Isolation and Identification of Novel Tocotrienols from Rice Bran with Hypocholesterolemic, Antioxidant, and Antitumor Properties

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Two novel tocotrienols were isolated from stabilized and heated rice bran, apart from the known α-, β-, γ-, and δ-tocopherols and tocotrienols. These new tocotrienols were separated by HPLC, using a normal phase silica column. Their structures were determined by ultraviolet, infrared, nuclear magnetic resonance, circular dichroism, and high-resolution mass spectroscopies and established as desmethyl tocotrienol [3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol] and didesmethy tocotrienol [3,4-dihydro-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol]. These tocotrienols significantly lowered serum total and LDL cholesterol levels and inhibited HMG-CoA reductase activity in chickens. They had much greater in vitro antioxidant activities and greater suppression of B16 melanoma cell proliferation than α-tocopherol and known tocotrienols. Results indicated that the number and position of methyl substituents in tocotrienols affect their hypocholesterolemic, antioxidant, and antitumor properties.

Keywords: *Stabilized and heated rice bran; novel tocotrienols; hypocholesterolemic; antioxidant and antitumor activities*

INTRODUCTION

The role of dietary plant constituents in the prevention and treatment of a wide variety of diseases and conditions has been established (National Research Council, 1989). For instance, α-tocotrienol isolated from barley extract effectively lowers serum cholesterol levels in various animal models and humans (Qureshi et al., 1986, 1997). Plant oils are common sources of tocols (tocopherols and tocotrienols). Some common cereals and legumes, such as corn, wheat, soybean, and peanut, contain predominantly tocopherols (vitamin E), whereas others, such as palm, rice bran, oat, and barley, contain mostly tocotrienols. These homologous forms of tocopherols and tocotrienols can be distinguished by the number and location of methyl groups on their chroman rings, which influences their biological activities (Qureshi and Qureshi, 1993). δ-Tocotrienol (8-methyl) is the most potent cholesterol inhibitor among the known tocotrienols, followed by γ-tocotrienol (7,8-dimethyl) and α-tocotrienol (5,7,8-trimethyl). These tocotrienols inhibit hepatic β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase (the rate-limiting enzyme for cholesterol synthesis) activity, whereas α-tocopherol increases HMG-CoA reductase activity without affecting serum cholesterol levels (Qureshi et al., 1996a).

Biological activities of tocols are generally believed to be due to their antioxidant action by inhibiting lipid peroxidation in biological membranes. α-Tocopherol has been labeled as the most efficient antioxidant for breaking free radical-driven chain reactions. However, recent results by a chemiluminescence assay in a liposome membrane system indicate that α-tocotrienol is at least 3-fold more efficient as a scavenger of peroxyl radicals than is α-tocopherol (Packer, 1995). Furthermore, the tocotrienols differ substantially in their capacity to suppress tumor cell proliferation (He et al., 1997). Tumor suppressing activities of tocotrienols lacking the 5-methyl substituent (γ- and δ-tocotrienols) are significantly greater than that of α-tocotrienol (Qureshi et al., 1999; He et al., 1997).

Rice bran, a byproduct of the rice milling process, constitutes about 10 wt % of rough rice grain and contains 18−22% oil, making it the richest oil source from a grain byproduct. The hypocholesterolemic activity of rice bran has been attributed to the presence of γ-oryzanols (ferulate esters of triterpene alcohols) and plant sterols (Sheetharamaiah and Chandrasekhara, 1988; Yoshino et al., 1989; Nicolosi et al., 1991; Rogers et al., 1993). Commercial rice bran and its oil also contain 95−125 and 750−830 mg/kg tocols, respectively. An endogenous lipase activity is activated by milling, resulting in rapid deterioration of the oil and rendering it unsuitable for human consumption. Lipase is inactivated by heating the rice bran at 130 °C for 90 s, which yields stabilized rice bran. Stabilized rice bran is a source of a stable food grade oil that also contains new tocols as well as known tocols in much higher concentrations than found in unheated rice bran (Qureshi et

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al., 1999). High-performance liquid chromatography (HPLC) analysis of methanol extracts of stabilized rice bran showed the presence of known tocols. Two additional peaks that eluted after the known tocols aroused our interest and caused us to explore the possibility that these might contain fewer methyl groups and may therefore have greater biological activities than the known tocotrienols. The present paper describes the isolation, structural identification, and biological activities of these peaks, named *d*-P_{21}-T3 (desmethyl tocotrienol) and *d*-P_{25}-T3 (didesmethyl tocotrienol), from stabilized rice bran. We discovered that these novel tocotrienols have superior efficacy with regard to cholesterol-lowering, antioxidant, and antitumor properties in comparison to the other known tocotrienols.

MATERIALS AND METHODS

Sources of chemicals, substrates, labeled substrates, enzymes, and diagnostic kits were identified previously (Qureshi et al., 1986, 1997). Aquasol scintillation solution and dl-β-hydroxy-β-methyl-[3-^{14}C]glutaryl-CoA were purchased from New England Nuclear, Boston, MA. Chemicals and solvents were of analytical grade. Stabilized rice bran (L-487, ML-63, Morris and Texamati; heat treated at 130 °C for 90 s) was supplied by Riviana Foods Inc., Abbeville, LA, and kernels of several other rice cultivars were obtained from USDA, ARS, Beaumont, TX. All the samples were ground with a UDY Cyclone Sample Mill. The samples were heated (160 °C) in a Precision vacuum oven connected with a water aspirator. A tocotrienol-rich fraction (TRF) from palm oil was obtained from Dr. Hj. Yusof Basiron (DG), Palm Oil Research Institute of Malaysia. The HPLC normal phase silica, Bond Elut amine, and Chiralpak-AD columns were obtained from Waters Corp. (Milford, MA), Analytical International (Harbor City, CA), and Chiral Technologies, Inc. (Exton, PA), respectively.

The mass spectral data were acquired using a model MS-902 manufactured by Associate Electrical Industries Ltd. (Manchester, U.K.). NMR ($CDCl_3$) spectra were recorded on a Bruker AM 300 spectrometer (Bruker Inc. Billerica, MA) at room temperature. IR spectra were done as thin film using a Perkin-Elmer instrument (Norwalk, CT). The mass spectra, NMR, CD (V-62DS, Aviv Inc., Jersey City, NJ) and IR were carried out in the Departments of Chemistry and Biochemistry, University of Wisconsin, Madison, WI.

Isolation and Analysis of Tocols. Stabilized rice brans (1 g) were extracted with 8 mL of hexane by shaking for 30 min at room temperature and centrifuged at 8800*g*. The hexane-soluble fraction (supernatant) was decanted and evaporated under vacuum at 40 °C in a Vortex evaporator. This dried hexane extract after solvent evaporation was redissolved in 4 mL of hexane and analyzed for tocol content by analytical HPLC as described in detail for cereal and serum samples (Lehmann et al., 1994; Qureshi et al., 1997). The remaining rice bran solid residue from the original extraction was heated at 160 °C under vacuum (1550mmHg) for 60 min and then extracted with 6 mL of methanol by shaking for 30 min at room temperature. After centrifugation at 15000*g*, the supernatant was decanted and removed under vacuum at 40 °C. The residue left after the solvent evaporation was redissolved in 2 mL of hexane and analyzed by HPLC. A preliminary identification of the peaks was done by comparison with pure standards obtained from Dr. B. C. Pearce (Pearce et al., 1992), and by running stop-flow spectra of each peak between 240 and 440 nm with a UV−visible LC-75 spectrometric detector (Perkin-Elmer, EG & G, Wellesley, MA) or 220−370 nm with an SPD-10 AV, UV−visible HPLC spectrometric detector (Shimadzu, Columbia, MD). Two peaks eluted that were not known tocols: one at 20 min with this analysis system is called P_{20} and was determined to be γ-oryzanols. Another at 25 min is called TRF_{25} (tocotrienol-rich fraction) and shown to be a mixture of the novel tocols, desmethyl- and didesmethyl tocotrienol (see below in Results).

