

Antioxidative Polyphenols Isolated from *Theobroma cacao*

Chiaki Sanbongi,[†] Naomi Osakabe,^{*,†} Midori Natsume,[†] Toshio Takizawa,[†] Shuichi Gomi,[‡] and Toshihiko Osawa[§]

Functional Food Research and Development Laboratory, Meiji Seika Kaisha, Ltd., 5-3-1 Chiyoda, Sakadoshi, Saitama, Japan; Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama, Kanagawa, Japan; and Department of Applied Biological Science, Nagoya University, Chikusa, Nagoya, Aichi, Japan

The antioxidant components of cacao liquor, which is a major ingredient of chocolate, were isolated with column chromatography and high-performance liquid chromatography. Quercetin and its glucoside were identified by spectrometric methods. Clovamide and deoxyclovamide were characterized by ¹H and ¹³C NMR and MS spectrometry. Their antioxidative activity was measured by peroxide value of linoleic acid and thiobarbituric acid reactive-substance value of erythrocyte ghost membranes and microsomes. In the bulk oil system, clovamide had the strongest antioxidative activity but was less active in the other experiments. In the case of the two hydrophilic systems, flavans such as quercetin and epicatechin were more potently effective than the glucosides. It is considered that chocolate is stable against oxidative deterioration due to the presence of these polyphenolic compounds.

Keywords: *Cacao liquor; polyphenols; antioxidant; catechin; clovamide*

INTRODUCTION

It was reported that unfermented cacao beans, the seeds of *Theobroma cacao*, are rich in polyphenols (Porter et al., 1991). However, in the process of fermentation and roasting to make cacao liquor, which is a major ingredient of chocolate, changes in polyphenols have not been understood well. We reported that cacao liquor extracted with hot water had strong antioxidative activity. It was suggested that the major antioxidative components were epicatechin and catechin, as determined by some models (Osakabe et al., 1997).

In the present work, we confirmed other antioxidative components by ethanol extraction. This paper reports the isolation and structure elucidation of a novel polyphenolic amide together with flavans such as catechins and quercetins from cacao liquor. Their antioxidative activities are estimated in model systems. Structure–activity relationships are also investigated.

MATERIALS AND METHODS

Cacao Liquor. Fermented and dried cacao beans were imported from Ghana and roasted and cracked at Meiji Seika Kaisha Ltd.

Extraction of Polyphenols from Cacao Liquor. One hundred grams of cacao liquor was defatted with *n*-hexane for 30 min twice and extracted with 80% v/v ethanol for 16 h twice. The extract was concentrated in vacuo, charged on a Diaion HP2MG column (Mitsubishi Chemical Co. Ltd., 35 mm ϕ \times 310 mm), and eluted with distilled water with stepwise increases in ratio of ethanol containing 0.5% v/v trifluoroacetic acid. These procedures are outlined in Figure 1.

* Author to whom correspondence should be addressed [telephone (0492) 84-5449; fax (0492) 84-7569; e-mail naomi_osakabe@meiji.co.jp].

[†] Functional Food Research and Development Laboratory.

[‡] Pharmaceutical Research Center.

[§] Department of Applied Biological Science.

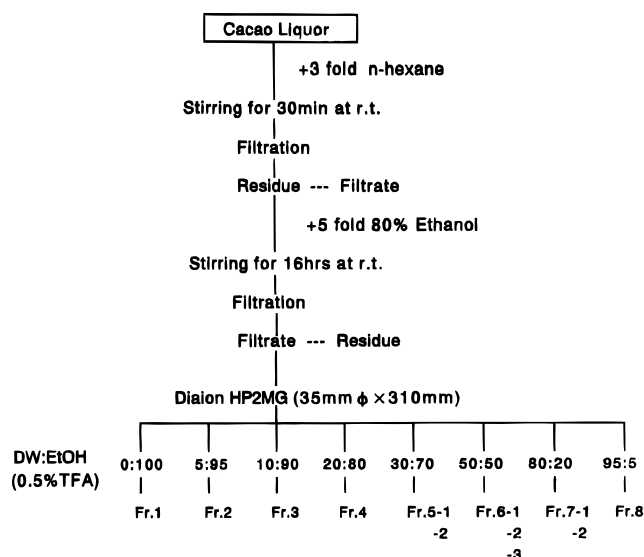


Figure 1. Scheme for isolation of polyphenolic substances from cacao liquor.

