Enzymatic Modification for Ascorbic Acid and Alpha-Tocopherol to Enhance their Stability in Food and Nutritional Applications

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Abstract: Antioxidants protect cells against the effects of harmful free radicals and play an important role in preventing many human diseases (e.g. cancer, atherosclerosis, neurodegeneration, inflammatory disorders, etc.) and aging itself. In addition, antioxidant molecules are employed to prevent unsaturated oil products from becoming rancid during storage and thus extend oil life. The modification –chemical or enzymatic- of natural antioxidants in order to increase their miscibility and/or stability towards the action of light and/or oxygen renders a series of "semisynthetic" antioxidants with great impact in the food and feed industries. In this review, we will discuss the enzymatic modifications of antioxidant vitamins C and E. L-Ascorbic acid (vitamin C) is the major water-soluble natural antioxidant acting as a free radical scavenger, and plays an important role in regenerating vitamin E. However, due to the low miscibility of ascorbic acid with α-tocopherol, it is necessary to use ascorbyl fatty acid derivatives. Thus, esters of L-ascorbic acid with long-chain fatty acids (esp. palmitic or stearic) are employed as additives (E-304) in foods rich in lipids. The enzymatic synthesis of acyl L-ascorbates offers some advantages compared with the current chemical process, such as its high regioselectivity and the moderate reaction conditions. Vitamin E enhances the oxidative stability of polyunsaturated fatty acids from peroxidation acting as a free radical scavenger and is generally administered in the form of *all-rac*-α-tocopheryl acetate or succinate to increase its stability. Several approaches have been described for the enzyme-catalysed synthesis of vitamin E acetate, based on the transesterification of vinyl acetate with vitamin E, or the regioselective hydrolysis of α-isophorone followed by reaction with isophytol. The above vitamin C and E derivatives may have impact not only as food preservatives but also as components of functional foods.

ANTIOXIDANTS IN HEALTH AND FOOD

Free radicals (e.g. superoxide, nitric oxide, hydroxyl radicals) and other reactive species (e.g. hydrogen peroxide, peroxynitrite hypochlorous acid) are produced in the body, primarily as a result of aerobic metabolism, causing the so-called oxidative stress. Oxidative inhibitors –called antioxidants- (e.g. glutathione, arginine, citrulline, taurine, creatine, selenium, zinc, vitamin E, vitamin C, vitamin A, tea polyphenols) and antioxidant enzymes (e.g. superoxide dismutase, catalase, glutathione reductase and glutathione peroxidases) exert synergistic actions in reducing free-radical lipid oxidation. Over the past three decades there has been growing evidence showing that malnutrition (e.g. dietary deficiencies of protein, selenium, or zinc) or excess of certain nutrients (e.g. iron, vitamin C, selenium) gives rise to the oxidation of biomolecules and cell injury [1].

Antioxidants are substances that when present at low concentration compared to that of an oxidable substrate markedly delay or prevent its oxidation. Antioxidants protect cells against the effects of harmful free radicals. There exists scientific evidence that the excessive production of free radicals in the organism, and the imbalance between their concentration and the antioxidant defenses, may be related to processes such as aging [2] and several diseases such as cancer [3,4], atherosclerosis [5], stroke [6], rheumatoid arthritis [7], neurodegeneration [8], inflammatory disorders [9] or diabetes [10]. The study of antioxidants is of great interest for the role they play in protecting living systems against lipid peroxidation and other anomalous molecular modifications [11]. The mechanisms that are involved include decompositions of peroxides, singlet oxygen inhibition and free-radical acceptors. Efficient suppression of lipid peroxidation is possible only by the concerted action of a chain-breaking antioxidant and a glutathione peroxidase. Depending on the type of lipid and biological compartment, different types of glutathione peroxidases could be involved in the suppression of these radical chain reactions. The reaction principle could also be similarly relevant for the control of inflammation-associated oxidative burst, for the maintenance of integrity of membrane lipids, as well as for the prevention of atherogenesis.

Most antioxidants are common food components, and have been used in the diet for thousands of years. Natural antioxidants can be classified in two groups: (i) those whose ingestion is essential in human feed -nutrients- comprising vitamins C and E and vitamin precursors (carotenoids); (ii) plant-derived compounds of low molecular weight, basically polyphenols (e.g. flavonoids), which have not been demonstrated to be essential for health . In last years, consumers have been increasingly confronted with the so-called functional food products, first introduced in Japan, which are claimed to promote health and well-being beyond their nutritive properties [12,13].