Purification of Novel Tocotrienols by Bond Elut Amine Column. Stabilized and heated rice bran (10 g) was extracted with methanol (100 mL), and the solvent was evaporated as described above but without prior hexane extraction. The methanol-soluble material was dissolved (10 mg/0.5 mL) in hexane and loaded on a Bond Elut amine column (1.0 mL), which was initially washed with 2 mL of methanol and 2 mL of hexane. After loading the sample, the column was then eluted with 1 mL of hexane, followed by 1 mL of 3% (v/v) 2-propanol in hexane. This step removed most of the known tocols. The column was successively eluted with 1 mL each of 5%, 6%, and 10% (v/v) 2-propanol in hexane. The composition of each fraction was determined by HPLC (Waters silica column, 1% (v/v) 2-propanol in hexane mobile phase). The fractions that were eluted from the Bond Elut amine column with 5% and 6% 2-propanol in hexane were combined, evaporated under vacuum at 40 °C, and redissolved in hexane. Compounds in this combined fraction were purified by preparative HPLC on a Beckman Ultrasphere Silica, 5 μm, 4.6 × 250 mm column, using 0.6% (v/v) 2-propanol in hexane as the mobile phase and a 1.3 mL/min flow rate. Peaks were detected by UV (295 nm) and fluorescence (excitation, 295; emission, 330 nm). The peaks were collected and further purified by analytical HPLC. The purity of these compounds was verified by observing a single symmetrical peak by fluorescence and a UV maximum absorption at 300 nm prior to carrying out mass spectral analyses.

Purification of Novel Tocotrienols Using a Chiralpak-AD Column. Two fractions were analyzed: (i) the fraction that eluted from the Bond Elut amine column with 5% 2-propanol and (ii) the peak that eluted after δ-tocotrienol with analytical HPLC. Each was dried under vacuum at 40 °C, redissolved in hexane, and analyzed by HPLC on a Chiralpak-AD column (10 μm, 4.6 × 250 mm). This column separates stereoisomers. The column was eluted with 3.0% (v/v) 2-propanol in hexane with a flow rate of 1.0 mL/min. Two peaks, identified as *d*-P_{21}-T3 and *d*-P_{25}-T3, were detected by fluorescence, collected, and rerun under the same conditions.

l-P_{21}-T3 and *l*-P_{25}-T3 were purified from synthetic *d*,*l*-P_{21}-T3 and *d*,*l*-P_{25}-T3 (provided by Dr. B. C. Pearce of Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, CT) on the Chiralpak-AD using the conditions described above. The two peaks purified by the Chiralpak-AD column were collected and analyzed by their mass spectra. Moreover, these two purified *d*-isomers from rice bran and the *l*-isomers of synthetic *d*,*l*-P_{21}-T3 and *d*,*l*-P_{25}-T3, purified by this column, were further characterized by circular dichroism analyses.

Preparative Purification of *d*-P_{21}-T3 and *d*-P_{25}-T3 by Flash Chromatography. Large quantities of *d*-P_{21}-T3 and *d*-P_{25}-T3 tocotrienols were prepared by flash chromatography. Stabilized rice bran (L-482 or Morris) was heated and then extracted in two 10-kg batches with 20 L of methanol at room temperature. The combined extracts were evaporated under vacuum at 50 °C, yielding a viscous oily residue containing tocols, polyphenols, γ-oryzanols, sterols, fatty acid esters, and triglycerides (587 g). The oily residue was extracted with hexane and left overnight at 0 °C to remove some of the sterols and triglycerides as precipitates. The hexane-soluble fraction was filtered and evaporated under vacuum at 50 °C, yielding 515 g of a mixture of tocols, γ-oryzanols, and some low molecular weight polyphenols.

Silica gel (Merck, 230−400 mesh, 60 Å, 500 g) suspended in 1000 mL of hexane was poured into a 1-L glass funnel with a fritted disk. The gel was washed with 2 L of hexane prior to being loaded with 100 g of the above material in 200 mL of hexane. The tocols were eluted with sequential application of 1-L mixtures of diethyl ether (5%, 10%, 12%, 14%, 16%, 18%, 20%, 22%, 25%, and 30% v/v) in hexane. This column (funnel) procedure was repeated five times using the same column packing. The absorbent was regenerated each time by washing with 600 mL of methanol, followed by 600 mL of ether, and then with 1 L of hexane. The eluates were dried under vacuum (40 °C), and the residues were redissolved in hexane and identified according to the retention time and absorption

profile using an analytical HPLC system as described earlier (Qureshi et al., 1997).

A fraction rich in tocotrienols with a very small level of α-tocopherol (6%) and free of γ-oryzanols was prepared from 500 g of a methanol extract of stabilized and heated rice bran by using the same procedure as described above except that, after removing most of the α-tocopherol with 10% (v/v) ether in hexane, the tocotrienol-rich fraction was eluted from the silica with 2 L of 30% ether. The eluate was evaporated under vacuum at 50 °C and yielded 9.2 g of tocols, free of γ-oryzanols.

Cholesterol-Related Enzyme Activities of Isolated Hepatocytes as Affected by Tocotrienols and γ-Oryzanols. Female white Leghorn chickens ($n = 10$) were housed at the University of Wisconsin Poultry Research Laboratory and fed a standard corn−soybean diet (protein 20.6%; metabolizable energy 3013 kcal/kg; Qureshi et al., 1996a). At 10 weeks of age, the chickens were fasted for 40 h and re-fed for 48 h before sacrifice. The hepatocytes were prepared following a standard procedure (Qureshi et al., 1986). The hepatocytes were incubated with various concentrations of α-tocotrienol, *d*-P_{21}-T3, *d*-P_{25}-T3, or P_{20} (γ-oryzanols) and assayed for activities of HMG-CoA reductase, fatty acid synthetase, and cholesterol 7α-hydroxylase as previously described (Philipp and Shapiro, 1979; Fitch et al., 1989).

Effects of Various Tocotrienols on Lipid Parameters in 6-Week-Old Male Chickens. The protocol was reviewed and approved by the University of Wisconsin−Madison College of Agriculture and Life Sciences Animal Care Committee. Eight groups ($n = 8$) of 2-week-old white Leghorn male chickens were fed the standard corn-soybean control diet (as described above), supplemented with 50 mg/kg of either TRF_{25}, α-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol, δ-tocotrienol, *d*-P_{21}-T3, or *d*-P_{25}-T3. A ninth group (unsupplemented) served as a control. All diets were isonitrogenous and isocaloric as described above. Each group was housed in a single brooder with continuous light and free access to water and diet. Following a 24-d feeding period, the birds were deprived of food for 40 h, re-fed for 48 h, and again deprived of food for 10 h before being killed by severing the carotid artery. The food deprivation and re-feeding segment of this regimen induces hepatic HMG-CoA reductase activity, and the terminal food deprivation facilitates chylomicron and VLDL clearance. The blood and liver were collected for analysis. The collected blood was incubated at 37 °C for 30 min and centrifuged at 8800*g* for 15 min, and the clear upper serum layer was removed. The livers were rinsed with saline, weighed, and homogenized, and the microsomal fraction was prepared by centrifugation as previously described (Qureshi et al., 1986). Serum and microsomal fractions were held at −20 °C for analysis. Serum total cholesterol and HDL and LDL cholesterol levels were determined by using a diagnostic kit. (Sigma Chemical Co., St. Louis, MO, Catalog No. 352) as described earlier (Qureshi et al., 1996a,b). Microsomal fractions were assayed in triplicate for HMG-CoA reductase activity, as previously described (Philipp and Shapiro, 1979; Assman et al., 1983).

Expression of Data and Statistical Anslysis. Enzyme data are presented as specific activities. StatView software (Abacus Concepts, Berkeley, CA) was used for the analysis of treatment-mediated effects. Treatment-mediated differences in HMG-CoA reductase activity and serum lipid parameters were identified with ANOVA and when the *F* test indicated a significant effect, the differences between the means were analyzed by a Fisher's Protected Least Significant Difference (LSD) test (Abacus Concepts, 1992). Differences were considered significant at $P < 0.05$.

Estimation of Antioxidant Activities of Tocols. Antioxidant activities of tocols were determined in a dipalmitoleylphosphatidyl choline liposome system. The generation of peroxyl radicals in this system was induced by 2,2-azobis(2,4-dimethylvaleronitrile) and was detected by monitoring the chemiluminescence of luminol at various concentrations as described earlier (Packer, 1995). The dose-dependent (5−200 μM) antioxidant activities of α-tocopherol; *d*-α-, *d*-γ-, and *d*-δ-tocotrienols; TRF (48% α-tocopherol); TRF (35% α-tocopherol); and TRF_{25} (6% α-tocopherol) from rice brans were also determined by measuring the coupled autoxidation of β-carotene and linoleic acid according to the reported method (Emmons et al., 1999). The 50% quenching concentrations (K_{50}) were then determined for tocopherol, tocotrienols, and various TRF.