High-Performance Liquid Chromatography (HPLC). Further purification of crude polyphenols was carried out by preparative HPLC, using an ODS column (Nomura Chemical Co. Ltd., 20 mm ϕ \times 250 mm). The elution solvent was 0.1% v/v trifluoroacetic acid/40% v/v methanol.

Instruments for Structure Elucidation. ¹H and ¹³C NMR spectra were obtained by a JEOL JNM-JSX 400 spectrometer. Chemical shifts were recorded in δ values using tetramethylsilane (TMS) as an internal standard. MS spectra were obtained by a Hitachi M-80B.

Antioxidative Activity. (1) *Linoleic Acid Oxidation.* Each sample was added at a final concentration of 0.5 mM to 1 g of linoleic acid and incubated at 30 °C; the degree of oxidation was measured by iodometry.

(2) *Erythrocyte Ghost System.* Erythrocyte ghosts were prepared from rat blood cells according to the method of Osawa et al. (1987). The induction of lipid peroxidation was carried

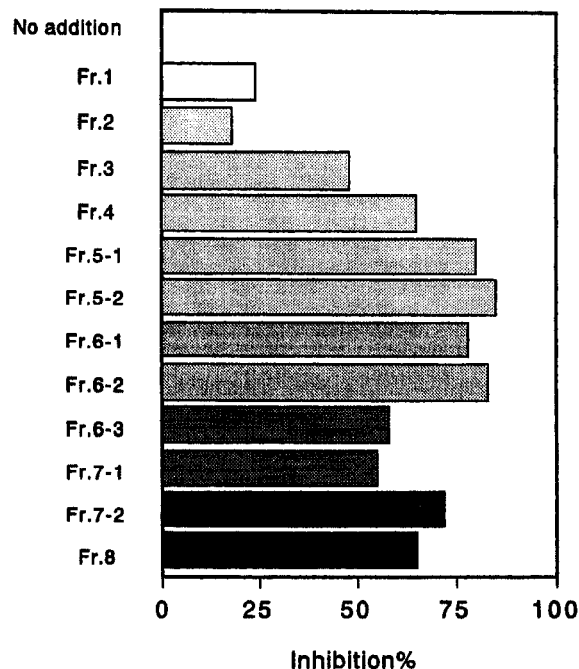


Figure 2. Antioxidative activity of crude fractions from cacao liquor on linoleic acid autoxidation.

out with *tert*-butylhydroxy peroxide (t-BHP) by following the method of Ames et al. (1981). Test chemicals were dissolved with methanol and added. After incubation, thiobarbituric acid-reactive substance (TBARS) was measured by reading its absorbance at 535 nm.

(3) *Microsomal Lipid Peroxidation*. Microsomes were prepared from rat liver. The induction of lipid peroxidation with ADP-Fe²⁺/NADPH or t-BHP was carried out according to the method of Pederson (1973). After incubation, TBARS were measured as described.

RESULTS

Isolation of Polyphenols from Cacao Liquor.

The crude fractions were obtained according to the outline in Figure 1. Each fraction was concentrated in vacuo and freeze-dried. These samples were then added to linoleic acid at a concentration of 100 µg/mL and incubated for 16 h; the peroxide value was then measured by iodometry. As shown in Figure 2, the fractions that are eluted with >20% ethanol had potent antioxidative activity. In particular, fractions 5, 6-1, and 6-2 showed almost 80% inhibition compared to the no-addition group.

An analytical HPLC was done for fraction 5; there were two major peaks detected. These peaks were identified as (+)-catechin and (-)-epicatechin according to mass and NMR data of commercial HPLC grade chemicals (Funakoshi, Japan) (data not shown).

Fraction 6 was similarly analyzed with HPLC; four unidentified peaks were revealed as shown in Figure 3.

Further purification of these peaks was carried out by preparative HPLC, collected individually.

