# APPENDIX

The work presented in this thesis has been published either wholly or in part in the following articles. Other papers are currently in preparation.

1. Goh, S.H., Hew, N.F. and Lee, M., "Stereochemistry of Bichromanyl Dimers from γ-Tocopherol and γ-Tocotrienol", *Tetrahedron Letters*, 1992, **33**:4613-4616.

2. Goh, S.H., Hew, N.F., Norhanom, A.W. and Yadav, M., "Inhibition of Tumour Promotion by Various Palm-Oil Tocotrienols", *International Journal of Cancer*, 1994, **57**:529-531.

3. Hew, N.F., Khor, H.T., Choo, Y.M., Yap, S.F. and Goh, S.H., "Unusual Accumulation of α-Tocopherol in Rabbits Fed Palm-Oil Diets Enhanced with Various Vitamin E Components", *Malaysian Journal of Science*, 1994, **15A**:73-78.

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# Stereochemistry of Bichromanyl Dimers from γ-Tocopherol and γ-Tocotrienol

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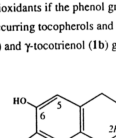
*Key Words* : γ-tocopherol, γ-tocotrienol, dimers, absolute configurations, 5,5'-bichromanyls

**Abstract:** Oxidation of natural γ-tocopherol and γ-tocotrienol gave mainly dimeric products, and their absolute configurations of diastereomeric 5,5'-bichromanyl dimers have been determined by NMR (NOE difference of OH to 2'-methyl) and chromatographic studies.

Tocopherols and tocotrienols have been the subject of intense research especially with respect to their roles as chemical[1-3] and biological antioxidants[4-6]. The tocotrienols, in particular, have recently come under close scrutiny with respect to the relationship of their chemical structure and biological activities including anticancer[7,8] and hypocholesterolemic properties[9-11]. Previous work has shown that the oxidation of tocopherols and tocotrienols lead to complex oxidation products[1,12,13]. In particular the γ and δ isomers of these chromanol derivatives containing unsubstituted aromatic positions provide dimeric as well as higher products which can also function as antioxidants if the phenol group is present.

Naturally occurring tocopherols and tocotrienols have the (*R*)-configuration at carbon-2. The oxidation of γ-tocopherol (**1a**) and γ-tocotrienol (**1b**) gives mainly dimeric products of two types, ether dimers (5-tocopheryltocopherol, 5-tocotrienoxyltocotrienol) and 5,5'-bichromanyl dimers (**2a, 2b, 3a, 3b**)[1,12,13]. The latter dimers are of interest since apart from the natural (2*R*)-configuration at carbons-2 and -2', diastereomers with (*R*)- and (*S*)-configurations are possible as the result of the restricted rotation of the 5,5'-bond in the bichromanyls .




**1a** : $R^1$ = $CH_3(CH_2CH_2)_3CH(CH_3)CH_2)_3H$

**1b** : $R^1$ = $CH_3(CH_2CH{=}C(CH_3)CH_2)_3H$

In a typical experiment **1a** or **1b** (5 mmol) in hexane (40 mL) were oxidized under nitrogen at room temperature by excess alkaline aqueous ferricyanide (120 mmol). After acidification and separation of the

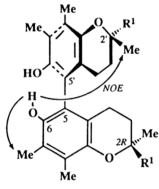
hexane layer the products were isolated by flash column chromatography (Silica gel 60) and normal phase HPLC. Typically the reaction provides ether dimers (45 and 60% from **1a** and **1b** respectively) and diastereomeric 5,5'-bichromanyls (20% **2a**, 20% **2b**; 20% **3a**, 15% **3b**)[14]. These diastereomeric dimers can readily be separated by chromatography (data given in Table 1) but they slowly isomerise to give mixtures of their diastereomeric forms. Some of the chromatographic and spectroscopic characteristics which can distinguish the diastereomers are given in the Table. Initial assignments[1] of the absolute configuration of (*R*)- & (*S*)- 5,5'-bi-(*2R,2'R*)-γ-tocotrienyls (**3a** & **3b**) based on the basis of chromatographic data. From molecular modelling studies (MM2, CAChe-Tetronix system), of the most stable conformer the 2-methyl group prefers an axial conformation and that the steric constraints in the bisected bichromanyls arise mainly from the C-4 methylene group rather than the OH group. This means that the (*S*)-bichromanyl dimer could assume a relatively more planar arrangement due to less steric interactions from 2 and 2'-methyls but less intramolecular hydrogen bonding between the phenolic groups as compared to the (*R*)-bichromanyl dimer which has a relatively more bisected conformation. As a consequence of the preferred conformational arrangements the (*R*)-bichromanyl dimer is more "polar" because the OH groups more exposed for chromatographic bonding, i.e. lower $R_f$ on normal phase silica gel TLC. Likewise the (*S*)-bichromanyl is actually more hydrophobic than the (*R*)-form due to the relative proximity of the long isoprenoid-hydrocarbon chain $R^1$ to the OH groups; modelling (MM2) does indicate that the OH groups are more "enclosed" by the $R^1$ isoprenoid chains in the (*S*)-stereoisomer. The structures are depicted in Figure 1 where the (*R*)- and (*S*)-bichromanyl configurations as shown are dependent on the (*2R*)-configurations present in the natural tocopherols/tocotrienols.

