.01$ by Student's *t*-test as compared to control group. [b] I.R. at 0.5 mg/ear. [c] Values taken from the literature (Yasukawa et al., 1998).

tanifolium (Alangiacae) (Otsuka et al., 1996), the *cis*-ferulates of C_{22}−C_{29} ω-hydroxy fatty acids in the bark of *Virola* species (Myristicaceae) (Kawanishi and Hashimoto, 1982), and taraxerol *cis*-*p*-coumarate in *Rhizophora apiculata* (Rhizophoraceae) (Kokpol and Chavasiri, 1990), has previously been reported.

To compare the inhibitory effects on TPA-induced inflammation in mice of free triterpene alcohols (CAR and 24-MCA) and sterols (from CEU to STAN; Table 1) with their *trans*- and *cis*-ferulates, we have prepared three *trans*-ferulates, *trans*-GRM, *trans*-CTR, and *trans*-STIG, and five *cis*-ferulates, *cis*-CEU, *cis*-GRM, *cis*-CTR, *cis*-24-MEC, and *cis*-STIG, from the corresponding free alcohols for their evaluation of the anti-inflammatory effects, among which *trans*-STIG is a known ferulate in rice bran oil (Endo et al., 1968). Table 1 summarizes the retention data on normal- and reverse-phase columns in the HPLC of 10 free alcohols (from CAR to STAN) and their *trans*- and *cis*-ferulates. Table 2 shows the ^{1}H NMR spectral data for two novel *trans*-ferulates, *trans*-CEU and *trans*-24-MEC, and, in addition, four known *trans*-ferulates, *trans*-GRM, *trans*-CTR, *trans*-STIG, and *trans*-STAN, since the full ^{1}H NMR data for the latter four are unavailable in the literature. The ^{1}H NMR spectral data for 10 *cis*-ferulates, from *cis*-CAR to *cis*-STAN (Table 1), are shown in Table 3.

Ten each of free alcohols and their *trans*- and *cis*-ferulates were evaluated with respect to their anti-inflammatory activity against TPA-induced inflammation in mice, and the inhibitory activities were compared with those of quercetin, a known antitumor-promoter, and indomethacin, a commercially available anti-inflammatory drug, as shown in Table 4. Whereas one free 4α-methylsterol, CEU (ID_{50} 1.9 mg/ear), and four free sterols, 24-MCO, 24-MEC, SITO, and STIG (1.7−2.7 mg/ear), exhibited inhibitory activities weaker than or almost equivalent with that of quercetin (1.6 mg/ear), the others inhibited markedly the TPA-induced inflammation with 0.1−1.0 mg/ear of the 50% inhibitory dose. While the activities of free 4α-methylsterols (0.7−1.9 mg/ear) and sterols (1.0−2.7 mg/ear) were weaker than those of triterpene alcohols (0.2−0.3 mg/ear), esterification with both *trans*- and *cis*-ferulic acids enhanced the activity as for the former two sterol groups (0.1−0.5 mg/ear) of which activity was almost comparable with that of indomethacin (0.3 mg/ear). As for the triterpene alcohols, CAR and 24-MCA, esterification with *trans*-ferulic acid exerted almost no influence (0.2 mg/ear) on the activity while with *cis*-ferulic acid reduced the activity (0.5−0.8 mg/ear).

trans-CAR has been proved in our recent study to possess marked antitumor-promoting activity in two-stage carcinogenesis in mouse skin (Yasukawa et al., 1998). Taking this into consideration, it is highly probable that the ferulates of 4α-methylsterols and sterols described in this paper also are effective anti-tumor-promoters. Rice bran which contains various triterpene alcohols and sterols as the feruloyl esters is suggested to have potential for the chemoprevention of cancer.

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