Chemical Structure. *Identification of CA1* [*N*-(3,4'-Dihydroxy-*trans*-cinnamoyl)-3-(3,4-dihydroxyphenyl)-L-alanine]: FD-MS, *m/z* 360 (M + H), 382 (M + Na); ¹H NMR (in DMSO-*d*₆) δ 8.62, 8.67, 9.07, 9.31 (4H, br, -OH), 8.75 (1H, d, *J* = 8.0 Hz, NH), 7.20 (1H, d, *J* = 15.7 Hz, H8), 6.93 (1H, d, *J* = 1.8 Hz, H2), 6.82 (1H, dd, *J* = 8.0, 1.8 Hz, H6), 6.74 (1H, d, *J* = 8.1 Hz, H5), 6.62 (1H, d, *J* = 1.8 Hz, H2'), 6.61 (1H, d, *J* = 7.8 Hz,

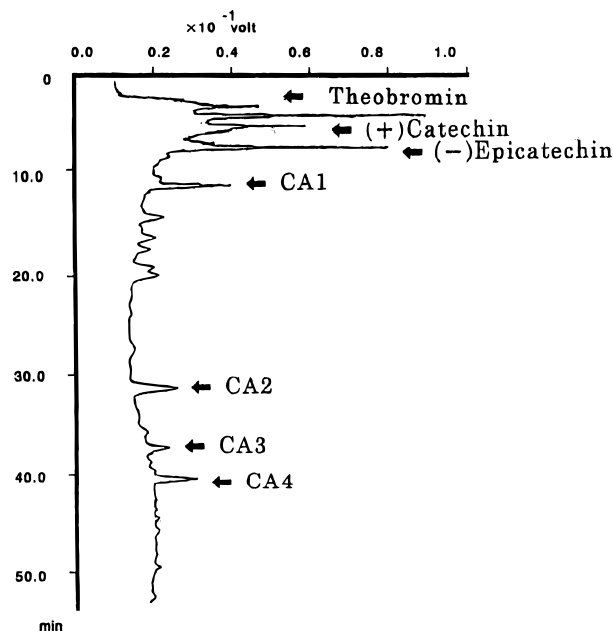


Figure 3. Typical HPLC chromatogram of fraction 6-2.

Table 1. ¹³C NMR Spectra for CA1, CA2, CA3, and CA4

carbon	CA1 ^a	CA2 ^b	carbon	CA3 ^c	CA4 ^c
1	128.1 (s)	127.7 (s)	2	156.1 ^d (s)	156.2 ^d (s)
2	115.8 (d)	130.7 (d)	3	133.3 (s)	133.7 (s)
3	144.9 (s)	116.7 (s)	4	177.4 (s)	177.5 (s)
4	147.2 (s)	160.6 (s)	4a	104.0 (s)	103.9 (s)
5	117.9 (d)	116.7 (d)	5	161.2 (s)	161.2 (s)
6	122.8 (d)	130.7 (d)	6	98.6 (d)	98.6 (d)
7	142.6 (d)	142.4 (d)	7	164.1 (s)	164.1 (s)
8	117.6 (d)	117.9 (d)	8	93.5 (d)	93.5 (d)
9	169.5 (s)	169.0 (s)	8a	156.3 ^d (s)	156.3 ^d (s)
1'	130.1 (s)	129.1 (s)	1'	121.1 (s)	121.1 (s)
2'	117.7 (d)	131.3 (d)	2'	116.2 (s)	115.7 (d)
3'	144.7 (s)	116.2 (s)	3'	144.8 (s)	144.9 (s)
4'	143.7 (s)	157.3 (s)	4'	148.4 (s)	148.5 (s)
5'	117.0 (d)	116.2 (d)	5'	115.2 (d)	115.3 (d)
6'	122.4 (d)	157.3 (d)	6'	121.6 (d)	122.0 (d)
7'	36.9 (t)	37.8 (t)	1''	100.8 (d)	101.4 (d)
8'	55.3 (d)	55.5 (d)	2''	74.1 (d)	70.7 (d)
9'	176.0 (s)	174.9 (s)	3''	76.5 ^d (d)	71.6 (d)
			4''	69.9 (d)	66.0 (d)
			5''	77.5 ^d (d)	64.2 (t)
			6''	60.9 (t)	

^a Parts per million from TMS in D₂O using dioxane at 67.4 ppm.