**Table 1: Spectral and Chromatographic Properties of Diastereomeric pairs 2a & 2b and 3a & 3b**

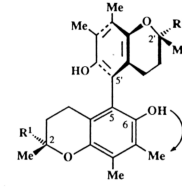
| | **2a** | **2b** | **3a** | **3b** |
|---|---|---|---|---|
| Configurational assignment | (*R*) | (*S*) | (*R*) | (*S*) |
| $R_f$ (TLC)[a] | 0.20 | 0.32 | 0.14 | 0.24 |
| HPLC retention time (min)[b] | 3.5 | 2.5 | 4.2 | 3.1 |
| NOE Difference (6-OH → 2'-Me) | 5.8% | 0 | 0.2% | 0 |
| Relative Energy (kcal/mole)[c] | 37.6 | 38.4 | 17.4 | 20.0 |
| $^{13}C$ NMR ($\delta$, $CDCl_3$) | | | | |
| C-2 Me | 23.98 | 23.74 | 23.80 | 23.67 |
| C-3 | 31.24 | 31.21 | 31.21 | 31.31 |
| C-4 | 20.66 | 20.59 | 20.58 | 20.56 |
| C-4a | 117.31 | 117.40 | 117.23 | 117.35 |
| C-8a | 144.64 | 144.53 | 144.65 | 144.57 |

[a] Silica gel 60; 3% EtOAc in hexane.
[b] Silica gel column, 25 cm x 4 mm; hexane-tetrahydrofuran-isopropanol (984:15:1).
[c] MM2 energy minimization on the CAChe Tektronix molecular mechanics system.

The chromatographic behaviour in the bichromanyl dimers (**2a** & **2b**) of γ-tocopherol have now been examined and a striking parallel has been observed with the (*R*)-bichromanyl dimers being chromatographi-




**2a**: (*R*)-5,5'-Bi-γ-tocopheryl



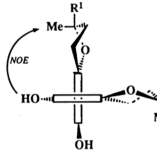

**2b**: (*S*)-5,5'-Bi-γ-tocopheryl


Tocopheryl : $R^1$ = $CH_2(CH_2CH_2{\overset{Me\ H}{\underset{}{C}}}CH_2)_3H$

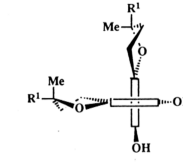
**3a**: (*R*)-5,5'-Bi-γ-tocotrienyl

**3b**: (*S*)-5,5'-Bi-γ-tocotrienyl


Tocotrienyl : $R^1$ = $CH_2(CH_2CH{=}{\overset{Me}{\underset{}{C}}}CH_2)_3H$




**2a'; 3a'** : (*R*)-configuration




**2b'; 3b'** : (*S*)-configuration

**Figure 1 : Stereochemistry of 5,5'-Bichromanyls of γ-Tocopherol and γ-Tocotrienol**

cally more "polar" than that of the (*S*)-dimer. The only difference between tocopherol and tocotrienol is that the latter compound has three double bonds in the terpenoid chain **R**[1] and it is expected that a parallel chromatographic behaviour should be observed. It is worthy to note that the relatively higher hydrophobicity of the tocopherol dimers (**2a** & **2b**) as compared to the tocotrienol dimers (**3a** & **3b**) as a result of differences in the isoprenoid chain, the triene being more compact than the saturated isoprene.

The absolute configurations of the bichromanyl dimers were assigned on the basis of modelling and NMR NOE-difference experiments. The MM2 energy minimizations on the molecules provide steric energies as given in the Table with the (*R*)-configuration of both pairs having lower energies. This is expected from an examination of models of the compounds (also summarised in simplified forms as **2a'** & **3a'** vs **2b'** & **3b'** pairs in Figure 1) whereby differences are from additional steric effects which arise from interactions of axial 2-methyl groups. Both forms will have intramolecular H-bonding of the the OH groups and it is likely that the (*R*)-configuration could have more intramolecular H-bonding as well.

The most convincing evidence for the assignment of configuration comes from the NMR NOE-difference experiment where it was observed that only the (*R*)-configuration produced an NOE between the phenoxyl protons to 2'-methyl protons. The data given in the Table indicates 0.2% to as high as 5.8% NOE enhancements for **2a** and **3a** respectively. Models of the pairs of diastereomers (also shown in Fig. 1) indicate that it is only in the (*R*)-configuration that OH → 2'-Me NOE effects may be observed because of the (*2R*)-configuration in the natural (*2R,4'R,8'R*)-tocopherol and (*2R*)-tocotrienol.

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14. All compounds were characterised by their $^{1}$H-NMR, $^{13}$C-NMR and mass spectra.

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UICC Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

# INHIBITION OF TUMOUR PROMOTION BY VARIOUS PALM-OIL TOCOTRIENOLS

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**Inhibition of tumour promotion by various vitamin E compounds (tocopherols and tocotrienols) and some of their dimers was examined by an *in vitro* assay utilizing the activation of Epstein-Barr virus (EBV) early antigen (EA) expression in EBV-genome-carrying human lymphoblastoid cells. The results reveal that γ- and δ-tocotrienols derived from palm oil exhibit a strong activity against tumour promotion by inhibiting EBV EA expression in Raji cells induced by 12-O-tetradecanoylphorbol-13-acetate (TPA). However, α- and γ-tocopherols and dimers of γ-tocotrienol or γ-tocopherol lack this activity.**


While major advances have been made in cancer therapy, they are costly and often inadequate when treatment is initiated at later stages of the disease. Thus, the best defences are early detection and commencement of treatment at an early stage of the disease. Cancer development is a multi-stage process involving initiation, promotion and progression. While it is difficult to protect against the action of environmental carcinogens, the next line of defence is to circumvent the tumour-promotion stage which some natural compounds can inhibit. These inhibitory agents include (-)-epigallocatechin gallate, sarcophytols, laxogenin and allixin (Nishino *et al.*, 1990; Fujiki *et al.*, 1990). It is of interest that some dietary items, *e.g.* garlic, can have protective effects against human cancers due to their inhibitory activity. A few studies using the 2-stage carcinogenesis test have shown that, after tumour initiation by 7,12-dimethylbenz(α)anthracene, inhibitory agents can counteract tumour promotion by TPA, a well-known, strong promoter of mouse skin tumour.