Fats, oils and lipid-based foods deteriorate through several degradation reactions both on heating and on long-term storage [14]. The main deterioration processes are oxidation reactions that result in decreased nutritional value and sensorial quality. The spontaneous reaction of atmospheric oxygen with lipids, known as autooxidation, is the most common process leading to oxidative deterioration (Fig. **1**) [15]. In

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addition to the prevention of oxygen access, the use of low temperatures, the inactivation of enzymes causing oxidation, or a suitable packaging, oxidation may be also inhibited by the use of specific additives (antioxidants) that may vary in their chemical structure and mode of action. Antioxidant molecules prevent unsaturated oil products from becoming rancid during storage, thus extending its shelf life [16,17]. Antioxidants were first used for food preservation before World War II. The most commonly used antioxidants for food preservation are listed in Table **1**.

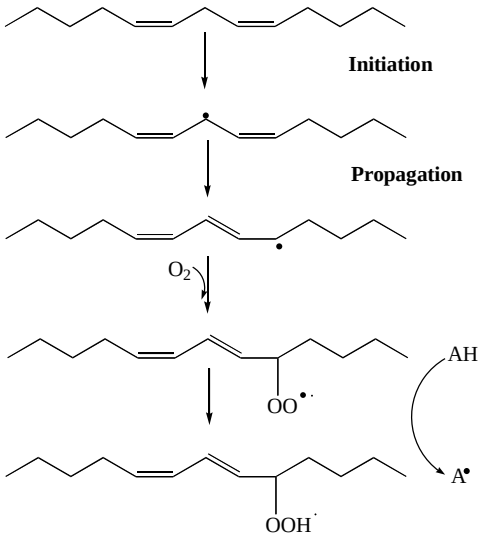

Fig. (1). Antioxidant (AH) action in the phases of lipid peroxidation.

Table 1. Antioxidants Most Commonly Used in Foods

Antioxidant	EC number
L-Ascorbic acid	E300
Sodium L-ascorbate	E301
Calcium L-ascorbate	E302
Potassium L-ascorbate	E303
Fatty acid esters of ascorbic acid esters I. Ascorbyl palmitate II. Ascorbyl stearate	E304
Mixed tocopherols concentrate (natural)	E306
Alpha-tocopherol (synthetic)	E307
Gamma-tocopherol (synthetic)	E308
Delta-tocopherol (synthetic)	E309
Propyl gallate	E310
Octyl gallate	E311
Dodecyl gallate	E312
Erythorbic acid	E315
tert-Butylhydroquinone (TBHQ)	E319
Butylated hydroxyanisole (BHA)	E320
Butylated hydroxytoluene (BHT)	E321
Lecithins	E322
Citric acid	E330
L-Tartaric acid	E334

The autooxidation of lipids starts with an initiation reaction during which free radicals are formed, followed by propagation reactions during which free radicals are converted into other radicals, and finally a termination reaction that involves the combination of two radicals, with the formation of stable products (Fig. **1**). The most common food antioxidants (primary antioxidants) interfere with lipid autoxidation by rapid donation of hydrogen atoms to lipid radicals (Fig. **1**). Alpha-tocopherol (vitamin E) suppresses the propagation of radical chain reactions at the stage of the peroxy radical.

Natural antioxidants such as vitamins C and E are the best accepted for food applications. Synthetic phenolic antioxidants, like *tert*-butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and propyl gallate are not so well accepted by consumers as dietary components, and there has been growing concern over their possible side-effects.

CHEMICAL AND ENZYMATIC MODIFICATIONS OF ANTIOXIDANTS

The modification of natural antioxidants (i) to improve their chemical, oxidative or heat stability, or (ii) to alter their hydrophile-lipophile balance (HLB), yields a series of "semisynthetic" antioxidants (e.g. tocopheryl acetate, L-ascorbyl palmitate) with great impact in the industry. These derivatives may have impact not only as food preservatives but also as components of functional foods.