^b Parts per million from TMS using a center peak of CD₃OD at 49.0 ppm.

^c Parts per million from TMS using a center peak of DMSO-*d*₆ at 39.5 ppm.

^d Interchangeable.

H5'), 6.57 (1H, d, *J* = 7.9, 1.8 Hz, H6'), 6.41 (1H, d, *J* = 15.7 Hz, H7), 4.48 (1H, m, H8'), 2.90 (H1, dd, *J* = 13.7, 4.9 Hz, H7'), 2.72 (H1, dd, *J* = 13.7, 9.1 Hz, H7'); data for the ¹³C NMR spectrum are shown in Table 1. Given these results and considering two-dimensional NMR spectra, HMBC, and decoupling (data not shown), CA1 was identified as *N*-(3',4'-dihydroxy-*trans*-cinnamoyl)-3-(3,4-dihydroxyphenyl)-L-alanine; its trivial name is clovamide (Yoshihara et al., 1974, 1977; Van Heerden et al., 1980; Roblot et al., 1988). The structure and ¹³C NMR spectra (Kelley et al., 1976) are very similar to those of rosmarinic acid, which has strong antioxidative activity (Cuvelier et al., 1992; Frankel et al., 1996). The only significant difference is the amide moiety, but not ester as confirmed by ¹³C NMR spectroscopy (data not shown). This compound was first isolated from red

clover (*Trifolium pratense*), but the antioxidative activity has not yet been reported.

Identification of CA2 [*N*-(4'-Hydroxy-*trans*-cinnamoyl)-3-(4-hydroxyphenyl)-L-alanine]: FD-MS, m/z 328 (M + H), 360 (M + Na); ^1H NMR (in DMSO- d_6) δ 9.19, 8.84 (4H, br, -OH), 8.18 (1H, d, $J = 8.2$ Hz, NH), 7.37 (2H, d, $J = 8.6$ Hz, H2',6'), 7.27 (1H, d, $J = 15.6$ Hz, H8), 7.02 (2H, d, $J = 8.6$ Hz, H2,6), 6.78 (2H, d, $J = 8.6$ Hz, H3',5'), 6.64 (2H, d, $J = 8.2$ Hz, H3,5), 6.48 (1H, d, $J = 15.6$ Hz, H7), 4.46 (1H, m, H8'), 2.97 (H1, dd, $J = 13.7$, 4.7 Hz, H7'), 2.79 (H1, dd, $J = 13.7$, 9.4 Hz, H7'); data for the ^{13}C NMR spectrum are shown in Table 1. Given these results and considering the two-dimensional NMR spectra, HMBC, and decoupling (data not shown), CA2 was identified as *N*-(4'-hydroxy-*trans*-cinnamoyl)-3-(3-hydroxyphenyl)-L-alanine. This compound was identified as a deoxy analogue of the clovamide as a minor metabolite (Van Heerden et al., 1980).

Identification of CA3 [*Quercetin 3-O- β -D-Glucopyranoside*]: FD-MS, m/z 465 (M + H); ^1H NMR δ 12.65 (1H, s, 5-OH), 7.58 (1H, dd, H6'), 7.57 (1H, d, H2'), 6.84 (1H, d, $J = 9.0$ Hz, H5'), 6.40 (1H, d, $J = 2.0$ Hz, H8), 6.20 (1H, d, $J = 2.0$ Hz, H6), 5.46 (1H, d, $J = 7.4$ Hz, G1), 3.58 (1H, m, G6), 3.32 (1H, m, G6), 3.23 (2H, m, G2, G3), 3.09 (2H, m, G4, G5); data for the ^{13}C NMR spectrum are shown in Table 1. These data were identical with those of an authentic sample of quercetin 3-*O*- β -D-glucopyranoside (Markham et al., 1978, 1987).