Epstein-Barr virus (EBV), a member of the herpes group, can readily transform normal human lymphocytes into lymphoblasts having an infinite capacity for replication. EBV EA is an EBV-associated product which appears soon after cell infection, and is first detectable by the indirect immunofluorescence method in certain lymphoblastoid cell lines abortively infected by EBV (Henle and Henle, 1966). Antibodies to the EA are commonly found in the sera of patients with nasopharyngeal carcinoma (NPC) which has been strongly associated with EBV infection for many years. In many parts of Asia, there is a fairly high incidence of NPC which may or may not be related to dietary habits or environmental effects. For instance, the high incidence of NPC in the southern region of China has been circumstantially linked to the distribution of plants such as *Aleurites fordii* L. and *Croton tiglium* L. which are commonly used as herbal medicines (Ito *et al.*, 1983). Many plants in Malaysia belonging to the Euphorbiaceae and Thymelaeaceae families have EBV-activating capacities (Yadav *et al.*, 1989). Faced with these potential cancer stimulators, we have also observed reports of natural substances which are anti-cancerous (Komiyama *et al.*, 1992; Nishino *et al.*, 1992) or which protect against cancer, derived from the widely grown oil palm, *Elaeis guineensis*. The major palm-oil vitamin E components, *viz.* α-tocotrienol (22%), γ-tocotrienol (38%) and α-tocotrienol (12%) (Goh *et al.*, 1990), rarely found in the other major vegetable oils, exert a number of biological activities including antioxidant, serum and low-density lipoprotein (LDL) cholesterol-lowering activities (Tan *et al.*, 1991). Earlier studies (Kato *et al.*, 1985; Komiyama *et al.*, 1989) have indicated that tocotrienols have an anti-tumour effect, while a Japanese study (Nishino *et al.*, 1992) indicated that palm oil carotenoids also possess anti-cancer activity.

In an effort to delineate the anti-cancer components, we have now purified various natural tocopherol and tocotrienol compounds, and examined the individual vitamin E compounds as well as some of their dimers for their *in vitro* activity against tumour promotion. We carried out the short-term *in vitro* assay (Ito *et al.*, 1981) by utilizing the activation of EBV EA expression in EBV-genome-carrying human lymphoblastoid cells by TPA, and observing the inhibitory effect exerted by individual tocopherols, tocotrienols and dimers of the γ-homologues at various concentrations.

## MATERIAL AND METHODS

### *Chemicals*

Vitamin E concentrate-containing tocotrienols were obtained from palm fatty acid distillate (PFAD). Briefly, saturated fatty acids in the PFAD were reduced by crystallization, and the remaining fatty acids were further removed by esterification and distillation to give a residual vitamin-E concentrate. Individual vitamin-E compounds were isolated from the concentrate by silica gel column chromatography, using 2-5% of ethyl acetate in n-hexane as eluting solvent (Goh *et al.*, 1990). Dimers derived from the oxidation of γ-tocotrienol or γ-tocopherol by alkaline ferricyanide were also purified by chromatographic methods (Goh *et al.*, 1990, 1992). All compounds used in testing for inhibition of tumour promotion are 97% HPLC pure. TPA and sodium n-butyrate (Sigma St Louis, MO), used as inducers of EBV, were dissolved in dimethylsulfoxide (Sigma) and further diluted with culture medium.

### *Cell lines and tissue culture medium*

The Raji cell line used in this study, comprising EBV-genome-carrying human lymphoblastoid cells, was kept in RPMI 1640 medium (Flow, Irvine, UK) supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (120 μg/ml), fungizone (50 μg/ml), 10% FCS (Flow) as a static suspension culture, and cultured at 37°C in a humidified atmosphere of 5% $CO_2$ in air.

### *Assay of EBV early antigens*

Raji cells were grown to a density of $1 \times 10^6$ cells/ml, pelleted by low-speed centrifugation and resuspended in culture medium containing 4 mM n-butyrate, 10 ng/ml TPA and varying amounts of the vitamin E compounds (*i.e.*, 0, 10, 20, 40, 80 and 160 μg/ml); each experiment was carried out in duplicate. The cells were incubated for 72 hr in a humidified incubator at 37°C with 5% $CO_2$ in air. The cells were then harvested, suspended in PBS, pH 7.2, and placed on a multi-well Teflon-coated slide, dried under cold air, then fixed in cold acetone (−20°C) for 10 min. EBV-positive serum (EA titre 1:1,280) obtained from NPC patients at Kuala Lumpur General Hospital, was used to detect the presence of EBV EA by means of the indirect immunofluorescence assay (Henle and Henle, 1966). The conjugate used was anti-human IgG (γ-chain) fluorescein-conjugate, prepared in rabbits (Behring, Marburg, Germany). Serum at a dilution of 1:40 was dispensed into the wells of the multi-test slide and incubated in a

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$\alpha$-T, $\alpha$-T$_3$ : $R^1 = R^2 = R^3 = Me$