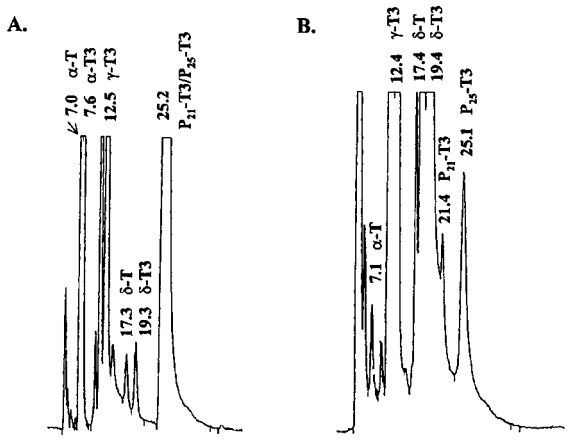


Figure 1. HPLC analysis of extracts from stabilized rice bran. Normal phase, 5 μm, 300 mm × 3.9 mm, silica column; mobile phase, 0.2% 2-propanol in hexane; loop size, 20 μL; flow rate, 1.3 mL/min; fluorescence detection, 295 nm excitation and 330 nm emission. (A) Hexane extract. (B) Methanol extract of heated (160 °C for 60 min under vacuum) residue from hexane extraction.

Estimation of Cell Proliferation Suppressive Activities of Novel Tocotrienols. The assays were carried out to determine the effects of *d*-P_{21}-T3 and *d*-P_{25}-T3 tocotrienols, TRF_{25}, and various TRF from other sources on proliferation of murine B16 melanoma cells (He et al., 1997). The IC_{50} concentrations that suppressed the increase in the cell population by 50% during a 48-h incubation period are reported.

RESULTS

Isolation of Novel Tocotrienols by HPLC from Stabilized and Heated Rice Bran. The HPLC elution profiles of the hexane extract of stabilized rice bran (L-487) showed the presence of α-tocopherol, α-, β-, γ-, and δ-tocotrienols, and δ-tocopherol (Figure 1A). Apart from these tocols, there was a major component that eluted at 25.2 min. This peak was later determined to be a mixture of *d*-P_{21}-T3 and *d*-P_{25}-T3. The yield of total tocols was 286 mg/kg. When the remaining solid residue after hexane extraction was heated under vacuum at 160 °C for 60 min and extracted with methanol, additional tocols were extracted (476 mg/kg). This methanol extract of stabilized, heated rice bran (Figure 1B) showed two peaks at 21.4 (*d*-P_{21}-T3) and 25.1 min (*d*-P_{25}-T3) in addition to α-tocopherol, α-, γ-, and δ-tocotrienol, and δ-tocopherol. If samples of rice bran were heated to above 160 °C under vacuum, two additional peaks appeared, one that eluted just after α-tocotrienol and the other that eluted after *d*-P_{21}-T3/*d*-P_{25}-T3. Both of them have a maximum UV absorption at 266 nm, which indicates that they are degradation products of ferulic acid (results not shown).

The separation of *d*-P_{21}-T3 and *d*-P_{25}-T3 depends on their concentrations in the bran extract. These compounds often appeared as a single peak, particularly when eluted from the 10-μm silica column; they were separated on the 5-μm column only when injected at low concentrations. HPLC analyses of several other rice

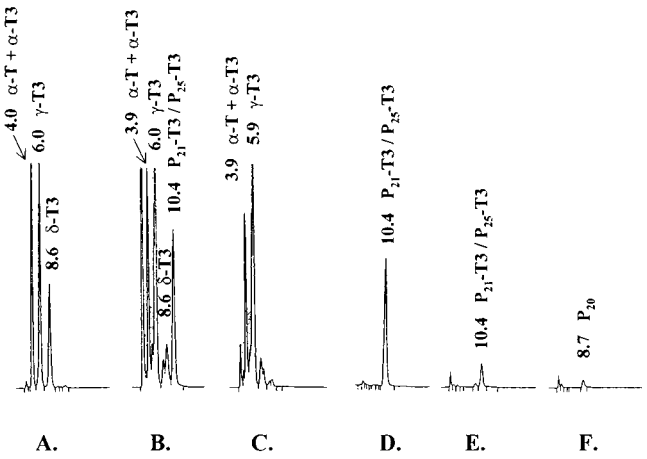


Figure 2. HPLC analysis of fractions eluted from a Bond Elut amine column**.** Normal phase silica column; mobile phase, 1.0% 2-propanol in hexane; fluorescence detection. (A) Standard TRF from palm oil. (B) Methanol-soluble fraction of stabilized rice bran. (C) Fraction eluted by 3% 2-propanol. (D) Fraction eluted by 5% 2-propanol. (E) Fraction eluted with 6% 2-propanol.

cultivars (at least 28) showed the presence of small concentrations of *d*-P_{21}-T3 and *d*-P_{25}-T3 in various ratios (data not shown). In subsequent experiments, the stabilized rice bran was heated and extracted with methanol without the prior hexane extraction to recover all the tocols (TRF_{25}) in one step.

Purification of Novel Tocotrienols from TRF_{25} with a Bond Elut Amine Column. From TRF_{25} (methanol-soluble fraction), *d*-P_{21}-T3 and *d*-P_{25}-T3 were purified from other components by elution from a Bond Elut amine column with 5% 2-propanol in hexane (Figure 2D). Additional *d*-P_{21}-T3 plus *d*-P_{25}-T3 and a small amount of P_{20} (γ-oryzanols) were eluted by 6% 2-propanol in hexane (Figure 2E). The fractions from the Bond Elut amine column were analyzed by HPLC using a mobile phase of 1% 2-propanol in hexane, which eluted all the tocols, including *d*-P_{21}-T3 and *d*-P_{25}-T3, within 11 min (Figure 2B) as compared to 25 min with 0.2% propanol (Figure 1) and did not separate α-tocopherol and α-tocotrienol.

The HPLC (mobile phase 0.6% 2-propanol in hexane) elution profile of the fractions eluted from the Bond Elut amine column with 5% and 6% 2-propanol in hexane was also detected by UV at 295 nm (Figure 3). A very large peak was eluted at 13.9 min, which has the characteristic UV spectrum of γ-oryzanols with maximum absorption at 315 nm and a shoulder at 295 nm. There were two very small peaks that eluted at 16.8 and 18.7 min showing the characteristic UV spectrum of tocotrienols with maximum absorption at 300 nm (Figure 3). By using UV detection, it was clear that the 5% and 6% 2-propanol eluted fractions from the Bond Elut amine column contained both γ-oryzanols and the novel tocols, which were not well separated by this analytical HPLC system.

The γ-oryzanols, *d*-P_{21}-T3, and *d*-P_{25}-T3 were separated by HPLC using the Beckman Ultrasphere silica 5-μm column and fluorescence detection (Figure 4). *d*-P_{21}-T3 and *d*-P_{25}-T3 were eluted at 16.8 and 18.7 min, respectively, and a small peak at 13.9 min was due to γ-oryzanols. These tocol peaks were repeatedly purified by HPLC, and the purity of these components was checked by HPLC analyses (Figure 5A−C) prior to mass spectrometric, IR, NMR, and CD analyses. Each of the

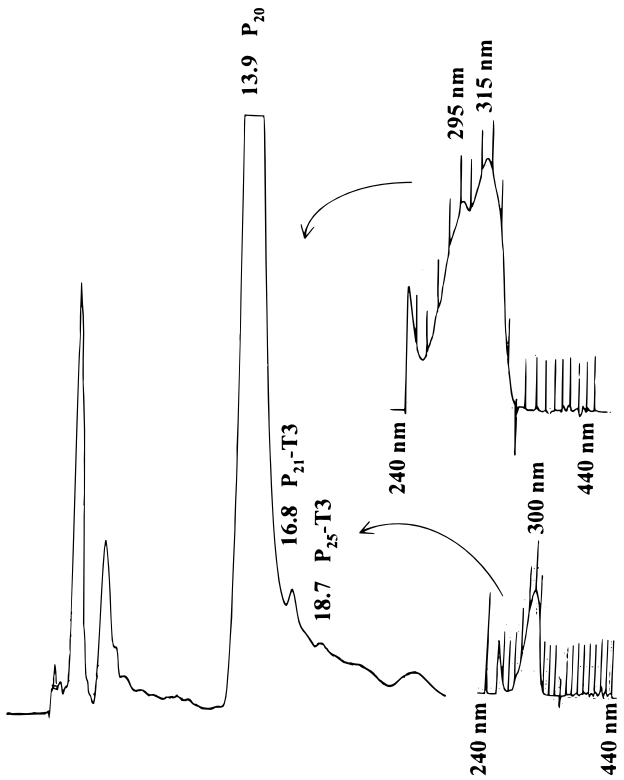


Figure 3. HPLC analysis and UV absorption spectra of combined 5% and 6% 2-propanol-eluted fractions from Bond Elut amine column. Normal phase silica column; mobile phase, 0.6% 2-propanol in hexane; UV (295 nm) detection; absorption spectra from 240 to 440 nm.