Identification of CA4 [*Quercetin 3-O- α -D-Arabinopyranoside*]: FD-MS, m/z 435 (M + H); ^1H NMR δ 12.65 (1H, s, 5-OH), 7.66 (1H, dd, $J = 2.3$, 8.0 Hz, H6'), 7.51 (1H, d, $J = 2.3$ Hz, H2'), 6.84 (1H, d, $J = 8.6$ Hz, H5'), 6.40 (1H, d, $J = 2.3$ Hz, H8), 6.20 (1H, d, $J = 2.0$ Hz, H6), 5.28 (1H, d, $J = 5.1$ Hz, G1), 3.75 (1H, dd, $J = 5.1$, 7.0 Hz, G6), 3.66 (1H, m, G4), 3.60 (1H, d, $J = 5.5$, 11.3 Hz, G5), 3.51 (1H, dd, $J = 3.1$, 7.0 Hz, G3), 3.21 (2H, dd, $J = 2.3$, 11.3 Hz, G5); data for the ^{13}C NMR spectrum are shown in Table 1. Given these results and considering the two-dimensional NMR spectra and HMBC, CA4 was identified as quercetin 3-*O*- α -D-arabinopyranoside (Markham et al., 1978, 1987).

Antioxidative Activity of Isolated Compounds.

Antioxidative activities of isolated compounds of CA1, CA2, CA3, and CA4 were measured using in vitro assay systems together with epicatechin (Sigma Chemical Co. Ltd.), catechin (Sigma), and quercetin (Extrasynthase S.A.) as follows.

(1) *Linoleic Acid Oxidation*. Clovamide showed a further prolonged induction period compared with linoleic acid alone as shown in Figure 5. Considering the strong antioxidative activity of structurally similar rosemarinic acid, these results are reasonable. Antioxidative activity was strong in the following order: clovamide > epicatechin > catechin > quercetin > quercetin 3-glucoside, quercetin 3-arabinoside, dideoxy-clovamide.

(2) *Erythrocyte Ghost System*. As shown in Figure 6, clovamide, epicatechin, catechin, and quercetin had almost similar inhibitory effects. The quercetin 3-glucoside appears to be only slightly less effective vs the first four cited compounds. Quercetin 3-arabinoside and dideoxyclovamide were less effective in the erythrocyte ghost system.

(3) *Effect on Microsomal Oxidation*. Figure 7 shows the antioxidative activity of isolated compounds, using rat liver microsomal oxidation induced by ADP- Fe^{2+} /NADPH or t-BHP. In this experiment, flavans such as

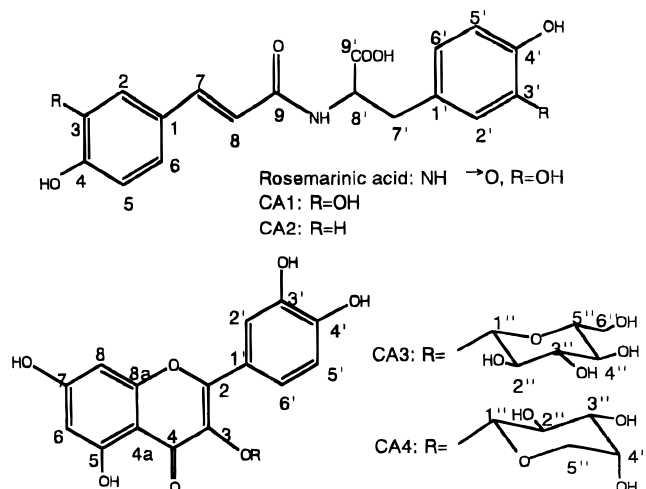


Figure 4. Chemical structures of antioxidative compounds isolated from cacao liquor.

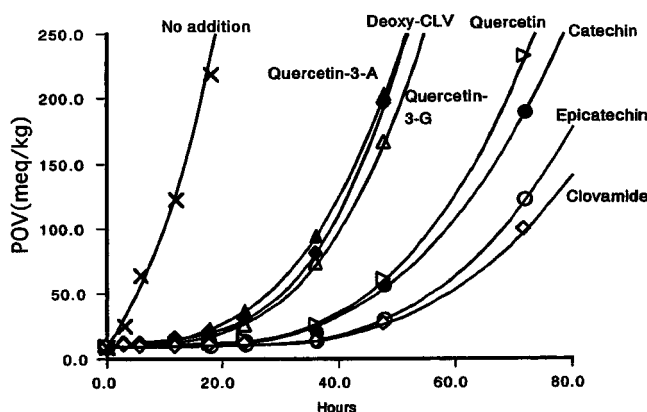


Figure 5. Effects of polyphenolic substances from cacao liquor on linoleic acid autoxidation.

