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Studies on Non-sesquiterpenoid Constituents of *Gaillardia pulchella*.[1] II. Less Lipophilic Substances, Methyl Caffeate as an Antitumor Catecholic

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A less lipophilic non-sesquiterpenoid antitumor constituent, methyl caffeate, along with D-fructose, was isolated from the aqueous methanol extract of the dried whole plant of *Gaillardia pulchella*. Among forty-three synthetic catechol analogues related to methyl caffeate tested for antitumor activity against Sarcoma 180 in mice, acetates of methyl catecholylpropionoid type showed stronger activity than the corresponding catechuic acid and catechualdehyde derivatives as well as simple benzaldehyde (so-called vidrol). None of the catecholic and related compounds tested showed significant antimicrobial activity *in vitro*.

Keywords——Compositae; *Gaillardia pulchella*; non-sesquiterpenoid; methyl caffeate; methanol extraction; synthetic catecholic; benzaldehyde; antitumor activity; antimicrobial activity; catecholylpropionoid

In our continuing studies on biologically active constituents in the chloroform extract of the dried flowering whole plant of *Gaillardia pulchella* Foug (Compositae), we have isolated several sesquiterpene lactones, and the biogenetic pathways were discussed.[2] They are the eudesmanolides pulchellins B, C, D, E and F,[3] the guaianolides gaillardin[4] and neogaillardin[2] (having antitumoral activity), and the 2,4-dihydroxypseudoguaianolides pulchellin[5] and neopulchellin (the main antitumor constituents).[6] Sesquiterpene lactone alkaloids of a new type, pulchellidine[5b,c] and neopulchellidine[6] (13-piperidino derivative of pulchellin and neopulchellin), having antitumoral and anti-inflammatory activity, together with a small amount of a modified pseudoguaianolide pulchellon[7] possessing a unique pseudotwistane skeleton, were also isolated from the chloroform extracts of different collections of the flowering plant. Furthermore, higher oxygenated pseudoguaianolides such as pulchelloid C,[8] (2,4,6-trihydroxy-4-angelate), pulchelloid A,[9] (2,4,6,9-tetrahydroxy-2-angelate) and pulchelloid B,[9] (2,4,6,9-tetrahydroxy-2-isovalerate) were also isolated as minor constituents from the aqueous methanol extract of the cultivar. Recently, we conducted further studies on more lipophilic substances in *G. pulchella* plant. Thus, three highly lipophilic non-sesquiterpenoid alkanols having plant growth regulating activity were isolated from the chloroform extract and were characterized as 1-octacosanol, 1-triacontanol and 1-dotriacontanol.[10]

According to our survey of the literature published since 1950, only five Compositae plants, *Tanacetum odessanum* (*klok.*) Tzvel,[11a] *Bedfordia solicina*,[11b] *Gochnatra rusbyana* Cabrera,[11c] *Artemisia apiacea* Hance,[11d] and *Pseudostiffita kingii* H. Robins,[11e] among various kinds of plants in fifteen families, were found to contain methyl caffeate on the basis of its isolation from methanol or even non-methanol extracts by the conventional plant extraction methods.

This paper describes the isolation and identification of methyl caffeate as a less lipophilic constituent from the aqueous methanol extract of the dried plant of *G. pulchella* as well as the biological activities (antitumor activity against Sarcoma 180 in mice and the *in vitro* antimicrobial activity) of methyl caffeate and related catecholics and analogues.

Isolation and Characterization

The air-dried chopped material of the flowering whole plant of *G. pulchella* was extracted with hot chloroform as described previously.[5a,c,10] The residuum was digested with aqueous methanol at ambient temperature for two weeks. Evaporation of the solvents at low temperature and reduced pressure afforded a gum, which was triturated with water and chloroform. The crude material obtained from the chloroform portion by the usual treatment was subjected to silica gel column chromatography. Elution with CH_2Cl_2–MeOH (19:1) afforded a crude crystalline mass as a non-sesquiterpenoid constituent in a yield of 0.01% based on the dried material. This product was identified as methyl caffeate, as described below.