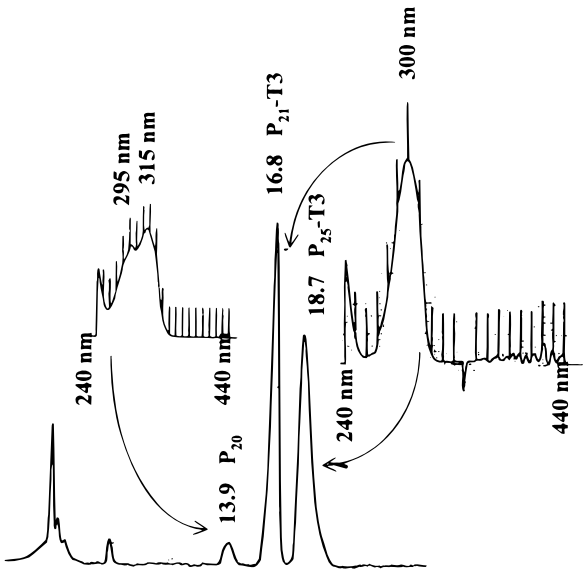


Figure 4. Purification and UV absorption spectra of combined 5% and 6% 2-propanol-eluted fractions from Bond Elut amine column. Ultrasphere SI-5 μm column; mobile phase, 0.6% 2-propanol in hexane; fluorescence detection; absorption spectra from 240 to 440 nm.

components showed a single peak (Figure 5A,C), with a maximum absorption at 300 nm (Figure 5B).

Structural Analyses of *d*-P_{21}-T3 and *d*-P_{25}-T3. The high-resolution mass spectra of these compounds showed molecular ions from *d*-P_{21}-T3 (M^+ 382) and *d*-P_{25}-T3 (M^+ 368) corresponding to molecular formulas $C_{26}H_{38}O_2$ and $C_{25}H_{36}O_2$, respectively (Figure 6A,B). The patterns were characteristic of fragmentations observed with α-, β-,

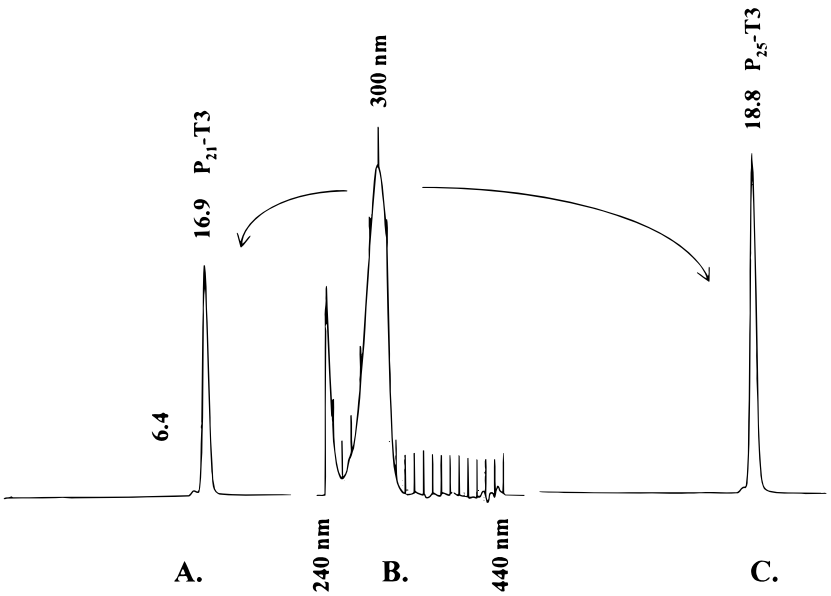


Figure 5. HPLC analysis and UV spectra of purified peaks collected from Ultrasphere SI column elution. Normal phase silica column; mobile phase, 0.6% 2-propanol in hexane; UV (295 nm) detection; absorption spectra from 240 to 440 nm.

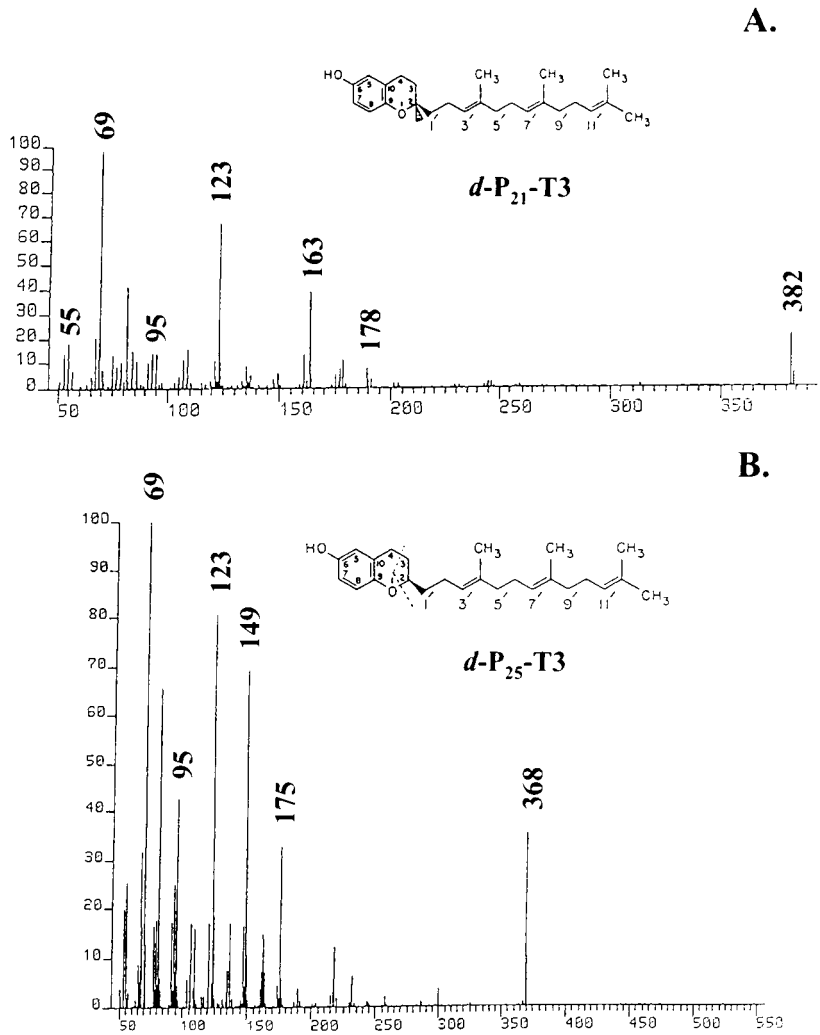


Figure 6. Mass spectra fragmentation patterns: (A) *d*-P_{21}-T3 (desmethyl tocotrienol); (B) *d*-P_{25}-T3 (didesmethyl tocotrienol).

and γ-tocotrienols (Rao and Perkins, 1972). No spectral deviation was recorded, thus indicating the purity of these samples. The mass spectrum of *d*-P_{21}-T3 showed a major peak at *m/e* 163^+ indicating loss of the side chain $(C_{16}H_{27})^+$ and giving rise to the ion $C_{10}H_{11}O_2$ (163.0759) (Figure 6A). The peak at *m/e* 123^+ ($C_7H_7O_2$, 123.0446) originated from cleavage of the side chain (loss of 40 mass units) accompanied by breakdown of

the chroman structure with a hydrogen rearrangement and loss of a methyl acetylene (CH_3—C≡CH) fragment. All this information is consistent with a structure for *d*-P_{21}-T3 (desmethyl tocotrienol) as 3,4-dihydro-2-methyl-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol.