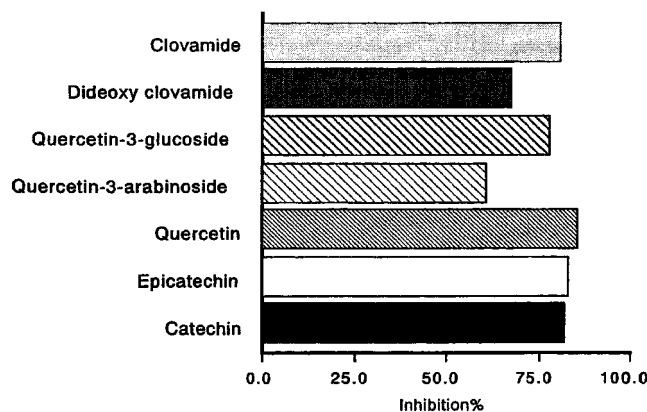


Figure 6. Effects of polyphenolic substances from cacao liquor on RBC ghost oxidation induced by t-BHP.

quercetin and epicatechin had a potent antioxidative activity. Approximately similar results were shown in the case of NADPH/ADP-dependent and t-BHP-induced oxidation. It is suggested that the antioxidative component mechanism using this study is not attributed to chelating ability.

DISCUSSION

Antioxidative fractions were obtained using column chromatography with 80% ethanol extraction of cacao liquor; it was verified that antioxidants other than

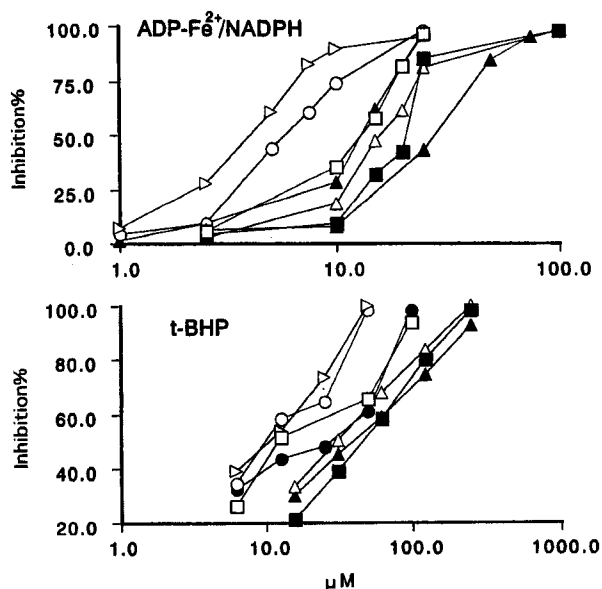


Figure 7. Effects of polyphenolic substances from cacao liquor on ADP-Fe²⁺/NADPH-dependent or t-BHP-induced oxidation of rat microsomes.

catechin and epicatechin exist in these fractions. Some of these antioxidants were characterized as quercetin, quercetin 3-glycoside, and quercetin 3-arabinside by using spectroscopic methods. Clovamide and deoxyclovamide identified in this study have caffeoyl and coumaroyl moieties in their structures, respectively. Clovamide, because of having a catechol part, exhibited stronger antioxidative activities in all systems compared to dideoxyclovamide. In a previous paper, such a structure-activity relationship was shown between caffeic acid and coumaric acid (Fereidoon et al., 1992). It is considered that the different antioxidative activities shown by these two hydroxycinnamic acids are due to the hydroxy group at the benzene rings of the chemical structure.

In the case of the hydrophilic system, such as using erythrocyte ghost and microsome fractions, quercetin had stronger antioxidative activities than its glycosides, quercetin 3-glucoside and quercetin 3-arabinside. In the bulk oil system, such as linoleic acid autoxidation, clovamide had the strongest activity but was less active in other experiments. Similar results were obtained for rosmarinic acid (Frankel et al., 1996).