$\gamma$-T, $\gamma$-T$_3$ : $R^1 = H, R^2 = R^3 = Me$

$\delta$-T, $\delta$-T$_3$ : $R^1 = R^2 = H, R^3 = Me$

Tocopherols (T) : R = $CH_2(CH_2CH_2CH_2)_3H$

Tocotrienols ($T_3$) : R = $CH_2(CH_2CH{=}CCH_2)_3H$

FIGURE 1 – Structures of tocopherols and tocotrienols.

humidity chamber at 37°C for 45 min. The slides were washed with PBS for 10 min, and reincubated with a 1:15 dilution of the fluorescein-isothiocyanate-conjugated IgG for 45 min. After washing with PBS, the slides were dried under cold air and mounted in glycerol buffer and the fluorescence was examined with a UV-microscope. The inhibitory activity was evaluated by the following grades according to the method of Ohigashi *et al.* (1992): (a) positive: total inhibition of EBV EA expression, (b) weakly positive: partial inhibition of EBV EA expression, and (c) negative: no inhibition of EBV EA expression.

## RESULTS

The Raji cells treated only with TPA and n-butyrate induced 30% EBV EA, and no spontaneous induction was noted. The inhibitory activity of vitamin E compounds against EBV activation has been evaluated by various concentrations of the compounds. Any inhibition in TPA-induced EBV EA expression by vitamin E compounds would indicate positive activity against tumour promotion. The results regarding inhibition of EBV EA expression by various vitamin E compounds are given in Table I. Total inhibition of the EBV EA was observed when pure $\gamma$- and $\delta$-tocotrienols were applied at levels as low as 20 $\mu$g/ml cell culture, whereas $\alpha$-tocotrienol provided inhibition only at a concentration of 80 $\mu$g/ml or higher. $\alpha$- and $\gamma$-tocopherols did not inhibit EBV EA at these concentrations (10-160 $\mu$g/ml), but $\delta$-tocopherol showed weak inhibition of tumour promotion at 80 $\mu$g/ml and enhanced inhibition at higher concentrations. Results also showed that all oxidative dimeric compounds derived from either $\gamma$-tocotrienol or $\gamma$-tocopherol did not cause any inhibition of the EBV EA expression.

TABLE I – INHIBITION OF TUMOUR PROMOTION BY VITAMIN E COMPOUNDS AND THEIR DIMERS

| Vitamin E[1] | Inhibition of EBV EA expression by vitamin E at various concentrations ($\mu$g/ml cell culture) | | | | |
|---|---|---|---|---|---|
| | 10 | 20 | 40 | 80 | 160 |
| $\alpha$-T | – | – | – | – | – |
| $\alpha$-T$_3$ | – | – | – | – | + |
| $\gamma$-T | – | – | – | – | – |
| $\gamma$-T$_3$ | – | weakly + | + | + | + |
| $\delta$-T | – | – | – | weakly + | + |
| $\delta$-T$_3$ | – | + | + | + | + |
| $\gamma$-TDED | – | – | – | – | – |
| (*R*)-bi-5,5′-$\gamma$-T | – | – | – | – | – |
| $\gamma$-T$_3$DED | – | – | – | – | – |
| (*S*)-bi-5,5′-$\gamma$-T$_3$ | – | – | – | – | – |
| (*R*)-bi-5,5′-$\gamma$-T$_3$ | – | – | – | – | – |

[1]T, tocopherol; $T_3$, tocotrienol (Fig. 1); TDED, tocopherol diphenyl ether dimer; bi-5,5′-$\gamma$-T, bi-5,5′-$\gamma$-tocopherol dimer; $T_3$DED, tocotrienol diphenyl ether dimer; bi-5,5′-$\gamma$-$T_3$, bi-5,5′-$\gamma$-tocotrienyl dimer. Inhibition of tumour promotion is presented as described in the text: positive (+) = complete inhibition of EBV EA expression: weakly positive (weakly +) = partial inhibition of EBV EA expression; and negative (–) = no inhibition of EBV EA expression.

## DISCUSSION

Tocopherols and tocotrienols are excellent antioxidants in inhibiting lipid peroxidation, and their chain-breaking antioxidant activity is associated with the properties of donating phenolic protons and formation of phenoxy free radicals (Goh *et al.*, 1990). In comparison with the tocopherols, the higher mobility of the corresponding tocotrienols due to a more compact isoprenoid chain with triunsaturation may partly explain the superior antioxidant activity of the tocotrienols (Serbinova *et al.*, 1991). The potential inhibitory activity against tumour promotion of the pure tocopherol and tocotrienol isomers has been studied to elicit the *in vitro* anti-tumour effect of the vitamin E compounds. Although all tocopherols and the corresponding tocotrienols have similar chromanol structures and possess comparable antioxidant activities, only tocotrienols (especially the $\gamma$- and $\delta$-homologues) exhibit inhibition of tumour promotion. The actual mechanism by which these compounds block multi-stage events in carcinogenesis is not known; it may not involve direct action of their antioxidant activity, but requires a unique structure which incorporates a less substituted chromanol moiety and a triunsaturated side chain, as present in the natural tocotrienols. These results indicate that the less methylated tocols ($\gamma$- and $\delta$-homologues) are substantially more active than the $\alpha$-homologues with a trimethylated chromanol moiety, and appeared to be inversely related to the antioxidant activity order (kinetic) of the vitamin E compounds (Burton and Ingold, 1981). To further examine the relationship between inhibition of tumour promotion and the antioxidant activity of the vitamin E compounds, we used dimers of $\gamma$-tocotrienol which also possess excellent antioxidant properties (Goh *et al.*, 1990) and the unchanged triunsaturated side chain but have a modified chromanol nucleus which is fully substituted because of dimerization at the 5-position. Although $\gamma$-tocotrienol has shown strong inhibition of EBV EA expression, its bulky dimers including $\gamma$-tocotrienol diphenyl ether dimer ($\gamma$-$T_3$DED) and (R)- and (S)-5,5′-bi-$\gamma$-tocotrienyls do not possess this activity. Likewise, dimers of $\gamma$-tocopherol, *i.e.* $\gamma$-tocopherol diphenyl ether dimer ($\gamma$-TDED) and (R)-5,5′-bi-$\gamma$-tocopheryl, also showed no inhibition of tumour promotion.