This structure was further supported by its NMR spectra in $CDCl_3$, which is identical to that of δ-tocotrienol, except that the methyl group on the benzene ring was missing at δ 2.2. NMR ($CDCl_3$) and showed signals at δ 1.26 (s, 3H), 1.50−1.84 (m, 4H), 1.58 (s, 9H), 1.66 (s, 3H), 1.90−2.15 (m, 10H), 2.69 (t, J = 6.7 Hz, 2H), 4.23 (s, 1H), 5.07 (m, 3H), 6.51−6.57 (m, 2H), and 6.63 (d, J = 8.5 Hz, 1H). Its IR spectra indicated the presence of a phenolic OH group at 3700; a benzene ring at 1650; the chroman ring at 1680; and other IR (film) peaks at 3400, 2990, 2950, 2876, 1500, 1458, 1240, and 750 cm^{-1}. The elemental analyses were calculated: C, 81.26; H, 10.01; O, 8.93%. Found: C, 81.68; H, 10.01; O, 8.29%, indicating a molecular weight of 382.59, which corresponds to $C_{26}H_{38}O_2$.

The mass spectrum of *d*-P_{25}-T3 showed a molecular ion at *m/e* 368^+ corresponding to the molecular formula $C_{25}H_{36}O_2$ (368.2715). The rest of the main fragmentation pattern, *m/e* 175^+, 149^+, and 123^+, was typical of δ- and desmethyl tocotrienol, except that the methyl group on the chroman ring at C2 next to the oxygen was missing (Figure 6B). The foregoing indicated, in turn, that *d*-P_{25}-T3 is didesmethyl tocotrienol (3,4-dihydro-2-(4,8,12-trimethyltrideca-3′(*E*),7′(*E*),11′-trienyl)-2*H*-1-benzopyran-6-ol).

This structure was further supported by its NMR ($CDCl_3$) spectrum, which showed δ 1.58 (s, 3H), 1.59 (s, 3H), 1.62 (s, 3H), 1.67 (s, 3H), 1.73 (m, 2H), 1.90−2.08 (m, 10H), 2.19 (m, 2H), 2.63−2.85 (m, 2H), 3.89 (m, 1H), 4.30 (br s, 1H), 5.07 (m, 2H), 5.14 (t, J = 7.9 Hz, 1H), 6.51 (d, J = 2.9 Hz, 1H), 6.56 (d of d, J = 2.9, 8.6 Hz, 1H), and 6.66 (d, J = 8.6 Hz, 1H). It is identical to the one obtained for desmethyl tocotrienol (*d*-P_{21}-T3). The IR spectrum showed similar functional groups (benzene ring, hydroxy group, and chroman ring) as were observed with desmethyl tocotrienol and other tocotrienols. The other main peaks in its IR (film) were 3388, 2924, 1494, 1450, 1352, 1280, 1218, and 1080 cm^{-1}. The elemental analyses were calculated: C, 81.47; H, 9.85; O, 8.685. Found: C, 81.19; H, 9.93; O, 8.87, indicating a molecular weight of 368.56, which equals $C_{25}H_{36}O_2$.

This is the first report that shows the presence of *d*-P_{21}-T3 and *d*-P_{25}-T3 from a natural source. Mass spectra were also obtained for synthetic desmethyl tocotrienol (*d,l*-P_{21}-T3) and didesmethyl tocotrienol (*d,l*-P_{25}-T3), which had similar fragmentation patterns as the natural compounds. Moreover, the UV absorption spectra and the elution times on HPLC were also identical for the synthetic and natural compounds from rice bran (Pearce et al., 1994).

Mass and Circular Dichroism of *d*-P_{21}-T3 and *d*-P_{25}-T3 and Isomers Purified by Chiralpak-AD column. *d*-P_{21}-T3 and *d*-P_{25}-T3 were separated from the 5% ether eluted fraction from the Bond Elut amine column by HPLC on a Chiralpak-AD column, which showed two peaks at 12.13 and 14.04 min (Figure 7A). Each of these peaks, when collected and rerun, showed a single peak at 12.12 and 14.03 min for *d*-P_{21}-T3 and *d*-P_{25}-T3, respectively (Figure 7B,C). The synthetic *d,l*-P_{21}-T3 and *d,l*-P_{25}-T3 were separated into their isomers *l*-P_{21}-T3 at 10.83 min, *d*-P_{21}-T3 at 12.12 min (Figure 7D), *l*-P_{25}-T3 at 12.25 min, and *d*-P_{25}-T3 at 14.02 min (Figure 7E) on the Chiralpak-AD column. High-resolution mass spectral analyses of the 5% ether fraction (Bond Elut amine column, mixture of *d*-P_{21}-T3 plus *d*-P_{25}-T3) showed two molecular ion peaks at *m/e* 382^+ (*d*-P_{21}-T3) and *m/e* 368^+ (*d*-P_{25}-T3) (Figure 8A). The individual purified peaks from the Chiralpak-AD column showed ion peaks at *m/e* 382^+ (Figure 8B) and *m/e* 368^+ (Figure 8C), respectively, with the same fragmentation pattern as observed earlier. Stereochemistry of these compounds was established as "*d*" by their circular dichroism (CD) spectra, which showed a strong (+) cotton effect at 233 nm and weak (−) cotton effect at 300 nm (Figure 9). The CD spectra of the "*d*" and "*l*" isomers were mirror images for both compounds (Figure 9A−C). The structures of the various tocotrienols including *d*-P_{21}-T3 and *d*-P_{25}-T3 are shown in Figure 10.

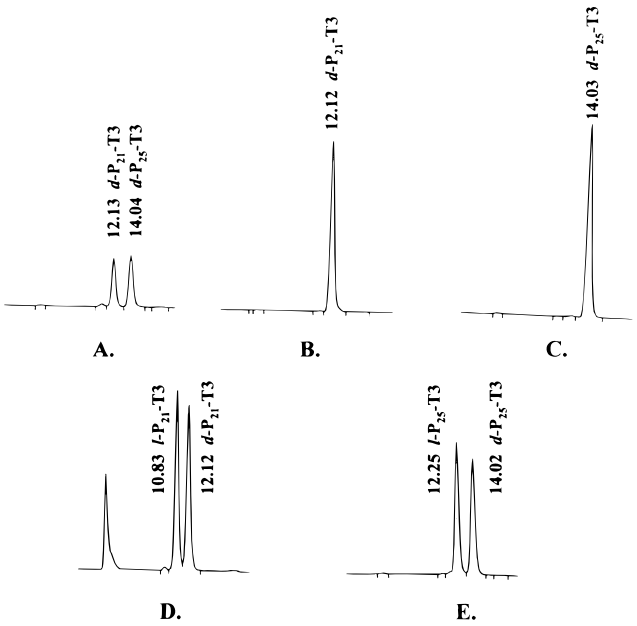


Figure 7. HPLC analyses of TRF_{25} and purified tocols. Chiralpak-AD column; mobile phase, 3% 2-propanol in hexane; fluorescence detection. (A) TRF_{25} (fraction eluted from Bond Elut amine column with 5% 2-propanol in hexane). (B) Purified *d*-P_{21}-T3. (C) Purified *d*-P_{25}-T3. (D) Synthetic *d,l*-P_{21}-T3. (E) Synthetic *d,l*-P_{25}-T3.

Preparative Purification of Tocols by Flash Chromatography. The compositions of the fractions obtained with each elution were as follows: 5% (v/v), ether; fatty acids, esters, and terpenes (473 g); 10%, α-tocopherol (3.1 g); 12%, a mixture of α-tocopherol and α-tocotrienol (0.7 g); 14%, α-tocotrienol (1.2 g); 16%, β-tocotrienol (0.8 g); 18%, γ-tocotrienol (2.8 g); 20%, a mixture of δ-tocopherol and δ-tocotrienol contaminated with *d*-P_{21}-T3 (0.7 g); 22%, *d*-P_{21}-T3 (0.8 g); 25%, *d*-P_{25}-T3 (0.6 g); and 30%, *d*-P_{25}-T3 (1.4 g). Washing with 100% ether gave a small quantity of γ-oryzanols (5.8 g). Each of these fractions contained more than 95% of its main component of the tocol.

The composition of the tocol-rich fraction that was purified by flash chromatography (short process, 30% ether elution after 10% ether wash) was 6% α-tocopherol, 12.5% α-tocotrienol, 33.5% γ-tocotrienol, 10% δ-tocopherol and δ-tocotrienol, 9.6% *d*-P_{21}-T3, 10.4% *d*-P_{25}-T3, and 8% unidentified tocol isomers. This preparation (TRF_{25}) was used for carrying out studies on biological activities.