Recrystallization from aqueous methanol gave colorless prisms, mp 161—162.5 °C, giving a positive ferric chloride test. The infrared (IR) spectrum (ν 1681 cm^{-1}) of the compound (I) revealed the presence of α,β-unsaturated ester. In the mass (MS) spectrum the molecular ion (*m/e* 194) ($C_{10}H_{10}O_4$) and prominent peaks at *m/e* 163 ($M^+ - OCH_3$) and *m/e* 135 ($M^+ - OCH_3$–CO) were observed. The proton magnetic resonance (^{1}H-NMR) spectrum revealed an ester methyl group (CH_3–CO–O) at 3.72 ppm, three aromatic protons (6.8—7.3 ppm) and a pair of doublets (J=16.0) at 6.28 and 7.56 ppm, probably attributable to a conjugated olefin. The signal at 8.22 ppm (2H) disappeared on addition of D_2O, probably indicating phenolic active hydrogen. Acetylation with acetic anhydride–pyridine in the usual manner yielded a diacetate (II), mp 79.5—81 °C, as colorless leaflets. This observation suggested that the structure of I should be formulated as a methyl ester of dihydroxy-*trans*-cinnamic acid; IR ν_{max}^{KBr} (cm^{-1}) 1776 ($OCOCH_3$), 1710 (–CH=CH–COO–), ^{1}H-NMR ($CDCl_3$) δ: 2.30 (6H, s, 2×$OCOCH_3$), 3.80 (3H, s, $COOCH_3$), 6.60 (1H, d, J=16 Hz, Ar–CH=CH–COO–), 7.1—7.5 (3H, s, aromatic protons), 7.66 (1H, d, J=16 Hz, Ar–CH=CH–COO–). The sample was identified as methyl 3,4-diacetoxy-*trans*-cinnamate by comparison (mixed melting point and IR) with an authentic specimen prepared from caffeic acid by acetylation followed by esterification with diazomethane.

In order to determine the biological activity of related catecholics, and to confirm further the location of the two hydroxy groups on the aromatic ring of naturally occurring methyl caffeate (I), the acetate (II) was subjected to ozonolysis followed by Jones oxidation to yield diacetoxybenzoic acid (III), mp 155—156 °C, as colorless prisms. The IR spectrum of III

Table I. Spectral Data for Diacetoxybenzoic Acids

Substitution	mp (°C)	IR ν_{max}^{KBr} (cm^{-1})	MS (I_{136}/I_{154})
2,3-	150—152	1774, 1763, 1694	2.32
2,4-	134—138	1776, 1682	1.80
2,5-	117—119.5	1760, 1728, 1705	1.57
2,6-	116[a]	—	—
3,4-	156—158	1772, 1688	0.18
3,5-	156—157[b]	—	—

a) Y. Ishii *et al.*, *J. Agr. Chem. Soc. Japan*, **26**, 510 (1952).
b) F. Mauthner, *J. Prakt. Chem.*, **136**, 205 (1933).

showed the presence of a typical carboxyl group (ν 2300—3250 and 1687 cm^{-1}) and an acetyl carbonyl (ν 1772 cm^{-1}). The molecular ion peak (*m/e* 238) ($C_5H_6O_4$) and prominent ion peaks at *m/e* 154, 136 and 44 were observed. The sample was found to be identical with 3,4-diacetoxybenzoic acid (III) when compared with authentic specimens of 2,3-, 2,4-, 2,5- and 3,4-diacetoxybenzoic acid prepared from the corresponding commercially available dihydroxybenzaldehydes by acetylation and subsequent Jones oxidation in the usual manner. All these compounds were also subjected to the biological assays described below for comparison. Table I shows some spectral data for these diacetoxybenzoic acids.