Earlier studies (Sundram *et al.*, 1989) have shown that rats fed palm-oil diets have a lower incidence of mammary cancers than those fed other dietary fats, and these results were partly attributed to the presence of carotenoids or tocopherols and tocotrienols. The inhibition of tumour promotion by tocotrienols rather than tocopherols or carotenoids is now shown to be an important factor in the suppression of carcinogenesis. $\gamma$- and $\delta$-tocotrienols present as major components of palm-oil vitamin E are better inhibitors than $\alpha$-tocotrienol, indicating that the triunsaturated isoprenoid side chain and a less substituted chromanol moiety (not methylated at 5-position or 5- and 7-positions) are essential structural features for tocotrienols to exhibit this activity. Tocotrienols occur in many plants and may partly explain why dietary intake of fruit and vegetables is protective against cancer (Kato *et al.*, 1985; Komiyama *et al.*, 1989, 1992) and other degenerative diseases. The present results suggest that a health benefit may be derived from the widespread use of palm oil as a dietary fat, particularly in Asian regions in view of the relatively high incidence of NPC caused by Epstein-Barr virus.

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# Unusual accumulation of α-tocopherol in rabbits fed palm-oil diets enhanced with various vitamin E components

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**ABSTRACT** The distributions of tocopherols and tocotrienols in rabbit plasma and liver were determined by feeding rabbits with various semisynthetic diets containing γ-tocotrienol, δ-tocopherol or α-tocopheryl acetate as the major vitamin E supplement. A high accumulation of α-tocopherol but not the other vitamin E compounds was observed in all groups. The ratios of tocotrienols: tocopherols and δ-tocopherol:α-tocopherol in the γ-tocotrienol and δ-tocopherol-supplemented diets, respectively, were drastically reduced in the plasma and liver of the rabbits. The low levels of γ-tocotrienol and δ-tocopherol were not due to bioconversion to the α-vitamer by the liver from radiotracer study, and may be due to their higher antioxidant reactivities in combination with the presence of a highly selective α-tocopherol-binding protein in the liver.

**ABSTRAK** Taburan tokoferol-tokoferol dan tokotrienol-tokotrienol dalam plasma dan hati arnab ditentukan dengan memberikan berbagai jenis diet semisintetik yang mengandungi γ-tokotrienol, δ-tokoferol atau α-tokoferil asetat sebagai vitamin E tambahan. Penghimpunan yang tinggi bagi α-tokoferol tetapi bukan komponen vitamin E yang lain diperhatikan dalam semua kumpulan. Nisbah-nisbah untuk tokotrienol:tokoferol dan δ-tokoferol:α-tokoferol dalam diet ditambahkan γ-tokotrienol dan δ-tokoferol masing-masing didapati berkurangan hebatnya dalam plasma dan hati arnab-arnab tersebut. Dari pengkajian radiotracer, paras rendah untuk γ-tokotrienol serta δ-tokoferol bukanlah disebabkan biokonversi kepada α-vitamer oleh hati, tetapi mungkin adalah sebab reaktiviti anti-pengoksidaannya yang lebih tinggi dan juga kehadiran protein pengikat α-tokoferol yang amat selektif dalam hati.

(vitamin E, palm oil, α-tocopherol, γ-tocotrienol, rabbit)

## INTRODUCTION

Natural vitamin E comprises two series of compounds, *viz.* α-, β-, γ- and δ-tocopherols (T) having a saturated side chain, and tocotrienols ($T_3$) having a triunsaturated side chain (Fig. 1). Each of the tocopherols can consist of up to eight stereoisomeric compounds derived from three chiral centres at carbons 2, 4' and 8' in the side chain. The natural *RRR*-α-tocopherol (α-T) and its stereoisomers have been widely studied in relation to their chemistry and biological activities, whereas the other vitamin E compounds, although having a wid distribution in plants and vegetable oils, have not re ceived sufficient attention previously. However, ther is growing interest concerning the biological impor tance of the tocotrienols, particularly their hypo cholesterolaemic [1-4] and anticancer [5-7] activities To explore the pharmacological uses of tocotrienols it is of importance to understand the bioavailability o

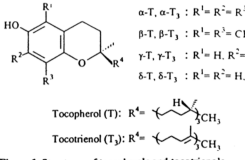


**Figure 1.** Structures of tocopherols and tocotrienols.

these compounds and their distribution in organs. Many studies on the distribution of tocopherols [8-11] have shown that the concentration of α-T is a few times higher than that of γ-tocopherol (γ-T) in human plasma despite intake of diets containing more γ-T than α-T. Various reasons have been advanced for this observation, including discriminating absorption [11], specific binding [12-15], retention [16] and secretion [17] differences among various vitamin E compounds. However, the distribution and fate of many vitamin E constituents, *e.g.* tocotrienols, in humans and animals have not been resolved. The present investigation using supplementation of γ-tocotrienol (γ-$T_3$) derived from palm oil and δ-tocopherol (δ-T) in semisynthetic diet, attempts to provide some information on the distribution and bioavailability of these vitamin E compounds in rabbits.