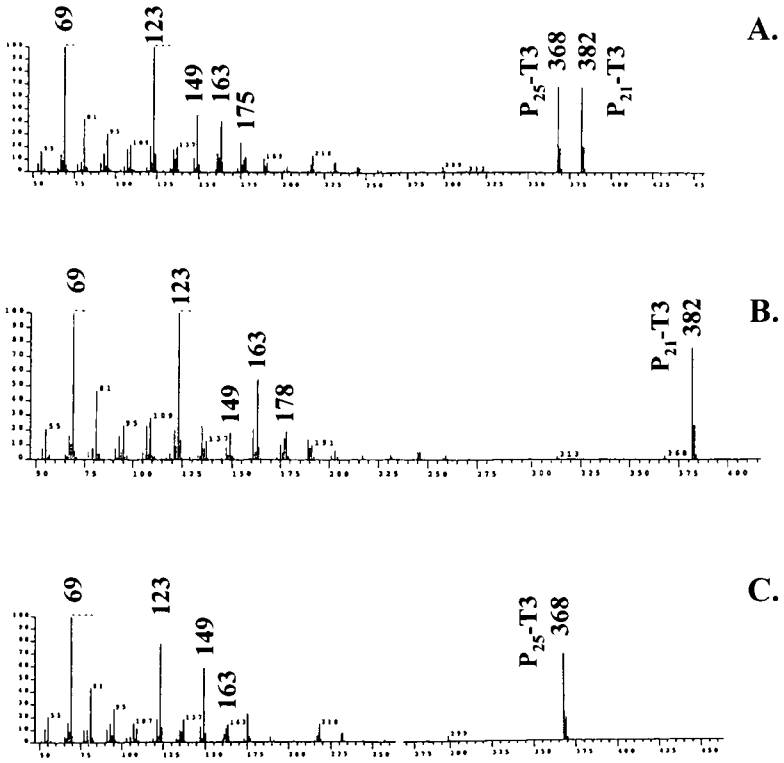


Figure 8. Mass spectra fragmentation patterns. (A) Fraction eluted from Bond Elut amine column with 5% 2-propanol in hexane. (B) Bond Elut amine purified *d*-P_{21}-T3. (C) Bond Elut amine purified *d*-P_{25}-T3.

Biological Activities of Novel Tocotrienols vs Known Tocotrienols. (a) Hypocholesterolemic Properties. α-Tocotrienol, *d*-P_{21}-T3, and *d*-P_{25}-T3 isolated from stabilized and heated rice bran inhibited HMG-CoA reductase and fatty acid synthetase activities but did not affect cholesterol 7α-hydroxylase activity of isolated chicken hepatocytes (Table 1). The inhibition of HMG-CoA reductase was dose dependent up to 20 μg/mL (α-tocotrienol) or 40 μg/mL (*d*-P_{21}-T3 and *d*-P_{25}-T3), whereas fatty acid synthetase activity declined to the highest concentrations tested. P_{20} (γ-oryzanols) at the concentrations tested did not affect HMG-CoA reductase activity (Table 1).

In the feeding study, average body weight gain (370 ± 11 g), feed consumption (992 ± 11 g), and feed efficiencies (0.394 ± 0.011) were not affected by any of these tocotrienols, including *d*-P_{21}-T3 and *d*-P_{25}-T3 or α-tocopherol. The serum total and LDL cholesterol in chickens fed tocol-containing diets (except α-tocopherol and β-tocotrienol) significantly ($P < 0.05$) decreased 13−31% and 17−48%, respectively, as compared to the control group (Table 2). There were no significant differences in the level of serum HDL cholesterol among all groups. Total and LDL cholesterol levels were lowered by the diet additions in the following order: *d*-P_{25}-T3 > *d*-P_{21}-T3 > δ-T_3 > γ-T_3 > α-T_3 > TRF_{25} (Table 2). Similar effects were also observed for the hepatic HMG-CoA reductase activities as shown in Table 2. Although α-tocopherol did not affect the levels of serum total cholesterol and LDL cholesterol, it caused an increase of 11% in HMG-CoA reductase activity.

(b) Antioxidant Properties. The dose-dependent antioxidant properties of α-tocopherol, *d*-α-, *d*-γ-, *d*-δ-, *d*-P_{21}-, and *d*-P_{25}-tocotrienols, and TRF_{25} in the liposome system are shown in Figure 11. The half quenching concentrations (K_{50}) were as follows: α-tocopherol, 200 nM; γ-tocotrienol, 60 nM; δ-tocotrienol, 50 nM; TRF_{25}, 25 nM; *d*-P_{21}-T3, 14 nM; and *d*-P_{25}-T3, 6 nM. These findings indicate that as compared to α-tocopherol, γ- and δ-tocotrienols were 4-fold; TRF_{25} was 8-fold; *d*-P_{21}-T3 was 14-fold; and *d*-P_{25}-T3 was 33-fold more efficient scavengers of peroxyl radicals in this system.

The K_{50} concentrations were also determined by a coupled autoxidation of β-carotene and linoleic acid procedure. They were α-tocopherol, 41 μM; α-tocotrienol, 32 μM; γ-tocotrienol, 30 μM; δ-tocotrienol, 28 μM; TRF (48% α-tocopherol), 35 μM; TRF (35% α-tocopherol), 33 μM; and TRF_{25} (6% α-tocopherol), 31 μM. These results indicate that the antioxidant activities of the tocotrienols and TRF were greater than that of α-tocopherol by the percentages indicated: α-tocotrienol, 22%; γ-tocotrienol, 27%; δ-tocotrienol 32%; TRF with 48% α-tocopherol, 15%; TRF with 35% α-tocopherol, 20%; and TRF_{25} with 6% α-tocopherol, 24%. The higher percentage of α-tocopherol in TRF reduces the antioxidant activity even in this least sensitive assay for measuring the antioxidant activity of various compounds.

(c) Antitumor Properties. Similarly, various tocotrienols showed concentration-dependent suppression of B16 melanoma cells proliferation (Table 3). The TRF_{25} isolated from the heated rice bran was a more potent antitumor agent than commercially produced TRF from rice bran, which contained higher percentages of α-tocopherol. Approximately 50 μM TRF_{25} had the same effect as 250 μM concentration of the other TRF prepared by other manufacturers from rice bran (Table 3). Moreover, the novel tocotrienols were at least 200-fold better suppressors of proliferation of these cancerous cells (Table 3). *d*-α-Tocopherol has minimal antitumor activity even at the very high concentration of 2 mM as compared to tocotrienols (data not shown).

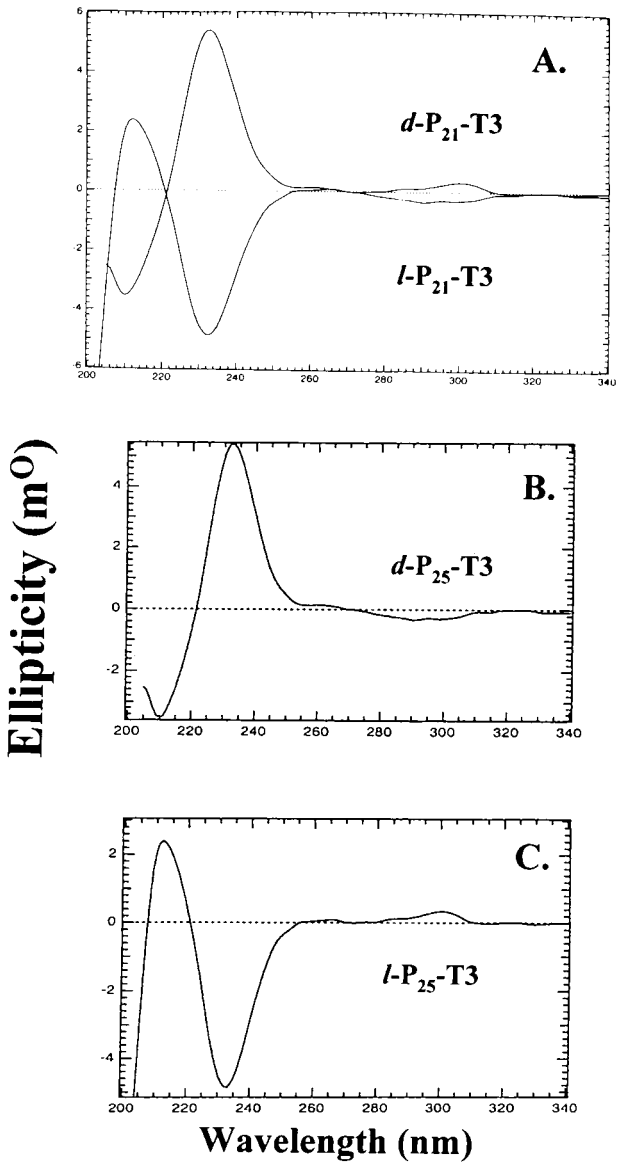
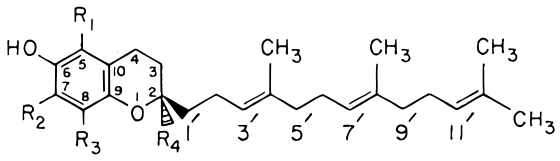


Figure 9. Circular dichroism spectra: (A) *d*-P_{21}-T3 (top) and *l*-P_{21}-T3 (bottom); (B) *d*-P_{25}-T3; (C) *l*-P_{25}-T3.