It is of interest to note here that the relative intensity ratio of the fragment peak of *m/e* 136 to 154 (I_{136}/I_{154}) is more than 1.0 in the salicylic acid type of dihydroxybenzoic acid congeners (2,3-, 2,4-, 2,5- and 2,6-). In sharp contrast to these catecholic analogues, those of the non-salicylic acid type of dihydroxybenzoic acid derivatives (3,4- and 3,5-) were found to be less than 1.0. This may be accounted for by the fragmentation scheme shown in Chart 1, in which the intermediary fragment at *m/e* 154 formed from the former derivatives undergoes easier dehydration yielding the characteristic keto ketene fragment ion peak at *m/e* 136. Thus, the degradation product (III) derived from I mentioned above was unequivocally determined to be 3,4-diacetoxybenzoic acid.

Thus, a non-sesquiterpenoid constituent isolated from *G. pulchella* plant was proved to be methyl 3,4-dihydroxy-*trans*-cinnamate (methyl caffeate).

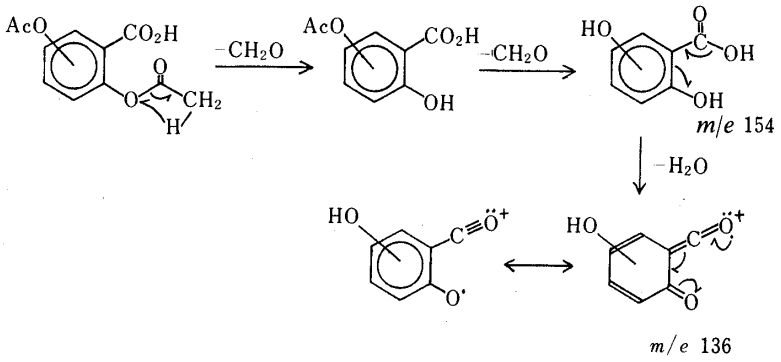


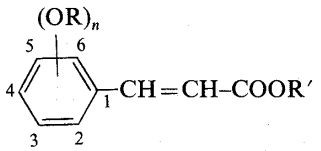
Chart 1. Possible Mass Fragmentation Scheme for Diacetoxybenzoic Acid Derivatives

Methyl caffeate and various catecholylpropionoids including related catecholics were examined for antitumor and antimicrobial activities. The three types of compounds, dihydroxycinnamic acid, dihydroxybenzoic acid and dihydroxybenzaldehyde, tested for antitumor activity are listed in Tables II, III and IV, respectively. The results are shown in Table V.

Results and Discussion

As far as we are aware from the literature since 1972, there are no reports concerning the antitumor activity of methyl caffeate or cinnamate among related phenylpropionoids. The antitumor activity of ether or ester derivatives of methyl caffeate, *i.e.* methyl ferulate (No. 10), methyl acetylferulate (No. 14), methyl acetylisoferulate (No. 16) and methyl diacetylcaffeate (No. 18), was found to be higher than that of methyl caffeate. In general, the degree of inhibition by the esterified catecholics was higher than that of non-esterified catecholics. Furthermore, catecholylpropionoids such as caffeic acid and its ester seem to possess higher activity than *p*-coumaric acid, cinnamic acid and their esters.

TABLE II. Catecholylpropionoids and Related Phenylpropionoids

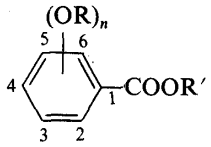


No.	R′	n	OR		
			3	4	5
1	H	0			
2	CH_3	0			
3	H	1		OH	
4	CH_3	1		OH	
5	H	2	OH	OH	
6	CH_3	2	OH	OH	
7	H	2	OH	OCH_3	
8	CH_3	2	OH	OCH_3	
9	H	2	OCH_3	OH	
10	CH_3	2	OCH_3	OH	
11	H	2	OCH_3	OCH_3	
12	CH_3	2	OCH_3	OCH_3	
13	H	2	OCH_3	$OCOCH_3$	
14	CH_3	2	OCH_3	$OCOCH_3$	
15	H	2	$OCOCH_3$	OCH_3	
16	CH_3	2	$OCOCH_3$	OCH_3	
17	H	2	$OCOCH_3$	$OCOCH_3$	
18	CH_3	2	$OCOCH_3$	$OCOCH_3$	
19	H	3	OCH_3	OH	OCH_3
20	CH_3	3	OCH_3	OH	OCH_3
21	H	3	OCH_3	$OCOCH_3$	OCH_3
22	CH_3	3	OCH_3	$OCOCH_3$	OCH_3