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## MATERIALS AND METHODS

New Zealand White rabbits fed *ad libitum* semisynthetic diets were divided into groups based on the supplementation of vitamin E components as follows: (a) refined-bleached-deodorized palm olein (RBDPO) depleted of α-T, plus 186 mg γ-$T_3$ per kg diet (RBDPO+γ-$T_3$), (b) RBDPO depleted of α-T, plus 863 mg δ-T per kg diet (RBDPO+δ-T), and (c) RBDPO enhanced with *dl*-α-tocopheryl acetate, α-TAc (RBDPO+α-T). Rabbits fed with commercial pellets were also used for comparison. RBDPO depleted of vitamin E was prepared by column chromatography using 0.063-0.200 mm neutral alumina (Merck) and n-hexane; the α-T and α-tocotrienol (α-$T_3$) contents in this oil were reduced to less than 10 and 5 ppm respectively. All the semisynthetic diets contained 5% (w/w) commercial rabbit pellets and 95% purified feed materials consisting of 15% RBDPO, 20% casein, 40% dextrose monohydrate, 20% fibre, and 0.5% cholesterol, the remainder being vitamins and mineral mixtures. Vitamin supplements specifically omitting vitamin E were used in the diets depleted of vitamin E, *i.e.* for RBDPO+γ-$T_3$ and RBDPO+δ-T groups. All the purified materials were purchased from US Biochemical Corporation. γ-$T_3$ was purified from a tocotrienol mixture isolated from palm fatty acid distillate (PFAD) [18,19]. δ-T was purchased from Sigma Chemical Co. (St. Louis, MO), and was purified by 40-63 μm (Merck) silica gel column chromatography prior to adding it to the diet. Compositions of the vitamin E compounds in various diets are given in Table 1. All rabbits were treated with respective dietary regimes for a duration of 12 weeks. At the end of dietary treatment, blood samples were collected from the overnight-fasted animals. Vitamin E compounds were extracted from plasma according to the method of Handelman *et al.* [20], vitamin E in the liver was extracted using the procedure of Lang *et al.* [21]. Analyses of the tocotrienols and tocopherols were carried out by normal phase high performance liquid chromatography (HPLC) using a 250 x 4.6 mm, 5 mm silica column and fluorescence detector (Waters 470); hexane-tetrahydrofuran-isopropanol (973.5:25: 1.5) was used as the mobile phase.

The following *in vitro* experiment was also attempted to investigate structural change of δ-tocotrienol (δ-$T_3$) incubated with rabbit's liver microsomal enzymes. Liver was excised from anaesthetized rabbit fed with commercial feed, quickly washed with ice-cold saline solution and homogenized at 4°C in 10 volumes of ice-cold 0.05 M Tris buffer (pH 7.4) containing 0.3 M sucrose, 10 mM EDTA, 50 mM NaCl and 1 mM dithiothreitol, using a motor-driven tissue homogeniser [22]. The homogenates were centrifuged at 600 g for 5 min to obtain the supernatant. Microsomal fraction was prepared by differential centrifugation at 15,000 g for 15 min using Beckman L8-80M ultracentrifuge equipped with a swing bucket rotor SW28 at 4°C to remove mitochondria and cell debris; the supernatant was further centrifuged at 105,000 g for 60 min to obtain sediment microsomal pellet, which was resuspended in 0.05 M Tris buffer, pH 7.4, at a concentration of 1.0 g equivalent of liver tissues per mL of suspension. Two mL of the microsomal suspension was mixed with 2 mg δ-$T_3$ in 100 μL 0.00625% of Tween 20 (0.25% w/v in acetone), 2.2 mg ATP, 3.06 mg NADPH and adenosyl-L-methionine-S-(methyl-$^{14}$C) 0.1 μCi (from NEN) [23]. The mixture was adjusted to a volume of 5 mL with buffer, incubated and gently agitated for 8 hr at 37°C. Aliquots (2.5 mL) were taken and vitamin E was extracted by ethanol-hexane (1:1). Various vitamin E compounds were separated by TLC using hexane-ethyl acetate (9:1) as mobile phase. Radioactivity was determined for each vitamin E fraction using a Packard Liquid Scintillation Analyser 2500TR.

**Table 1.** Composition of the vitamin E compounds in various diets (in ppm).

| Vitamin E | Diets | | | |
|---|---|---|---|---|
| | RBDPO+γ-$T_3$ | RBDPO+δ-T | RBDPO+α-T | Commercial feed |
| α-TAc[a] | 0 | 0 | 176 | 0 |
| α-T | 6 | 15 | 20 | 22 |
| α-$T_3$ | 3 | 9 | 14 | 5 |
| γ-T | 0 | 34 | 0 | 0 |
| γ-$T_3$ | 186 | 32 | 22 | 16 |
| δ-T | 0 | 863 | 0 | 0 |
| δ-$T_3$ | 14 | 12 | 5 | 5 |
| Total | 209 | 965 | 240 | 48 |
| % α-T | 2.9 | 1.6 | 81.7 | 45.8 |
| $T_3$/T[b] | 1:0.03 | 1:17.2 | 1:4.4 | 1:0.85 |

[a]the weight of *dl*-α-tocopheryl acetate (α-TAc) is given in α-T equivalent.
[b]$T_3$/T is the ratio of total tocotrienols to tocopherols.