		R_1	R_2	R_3	R_4	Mw
α-T_3	α-Tocotrienol	CH_3	CH_3	CH_3	CH_3	424
β-T_3	β-Tocotrienol	CH_3	H	CH_3	CH_3	410
γ-T_3	γ-Tocotrienol	H	CH_3	CH_3	CH_3	410
δ-T_3	δ-Tocotrienol	H	H	CH_3	CH_3	396
P-21	Desmethyl-tocotrienol	H	H	H	CH_3	382
P-25	Didesmethyl-tocotrienol	H	H	H	H	368

Figure 10. Molecular formulas of known tocotrienols including *d*-P_{21}-T3 and *d*-P_{25}-T3.

DISCUSSION

The release of *d*-P_{21}-T3 (desmethyl tocotrienol), *d*-P_{25}-T3 (didesmethyl tocotrienol), and known tocols by heating rice bran demonstrates that tocotrienols and tocotrienol-like compounds are bound to insoluble cellular components. The methanol extract of the residue of hexane-extracted rice bran, when heated under vacuum (160 °C for 60 min), contained 2-fold higher tocols than were in the hexane extract. The bound tocols were not released by methanol extraction without prior heating (data not shown). Heating at 110 °C in a conventional oven for 60 min destroyed 50−60% of the tocols (Emam et al., 1995; Oomah et al., 1998; Chow and Draper, 1974; Moreau et al., 1999), suggesting that they are readily oxidized under these conditions. However, microwave irradiation for 1−3 min resulted in a 2−4-fold increase in extractable tocols (Wang et al., 1995; Emam et al., 1995).

The high concentrations of γ-oryzanols (> 1.2%) in stabilized and heated rice bran made purifying *d*-P_{21}-T3 and *d*-P_{25}-T3 difficult. Purification was achieved by one of three methods. Prior hexane extraction to remove free fatty acids, γ-oryzanols, and terpenes and subsequent extraction of the heated residue with methanol yielded *d*-P_{21}-T3 and *d*-P_{25}-T3. On an analytical scale, the novel tocols could be separated from γ-oryzanol on a Chiralpak-AD column. For preparative work, flash chromatography on silica gel produced a tocotrienol-rich fraction free of γ-oryzanol but containing 6% α-tocopherol.

The designation of desmethyl tocotrienol as *d*-P_{21}-T3 and didesmethyl tocotrienol as *d*-P_{25}-T3 reflects the elution time of each component with a particular mobile phase (0.2% 2-propanol in hexane). The actual elution time depends on the concentration of 2-propanol (0.2−1.0%) and on the age of the column. Higher concentrations of 2-propanol and older columns result in faster elution of these tocols.

The structural characteristics of tocotrienols and tocotrienol-like compounds affect their specific biological activities and their elution profiles. Structurally, all the

Table 1. Cholesterol-Related Enzyme Activities of Isolated Hepatocytes as Affected by Tocotrienols and γ-Oryzanols

concn (μg/mL)	HMG-CoA reductase (pmol min^{-1} mg^{-1})	fatty acid synthetase (nmol min^{-1} mg^{-1})	cholesterol 7α-hydroxylase (pmol min^{-1} mg^{-1})
		α-Tocotrienol	
0	26.8 (100)[a]	17.2 (100)	2.31 (100)
10	21.4 (79.9)	15.2 (88.4)	2.12 (91.8)
20	17.3 (64.6)	14.3 (83.3)	2.27 (98.7)
40	16.7 (62.3)	12.4 (72.1)	2.34 (101.3)
		d-P_{21}-T3	
0	26.8 (100)	17.2 (100)	2.31 (100)
10	20.4 (76.1)	14.3 (83.1)	2.41 (104.3)
20	16.7 (62.3)	12.7 (73.8)	3.40 (103.9)
40	14.2 (52.9)	11.8 (68.6)	2.39 (103.5)
80	14.3 (53.4)	10.2 (59.3)	1.37 (102.6)
		d-P_{25}-T3	
0	26.8 (100)	17.2 (100)	2.31 (100)
10	19.0 (70.9)	16.2 (94.2)	2.33 (100.9)
20	15.3 (57.1)	14.3 (83.1)	2.37 (102.6)
40	12.4 (46.3)	12.7 (73.8)	2.44 (105.6)
80	14.3 (53.4)	12.1 (70.3)	2.37 (102.6)
		P_{20} (γ-Oryzanols)	
0	28.8 (100)		
25	29.2 (101.5)		
50	28.9 (100.3)		
100	28.2 (98.0)		

[a] Percentages of control values are shown in parentheses.

Table 2. Effects of Feeding Tocols on Serum Lipid Parameters and Hepatic HMG-CoA Reductase Activities[a]

diets[a]	total cholesterol (mmol/L)	HDL cholesterol (mmol/L)	LDL cholesterol (mmol/L)	HMG-CoA reductase (pmol mg^{-1} min^{-1})
CD	4.22 ± 0.17[d] (100)[c]	2.09 ± 0.06[d] (100)	1.84 ± 0.05[d] (100)	411.6 ± 17.8[d] (100)
CD + TRF_{25}[b]	3.67 ± 0.12[e] (87)	2.01 ± 0.05[d] (96)	1.53 ± 0.09[e] (83)	346.7 ± 15.4[e] (84)
CD + α-T[b]	4.27 ± 0.21[d] (101)	2.17 ± 0.09[d] (104)	1.91 ± 0.09[d] (104)	456.3 ± 19.7[d] (111)
CD + α-T3[b]	3.57 ± 0.13[e] (85)	2.07 ± 0.10[d] (99)	1.32 ± 0.07[e] (72)	322.8 ± 16.3[e,f] (78)
CD + β-T3	4.16 ± 0.16[d] (99)	2.04 ± 0.12[d] (98)	1.81 ± 0.07[d] (99)	401.4 ± 18.6[d] (98)
CD + γ-T3	3.44 ± 0.11[e,f] (82)	1.97 ± 0.14[d] (95)	1.32 ± 0.07[e] (72)	305.6 ± 14.9[f] (74)
CD + δ-T3	3.28 ± 0.13[f] (78)	1.99 ± 0.12[d] (95)	1.14 ± 0.06[f] (62)	294.7 ± 15.4[f] (72)
CD + *d*-P_{21}-T3	3.13 ± 0.12[f,g] (74)	2.04 ± 0.11[d] (98)	1.09 ± 0.06[f,g] (59)	281.5 ± 12.8[f,g] (68)
CD + *d*-P_{25}-T3	2.89 ± 0.10[g] (69)	2.07 ± 0.11[d] (99)	0.96 ± 0.05[g] (52)	262.3 ± 14.6[g] (64)

[a] Values not sharing a common superscript letter (d−f) are different at $P < 0.05$. The composition of control diet (CD) was same as that described for Table 1. [b] All additions to the control diet were 50 mg/kg of each α-tocopherol (T) or TRF_{25} or tocotrienol (T3). [c] Percentages of control values are shown in parentheses.