Although it has been reported that benzaldehyde (so-called "vidrol") has antitumor activity,[12] it produced inhibition of no more than 10.0% in this experimental system. Hydroxy and dihydroxy derivatives of benzaldehyde and benzoic acid including the corresponding catecholics had lower antitumor activity than the above-mentioned catecholylpropionoids.

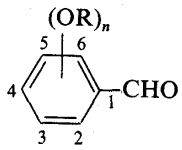
Several catecholylpropionoids including methyl caffeate (No. 6) were tested for *in vitro* antimicrobial activity against the following 27 pathogenic microorganisms: *Streptococcus haemolyticus* Group A-089, *Staphylococcus aureus* 209-P JC-1, SMITH and 72R, *Streptococcus epidermidis* ATCC-12228, *Escherichia coli* NIHJ JC-2, K-60 and K-74, *Salmonella enteritides* 1891, *Sal. typhimurium* K-52, *Sal. Pullorum* TYUYU, *Enterobacter cloacae* IID 977, *En. aerogenes* IID 972, *Serratia marcescens* IID 620, *Proteus vulgaris* OX-19, *Pro. morganii* IFO 3168, *Pro. rettgeri* IFO 13501, *Pro. inconstans* IFO 12930, *Klebsiella pneumoniae* ST-101, IID 875 and JID 979, *Shigella flexneri* IID 642, *Shi. sonnei* IID 969, *Yersinia enterocolitica* IID 981, *Pseudomonas aeruginosa* 347 (ATCC-17662) and K-99 (ATCC-17662), and *Ps. maltophilia* IID 1275. Catecholylpropionoids with a 3,4-disubstituted acid moiety and methyl esters possessing a free hydroxy group at C_4 and either a hydroxy (Nos. 5 and 6) or methoxy group at C_3 (Nos. 9 and 10) exhibited almost no activity against these organisms. No activity was shown by the following C_4-substituted catecholics: 3-hydroxy-4-methoxy- (No. 7), 3,4-dimethoxy- (No. 11), and 3,4-diacetoxy-cinnamic acid (No. 17) and their methyl esters (Nos. 8, 12 and 18), as well as less-substituted phenylpropionoids (Nos. 1, 2, 3 and 4). Further,

TABLE III. Protocatechuic Acid and Related Dihydroxybenzoic Acid Derivatives



No.	R′	n	OR			
			2	3	4	5
23	H	2	OH	OH		
24	CH_3	2	OH	OH		
25	H	2	OH		OH	
26	CH_3	2	OH		OH	
27	H	2	OH			OH
28	CH_3	2	OH			OH
29	H	2		OH	OH	
30	CH_3	2		OH	OH	
31	CH_3	2	OCH_3	OCH_3		
32	CH_3	2	OCH_3		OCH_3	
33	CH_3	2	OCH_3			OCH_3
34	CH_3	2		OCH_3	OCH_3	
35	H	3		OCH_3	OH	OCH_3
36	CH_3	3		OCH_3	OH	OCH_3