## RESULTS

The concentration of vitamin E compounds in plasma of rabbits fed various diets is presented in Table 2. All rabbits fed semisynthetic diets fortified with various vitamin E showed elevated level of total plasma vitamin E which was 7- to 9-fold higher than those fed commercial pellets; the major increase was contributed by tocopherols, particularly α-T which constituted more than 94% of the total vitamin E. For rabbits fed diet with low content in α-T but enriched with $\gamma$-$T_3$ (*i.e.* RBDPO+$\gamma$-$T_3$ diet group), the level of α-T (20.78 ± 4.34 μg/mL) was disproportionately high; whereas the plasma $\gamma$-$T_3$ level was very low (0.04 μg/mL) similar to those found in other groups without $\gamma$-$T_3$ supplement. Despite the absence of γ-T in the diet, plasma γ-T level for the RBDPO+$\gamma$-$T_3$ rabbits was increased to 0.40 μg/mL, which was significantly higher than that for RBDPO+α-T rabbits (0.08 μg/mL) treated with lesser $\gamma$-$T_3$ supplement. For rabbits fed a diet low in α-T content but enriched with δ-T (*i.e.* RBDPO+δ-T dietary group), giving a δ-T:α-T ratio = 1:0.017, most of the δ-T appeared to have disappeared whereas high level of α-T (26.10 ± 13.75 μg/mL; δ-T:α-T = 1:65) was found in the plasma. Although very much lesser amount of γ-T was given to rabbits with a RBDPO+δ-T diet, *i.e.* 34 mg γ-T as compared to 863 mg δ-T per kg diet, the plasma γ-T level (1.07 ± 0.45 μg/mL) was significantly ($p < 0.01$) higher than the δ-T level (0.40 ± 0.14 μg/mL); this γ-T level was also significantly ($p < 0.05$) higher than that in RBDPO+$\gamma$-$T_3$ and RBDPO+α-T groups.

The concentration of vitamin E compounds in the liver is given in Table 3. The total vitamin E level was remarkably increased following supplementation of either normal dosage of $\gamma$-$T_3$ or high dosage of δ-T. However, the major vitamin E was α-T regardless of the type of vitamin E consumed. The concentration of α-T in the liver of rabbits fed RBDPO+δ-T diet (fed 863 mg δ-T per kg diet) was about two-fold higher than that in rabbits fed RBDPO+$\gamma$-$T_3$ diet (fed 186 mg $\gamma$-$T_3$ per kg diet), indicating that increase of α-T was disproportionately dose-dependent. Compositional changes of vitamin E compounds in the plasma and liver as compared to the feed for RBDPO+$\gamma$-$T_3$ and RBDPO+δ-T rabbits are shown by HPLC profiles in Figures 2 and 3, respectively, showing an obvious similarity of vitamin E composition in plasma and liver of each group.

Results of the *in vitro* experiment using radiolabelled S-adenosyl-$^{14}$C-methyl-methionine as the tracer are given in Table 4. There was no obvious increase of radioactivity in the α-T and other vitamin E components.

## DISCUSSION

The compositions of tocopherols and tocotrienols in the plasma of rabbits fed diets enchanced with tocotrienols show consistently very low levels of tocotrienols in contrast to an apparent differential retention of tocopherols, with α-T being predominant. Low levels of tocotrienols in the plasma of humans and hamsters after

**Table 2.** Composition of the vitamin E compounds (μg/mL) in plasma of treated rabbits.

| Vitamin E | Diets | | | |
|---|---|---|---|---|
| | RBDPO+$\gamma$-$T_3$ (n=5) | RBDPO+δ-T (n=6) | RBDPO+α-T (n=6) | commercial feed (n=3) |
| α-T | 20.8 ± 4.3 | 26.10± 13.7 | 27.5 ± 4.2 | 3.2 ± 1.3 |
| α-$T_3$ | 0 | 0 | 0 | 0 |
| γ-T | 0.40 ± 0.26 [a,c] | 1.1 ± 0.4 [b,c] | 0.08 ± 0.06 [c] | 0.03 ± 0.01 |
| γ-$T_3$ | 0.04 ± 0.02 | 0 | <0.01 | <0.01 |
| δ-T | 0.03 ± 0.03 [a] | 0.40± 0.14 [b] | 0.02 ± 0.02 | <0.01 |
| δ-$T_3$ | <0.01 | 0 | 0 | 0 |
| Total | 21.2 ± 4.5 | 27.6 ± 14.3 | 27.6 ± 4.2 | 3.2 ± 1.3 |
| % α-T | 97.8 ± 1.0 | 94.3 ± 1.1 | 99.62 ± 0.22 | 98.9 ± 0.5 |
| $T_3$:T | 1:>424 | nil:1 | 1:>2759 | 1:>320 |

n, number of rabbits in each group. Statistically significant difference at $p < 0.01$ [a,b] or $p < 0.05$ [c,d].

**Table 3.** Composition of the vitamin E compounds (μg/g) in the liver of rabbits.

| Vitamin E | Diets | | |
|---|---|---|---|
| | RBDPO+$\gamma$-$T_3$ | RBDPO+δ-T | Commercial feed |
| α-T | 29.8 ± 8.9 [a] | 53.7 ± 15.1 [a] | 9.6 ± 0.3 |
| α-$T_3$ | 0 | 0 | 0 |
| γ-T | 0.56 ± 0.55 [b,c] | 2.52 ± 0.72 [c,d] | 0.02 ± 0.005 |
| γ-$T_3$ | 0.02 ± 0.04 [b] | 0.03 ± 0.05 | 0 |
| δ-T | 0.01 ± 0.03 [d] | 1.37 ± 0.48 [d] | 0 |
| δ-$T_3$ | 0 | 0 | 0 |
| Total | 30.4 ± 9.3 [a] | 57.6 ± 15.8 [a] | 9.6 ± 0.3 |
| % α-T | 98.4 ± 1.4 | 93.1 ± 1.4 | 99.7 ± 0.1 |
| $T_3$:T | 1:1520 | 1:1920 | nil:1 |

Values are averages ± standard deviation. Statistically significant difference at $p < 0.05$ [a,c] or $p < 0.01$ [b,d].