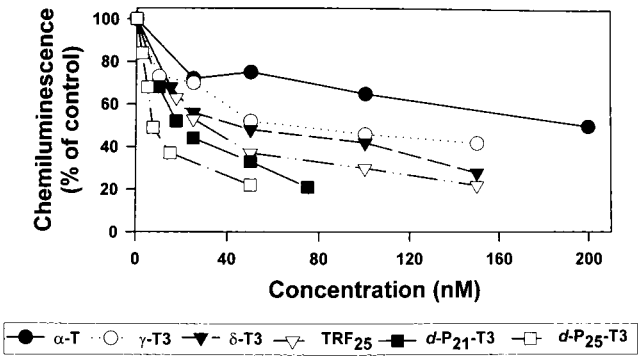


Figure 11. Peroxyl radical scavenging activities of α-tocopherol, known, and novel tocotrienols of rice bran. The antioxidant activities of α-tocopherol, γ-tocotrienol, δ-tocotrienol, TRF_{25} (rice bran; 6% α-tocopherol), *d*-P_{21}-T3, and *d*-P_{25}-T3 were carried out as described in the Experimental Methods.

tocotrienols share three features: (i) a hydrogen donor group attached to an aromatic ring system, (ii) an atom having at least one lone pair of electrons in conjunction with the aromatic system, and (iii) a side chain comprising one or more isoprenoid-like units attached to a position adjacent to that atom. With normal-phase HPLC, these tocotrienols are eluted in descending order according to the number of methyl groups on the chroman rings.

Parker et al. (1993) reported that tocotrienols inhibit cholesterogenesis by suppressing HMG-CoA reductase through a novel post-transcriptional mechanism. *d*-P_{25}-T3 exhibited greater cholesterol lowering activity in chickens than any of the other known tocotrienols including *d*-P_{21}-T3, α-, β-, γ-, or δ-tocotrienols. Most tocotrienols (except β-tocotrienol) significantly lower serum total (>30%) and LDL cholesterol (>45%) in parallel with an inhibition of HMG-CoA reductase activity. *d*-P_{25}-T3 caused maximum enzyme inhibition as compared to other tocotrienols.

The reason fewer alkyl substituents are associated with greater activity may be because of steric hindrance caused by the methyl groups that inhibit the penetration of the compounds into the membrane. As a result, the *d*-P_{25}-T3 with no methyl groups demonstrates increased biological activity as compared to other known tocotrienols, particularly with regard to lowering the serum total and LDL cholesterol levels.

Tocotrienols are natural unsaturated forms of vitamin E, the major chain-breaking antioxidant in cellular membranes. They quench free radicals in cell membranes and protect them against lipid peroxidation. Antioxidants help to decrease the oxidation of LDL cholesterol, thus reducing the buildup of cholesterol and

Table 3. Concentration-Dependent Suppression of the Proliferation of B16 Melanoma Cells by Novel Tocotrienols

compound	concn (μM)	% of net growth
control		100
α-tocopherol (vitamin E from soybean)[a]	250	88
	500	86
	1000	92
TRF (rice bran, 35% α-tocopherol)[b]	50	64
	100	31
	250	−16
TRF (rice bran, 48% α-tocopherol)[c]	50	71
	100	20
	250	−14
TRF_{25} (heated rice bran, 6% α-tocopherol)[d]	50	0
	100	−16
	250	−16
d-γ-tocotrienol	10	89
	25	−16
	50	−16
	100	−16
d-P_{21}-tocotrienol	0.25	95
	0.5	84
	1	63
	2	35
	5	−5
	10	−16
	25	−16
	50	−16
d-P_{25}-tocotrienol	0.25	79
	0.5	61
	1	38
	2	25
	5	1
	10	−16
	25	−16
	50	−16

[a] Archer Daniels Midland Company, Box 1470, Decatur, IL 62525. [b] Eastman TRF supplied by American River Nutrition, Inc, 31 Campus Plaza Road, Hadley, MA 01035. [c] Japanese TRF, American River Nutrition, Inc. [d] TRF_{25}, Advanced Medical Research, Madison, WI 53719.

fatty acid plaques on artery walls and preserving the function of cells that line arteries. All of these effects can help counter the deterioration that characterizes coronary artery disease. The antioxidant activity of the α-tocotrienol homologue has been shown to be more than 3-fold greater than that of α-tocopherol (Packer, 1995). Using an in vitro liposome system, antioxidant activities for several tocotrienols were 4−33-fold higher than that for α-tocopherol. The order of activity was *d*-P_{25}-T3 > *d*-P_{21}-T3 > TRF_{25} > δ-tocotrienol > γ-tocotrienol > α-tocotrienol > α-tocopherol. These results collectively indicate that in intact membranes, including LDL particles, tocotrienols may have a significantly

greater antioxidant effect than tocopherols and they may provide greater protection against coronary artery disease. *d*-P_{25}-T3 is the most potent tocol homologue of all natural forms of vitamin E tested.

These findings were further confirmed by determining the antioxidant activities using a different method, the coupled autoxidation of β-carotene and linoleic. The antioxidant activities of known α-, γ-, and δ-tocotrienols and TRF_{25} (prepared from stabilized and heated rice bran with 6% α-tocopherol) were 22%, 27%, 32%, and 24% better than α-tocopherol, respectively. The possible mechanism for this superior efficacy of tocotrienols compared to tocopherols has been reported (Packer, 1995).

The tocotrienol homologues differ substantially in their capacity to suppress tumor cell proliferation (Komiyama et al., 1989). γ-Tocotrienol has been shown to suppress tumor cell proliferation by initiating apoptosis and arresting cells in the G1 phase of the cell cycle (Mo and Elson, 1999). This action is traced to the suppression of HMG-CoA reductase activity. The IC_{50} values for the suppression of B16 melanoma cell proliferation by α-tocopherol; α-, γ-, and δ-tocotrienol; and P_{25}-T3 are >1600, 110, 20, 10, and 0.9 μM, respectively (He et al., 1997). The suppression of B16 melanoma cell proliferation by TRF_{25} was 5-fold better than other commercially available TRFs of rice bran. This was because the commercial TRFs contained large concentrations of α-tocopherol (35−48% vs only 6% for TRF_{25}). Moreover, the novel tocotrienols (only "*d*" isomers) were 50-fold better than TRF and 200-fold better than α-tocopherol. The *l*-isomers of these tocotrienols showed no effects on these cells (data not shown). The mechanism of action of various tocotrienols for the suppression of antitumor activity has been reported recently. (Mo and Elson, 1999).

The present results demonstrate that the ratios of the individual tocopherols and tocotrienols play an important role in determining the hypocholesterolemic, antioxidant, and antitumor properties of the tocotrienol-rich fraction from palm oil, commercial rice bran (TRF), and stabilized and heated rice bran (TRF_{25}). α-Tocopherol stimulates HMG-CoA reductase activity in the liver, and more than 20% α-tocopherol in TRF results in an attenuation of the hypocholesterolemic effect of tocotrienols (Qureshi et al., 1996b; Khor and Ng, 1999). Similar differences in the effects of TRF_{25} of stabilized rice bran and other commercial TRF were observed in antioxidant and antitumor assays.

Chemical syntheses of all the tocotrienols have been achieved, but the process yields mixtures of both the active *d* and inactive *l* isomers (Pearce et al., 1994). These can be separated on a Chiralpak-AD column, although this process is not commercially feasible.

This is the first report that demonstrates the isolation and structural determination of two new tocotrienols with high biological activity from stabilized and heated rice bran. Moreover, TRF_{25}, *d*-P_{21}-, and *d*-P_{25}-tocotrienols from rice bran are much more potent as hypocholesterolemic, antioxidant, and antitumor agents as compared to the known tocotrienols. One possible approach to develop a commercial source of these compounds is by genetically engineering rice to overexpress the genes involved in coding for specific tocotrienol synthesis (Shintani and DellaPenna, 1998). Alternatively, yeast or *Escherichia coli* mutants can be produced that will provide not only the pure components but also the right isomers of these tocotrienols during fermentation. A yeast mutant has been developed that overexpresses geranylgeranyl pyrophosphate in the presence of an appropriate precursor in glucose-rich media. This mutant broth produces over 40% of the total tocols as *d*-P_{21}-tocotrienol after 72 h of fermentation. This work is in progress and will be described in a separate manuscript.

ABBREVIATIONS USED

HMG-CoA, β-hydroxy-β-methylglutaryl coenzyme A; HPLC, high-performance liquid chromatography; T, tocopherol; T3, tocotrienol; TRF, tocotrienol-rich fraction of palm oil; TRF_{25}, tocotrienol-rich fraction of stabilized and heated rice bran; *d*-P_{21}-T3, desmethyl tocotrienol of stabilized and heated rice bran; *d*-P_{25}-T3, didesmethyl tocotrienol of stabilized and heated rice bran; P_{20}, γ-oryzanols; LDL, low-density lipoprotein; HDL, high-density lipoprotein; VLDL, very low-density lipoprotein.

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