TABLE IV. Protocatechualdehyde and Other Dihydroxybenzaldehyde Analogues



No.	n	OR			
		2	3	4	5
37	2	OH	OH		
38	2	OH		OH	
39	2	OH			OH
40	2		OH	OH	
41	2		OH	OCH_3	
42	2		OCH_3	OH	
43	3		OCH_3	OCH_3	OCH_3

protocatechuic acids and methyl esters (Nos. 29 and 30) and other dihydroxy isomers such as 2,3-dihydroxy- (Nos. 23 and 24) and 2,4-dihydroxybenzoic acid and -benzoate (Nos. 25 and 26) as well as 2,3- (No. 31), 2,4- (No. 32), 2,5- (No. 33), 3,4-dimethoxybenzoate (No. 34) and 3,5-dimethoxy-4-hydroxybenzoic acid and its methyl ester (Nos. 35 and 36) showed no appreciable activity. Methyl 2,5-dihydroxybenzoate (No. 28) was found to have a weak antimicrobial activity, especially against *K. pneumoniae* ST-101 at the concentration of 25 ppm; its activity was slightly higher than that of the corresponding acid (No. 27).

TABLE V. Antitumor Activity of Methyl Caffeate, Related Catecholics and Dihydroxyphenyl Derivatives against Sarcoma 180

No.	Inhibition rate (%)	No.	Inhibition rate (%)
1	21.3	23	12.3
2	23.6	24	20.1
3	17.2	25	3.2
4	16.0	26	27.9
5	21.4	27	12.3
6	41.2	28	23.7
7	38.3	29	7.3
8	37.4	30	34.8
9	23.7	31	18.4
10	44.4	32	8.4
11	33.0	33	20.7
12	34.0	34	20.7
13	27.9	35	13.2
14	49.7	36	29.2
15	26.2	37	25.0
16	49.8	38	14.3
17	35.5	39	16.1
18	51.8	40	19.6
19	16.6	41	20.1
20	15.5	42	11.0
21	24.9	43	14.6
22	18.2		

Experimental

Extraction and Separation from *Gaillardia pulchella*——Whole plants of *Gaillardia pulchella* in the flowering stage, cultivated at Koganei City near Tokyo, were harvested in September 1977. The air-dried and chopped material (16 kg) was percolated with hot $CHCl_3$ and the $CHCl_3$ extract was treated as previously described to yield pulchellin[5] and neopulchellin[6] as the major sesquiterpenoid principles.[5a,c,10] The residuum was further extracted with MeOH–H_2O (1 : 1) at room temperature for 2 weeks. Flash evaporation of the solvent at about 40—50 °C under reduced pressure afforded a brown gum (300 g), which was dissolved in water (600 ml) and extracted three times with $CHCl_3$ (1 l). The dried $CHCl_3$ extract left, on evaporation at reduced pressure, a brown residue (8 g), which was subjected to column chromatography on silica gel. The fraction eluted with CH_2Cl_2–MeOH (95 : 5) afforded a crystalline mass (1.8 g, 0.01% based on the dried plant). Recrystallization from H_2O–MeOH gave methyl 3,4-dihydroxy-*trans*-cinnamate (I) (500 mg, 0.003%). mp 161—162.5 °C (colorless prisms); $FeCl_3$ test (+); UV ν_{max}^{EtOH} nm (ε): 332 (24497), 300 (20738), 217 (20134); IR ν_{max}^{KBr} cm^{-1}: 3463 (OH), 1681 (α,β-unsaturated ester); ^{1}H-NMR ($(CD_3)_2CO$) δ: 3.72 (3H, s, $COOC\underline{H}_3$), 6.28 (1H, d, J = 16.0 Hz, Ar–CH = C$\underline{H}$–COO–), 6.8—7.3 (3H, m, aromatic protons), 7.56 (1H, d, J = 16.0 Hz, Ar–C$\underline{H}$ = CH–COO–), 8.22 (2H, s, 2 × O$\underline{H}$); MS (*m/e*): 194 (M^+), 163 ($M^+ - OCH_3$), 135 ($M^+ - OCH_3$–CO); m-MS *m/e*: Calcd for $C_{10}H_{10}O_4$, 194.187, Found 194.057.