Table 4. Incubation of liver microsomal fraction with S-adenosyl-$^{14}$C-methyl-methionine.

| Vitamin E fraction | TLC $R_f$ | without -$T_3$ | with -$T_3$ |
|---|---|---|---|
| α-T | 0.65 | 15 | 77 |
| α-$T_3$ | 0.54 | 27 | 17 |
| γ-T | 0.42 | 30 | 94 |
| γ-$T_3$ | 0.30 | 243 | 134 |
| δ-T | 0.20 | 19 | 21 |
| δ-$T_3$ | 0.11 | 57 | 39 |

Values given are counts per minute average, value for a blank solution is 21, for radiolabelled S-adenosyl-$^{14}$C-methyl-methionine, 0.1 μCi, is 1.02x10$^3$.

supplementation of palm-oil vitamin E, containing both tocotrienols and tocopherols, have also been noted by previous workers [24-26]. Previous reports on the absorption of α-T, γ-T and radiolabelled tocols showed that there was no absorptive discrimination of various tocopherols [8, 17, 27]. The preferential accumulation of α-T over γ-T in human or hamster liver has been observed before [20, 28], and this was unlikely to be due to a preferential excretion of the γ-T into bile [17, 26]. Vitamin E deposited in adipose tissues had more tocotrienols than tocopherols (for hamsters fed diets supplemented with tocotrienols) [26], because the turnover or release rate of vitamin E in adipose tissue is much slower than in other organs [29, 30]. Discrimination of tocopherol homologs based on the structure of chroman ring and stereochemistry of phytyl side chain [31-33] may be possible in the liver as a highly specific hepatic binding protein which exhibited high affinity for α-T has been reported [12-15]; however, these metabolic pathways do not fully explain the disappearance of tocotrienols and unusual elevated level of α-T in the present study. Our results suggest that there is a clearance of tocotrienols from plasma, possibly by the liver, since transport of various vitamin E compounds is regulated by the liver [34], and similar compositions of vitamin E compounds in the plasma and liver were also observed in this study. Distribution data of various vitamin E compounds in the liver also showed that there was an accumulation of α-T in liver of rabbits even though γ-$T_3$ and δ-T were supplemented to be the major vitamin E components. The presence of γ-T in plasma and liver of rabbits fed RBDPO+γ-$T_3$ diets may indicate that reduction of the unsaturated side chain of γ-$T_3$ gave rise to γ-T which could undergo biomethylation to form α-T. In rabbits fed with a semisynthetic diet depleted of α-T but supplemented with δ-T, remarkable changes of the ratio α-T:γ-T:δ-T = 100:227:5753 in the diet to 100:4.1:1.5 in the plasma and 100:4.7:2.6 in the liver would also suggest a methylation of δ-T to α-T via γ-T (see Fig. 4). However, such transformations are unprecedented even though a slow methylation of γ-T to α-T in Wistar rats was shown after a few generations [35]. However, the present *in vitro* experiment using liver microsomal fraction did not provide evidence for methylation of δ-$T_3$ to γ-$T_3$ or α-$T_3$.

Results of the present study are explained as due to

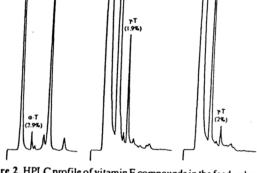


**Figure 2.** HPLC profile of vitamin E compounds in the feed, plasma and liver of rabbits treated with RBDPO+ γ-$T_3$. Values are the average percentage (by weight) of individual vitamin E compounds in the samples.

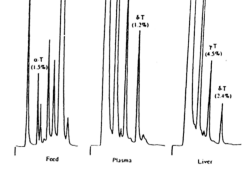


**Figure 3.** HPLC profile of vitamin E compounds in the feed, plasma and liver of rabbits treated with RBDPO+ δ-T. Values are the average percentage (by weight) of individual vitamin E compounds in the samples.

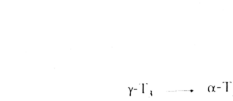


**Figure 4.** Possible methylation and reduction pathways in bioconversion of tocotrienols to α-tocopherol.

a selective accumulation of α-T while a rapid clearance of tocotrienols occurred. Possible explanations for these observations are that preferential retention of α-T is caused by a specific binding protein which is extremely specific for α-T, whereas tocotrienols which have *in vivo* reactivities much higher than α-T are selectively lost by oxidation. Data of Table 2 seems to indicate a bioconversion of tocotrienols to tocopherols although the present *in vitro* experiment made this less likely. It is observed that α-T is persistently high even when the animals were fed $\gamma$-$T_3$ and δ-T; this could mean that even though there is no bioconversion of vitamin E components to α-T in the liver, the process could be effected by microflora in the rabbit's alimentary tracts.

Further studies on the metabolism of tocotrienols in other organs would be needed to justify the bioavailability of tocotrienols and the actual mechanism of their *in vivo* activities. This is of importance if their radical-scavenging properties [18] and anticancer activities [5-7] are to be realized.

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