As unfermented cacao beans have high molecular polyphenols, such as polymeric procyanidin (Porter et al., 1991), further studies to investigate other antioxidative compounds in CLP are in progress.

ABBREVIATIONS USED

CLP, cacao liquor crude polyphenols; HPLC, high-performance liquid chromatography; MS, mass spec-

trometry; NMR, nuclear magnetic resonance; t-BHP, *tert*-butylhydroxy peroxide; TBARS, thiobarbituric acid-reactive substance; TMS, tetramethylsilane.

LITERATURE CITED

- Ames, B. N.; Cathcart, R.; Schwiers, E.; Hochstein, P. Uric acid provides an antioxidant defense in humans against oxidant- and radical-caused aging and cancer: A hypothesis. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78* (11), 6858-6862.
- Cuvelier, M. E.; Richard, H.; Berset, C. Comparison of the antioxidative activity of some acid-phenols: structure-activity relationship. *Biosci., Biotechnol., Biochem.* **1992**, *56* (2), 324-325.
- Fereidoon, S.; Wanasundara, P. K. J. P. D.; Hong, C. Antioxidant activity of phenolic compounds in meat model system. *ACS Symp. Ser.* **1992**, *506*, 214-222.
- Frankel, E. N.; Huang, S. W.; Aeschbach, R.; Prior, E. Antioxidant activity of a rosemary extract and its constituents, carsonic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *J. Agric. Food Chem.* **1996**, *44*, 131-135.
- Kelley, C. J.; Harruff, R. C.; Carmack, M. The polyphenolic acid of *lithospermum ruderale* II. *J. Org. Chem.* **1976**, *41*, 449-455.
- Markham, K. R.; Ternai, B.; Stanley, R.; Geiger, H.; Mabry, T. J. Carbon-13 NMR studies of flavonoids-III. *Tetrahedron* **1978**, *34*, 1389-1397.
- Markham, K. R.; Whitehouse, L. A.; Webby, R. F. Luteolin 7-*O*-[6-*O*-1-arabinofuranosyl]-*D*-glucopyranoside and other new flavonoid glycosides from New Zealand *Dacrydium* species. *J. Nat. Prod.* **1987**, *50* (4), 660-663.
- Osakabe, N.; Yamagish, M.; Sanbongi, C.; Natsume, M.; Takizawa, T.; Osawa, T. The antioxidant substances in cacao liquor. *J. Nutr. Sci. Vitaminol.* **1997**, in press.
- Osawa, T.; Ide, A.; Su, J. D.; Namiki, M. Inhibition of lipid peroxidation by ellagic acid. *J. Agric. Food Chem.* **1987**, *35*, 808-812.
- Pederson, T. C.; Buege, J. A.; Aust, S. D. Microsomal electron transport. *J. Biol. Chem.* **1973**, *25*, 7134-7141.
- Porter, L. J.; Ma, Z.; Chan, B. G. Cacao procyanidines: Major flavanoids and identification of some minor metabolites. *Phytochemistry* **1991**, *30*, 1657-1663.
- Roblot, G.; Wylde, R. Nouvelle synthese du trans(-)clovamide. *J. Nat. Prod.* **1988**, *51* (3), 596-598.
- Van Heerden, F. R.; Brandt, E. V.; Roux, D. G. Isolation and synthesis of *trans*- and *cis*-(-)-clovamides and their deoxy analogues from the bark of *Dalbergia melanoxylon*. *Phytochemistry* **1980**, *19*, 2125-2129.
- Yoshihara, T.; Yoshikawa, H.; Sakamura, S.; Sakuma, T. Clovamides; l-Dopa conjugated with *trans*- and *cis*-caffeic acids in red clover. *Agric. Biol. Chem.* **1974**, *38*, 1107-1109.
- Yoshihara, T.; Yoshikawa, H.; Kunimatsu, S.; Sakamura, S.; Sakuma, T. New amino acid derivatives conjugated with caffeic acid and DOPA from red clover. *Agric. Biol. Chem.* **1977**, *41*, 1679-1684.

Received for review July 7, 1997. Revised manuscript received November 24, 1997. Accepted November 25, 1997.

JF970575O