Methyl 3,4-Diacetoxy-*trans*-cinnamate (3,4-Diacetoxy Caffeate)——I (194 mg) was treated with acetic anhydride–pyridine in the usual manner. Recrystallization from H_2O–MeOH afforded the diacetate (II) quantitatively (colorless leaflets). mp 79.5—81 °C; IR ν_{max}^{KBr} cm^{-1}: 1776 ($OCOCH_3$), 1710 (–CH = CH–COO–); ^{1}H-NMR ($CDCl_3$) δ: 2.30 (6H, s, 2 × $OCOC\underline{H}_3$), 3.80 (3H, s, $COOC\underline{H}_3$), 6.60 (1H, d, J = 16 Hz, Ar–CH = C$\underline{H}$–COO–), 7.1—7.5 (3H, s, aromatic protons), 7.66 (1H, d, J = 16 Hz, Ar–C$\underline{H}$ = CH–COO–).

The sample was identified as 3,4-diacetoxy-*trans*-cinnamic acid methyl ester by comparison (mixed mp and IR) with an authentic specimen prepared from 3,4-dihydroxycaffeic acid by acetylation followed by esterification with CH_2N_2, as usual.

Ozonolysis of II——A solution of II (110 mg) in AcOEt (30 ml) was ozonized at −10——−15 °C for 1 h. After evaporation of the solvent *in vacuo*, the residue (200 mg) was dissolved in acetone (15 ml), and Jones reagent (1 ml) was added under ice-cooling. The resulting mixture was stirred at room temperature for 20 min, poured into ice-water and extracted with CH_2Cl_2. The CH_2Cl_2 solution was extracted with 5% $NaHCO_3$ and the alkaline layer was neutralized with 35% HCl, then extracted with CH_2Cl_2. The CH_2Cl_2 solution was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give a colorless powder, 35 mg (37.3%). Recrystallization from ether afforded

3,4-diacetoxybenzoic acid (III), mp 155—156 °C as colorless prisms; IR ν_{max}^{KBr} cm^{-1}: 1772 ($OCOCH_3$), 1687 (COOH), ^{1}H-NMR ($CDCl_3$) δ: 2.17 (6H, s, $2\times OCOC\underline{H}_3$), 7.19 (1H, d, J=4.2 Hz, aromatic proton), 7.59—7.88 (1H, S_{br}, $COO\underline{H}$), 7.8—8.2 (2H, m, aromatic protons). III was identical with authentic 2,3-diacetoxybenzoic acid (mixed mp and IR comparison).

Antitumor Activity Assay——a) Chemicals: The synthetic catechols related to methyl caffeate were prepared as indicated above and used for assay of antitumor activity as described below. They are listed in Tables II (cinnamic acid type), III (benzoic acid type) and IV (benzaldehyde type).

b) Animals: Male ICR mice, weighing 20—25 g, were purchased from the Shizuoka Agricultural Cooperative for Experimental Animals, Hamamatsu. They were housed in plastic cages (five to a cage), kept in an air-conditioned room, and given food and water *ad libitum*. Each experimental group consisted of 10 mice.

c) Tumor: Sarcoma 180 was maintained in the peritoneal cavities of ICR mice by weekly sequential transplantation. The tumor cells were harvested from the peritoneal cavity and adjusted to the required concentrations in sterile physiological saline.

d) Assay of Antitumor Activity: Tumor cells were inoculated subcutaneously ($1—2\times10^6$ cells/mouse) into the left groin of ICR mice on day 0, and then a test substance dissolved or suspended in carboxymethyl cellulose in saline was administered intraperitoneally on days +1 to +10 at a total dose of 100 mg/kg. The mice were killed 21 d after the tumor inoculation and the tumor weight was assessed. Saline alone was given to control mice. The inhibition of tumor growth was calculated by means of the following formula:

$$\text{inhibition (\%)} = \left(1 - \frac{\text{mean tumor weight of treated mice}}{\text{mean tumor weight of control mice}}\right) \times 100$$

Antimicrobial Activity Assay——The *in vitro* antimicrobial activity testing was performed by the conventional screening method recommended by the Chemotherapeutic Society of Japan against the organisms described above.

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