The antioxidant derivatives are generally prepared using strongly corrosive acids such as sulfuric acid or hydrogen fluoride followed by a re-esterification step [18]. In this context, the chemical synthesis of benzoic and phenolic esters is commonly performed with acid or basic catalysts under reflux [19], but these procedures do not fulfill the requirements needed for food applications. To overcome the shortcomings of conventional processes, new approaches based on the so-called "green chemistry" are being developed.

Green chemistry is defined as the design, development, and application of chemical processes and products to reduce or eliminate the use and generation of substances hazardous to human health and the environment [20]. Biocatalytic processes fully participate in the "green chemistry" concept that was introduced in the 90's and its impact on sustainability is now well established [21]. Biocatalysts (either enzymes or whole-cells) constitute a greener alternative to traditional organic synthesis [22], offering appropriate tools for the industrial transformation of natural (e.g. antioxidants) or synthetic materials under mild reaction conditions, low energy requirements and minimising the problems of isomerisation and rearrangement [23]. In addition, biocatalysts are biodegradable and display chemo-, regio- and/or stereospecificity resulting in decreased by-product formation thus avoiding the need for functional group protection and activation. The relatively recent development of novel recombinant DNA technologies, e.g. (meta)genomics [24] and molecular directed evolution [25], are exerting a profound positive effect in the expression and production of large amounts of recombinant proteins (grams to kilograms, which means more competitive prices), with new- or tailored catalytic activities.

The enzymatic methods to modify the two major vitamin antioxidants C and E will be further reviewed.

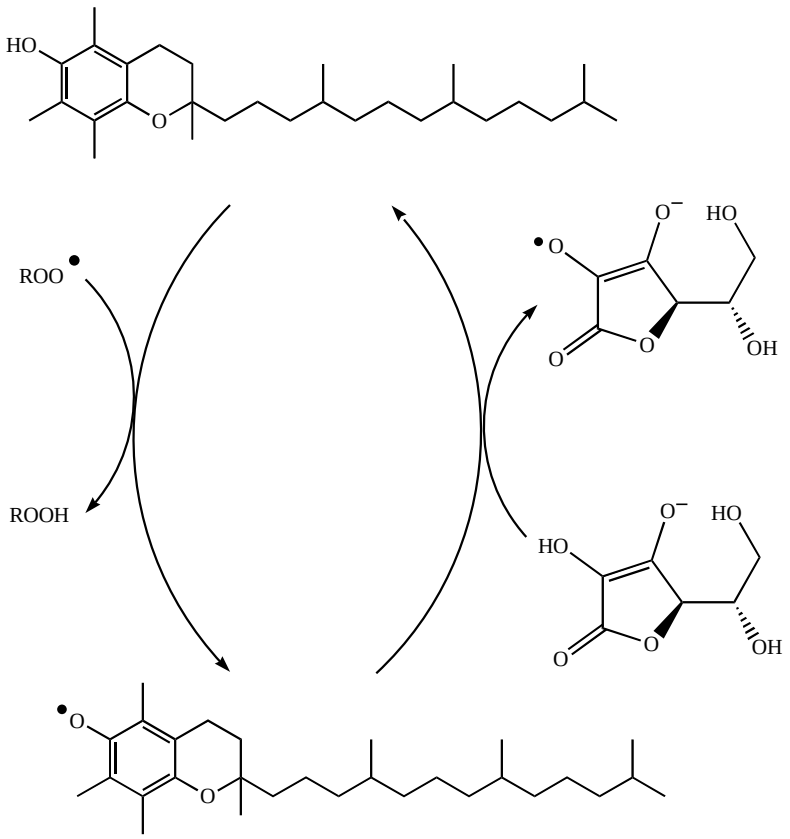

Fig. (2). Vitamin E recycling system.

ENZYMATIC MODIFICATIONS OF L-ASCORBIC ACID

L-ascorbic acid (vitamin C) belongs to the group of vitamin-based antioxidants and is the major water-soluble natural antioxidant. Acting as a free radical scavenger, L-ascorbic acid and its derivatives react with oxygen, thus removing it in a closed system. The combination of L-ascorbic acid and primary antioxidants like α-tocopherol [26,27] makes a synergic effect that results in the *"vitamin E recycling system"* (Fig. **2**) [28]. This mixture of vitamins is usually added to cookies, pastes, meat products and other fatty products to maintain their quality and extend their shelf-life. However, due to the low miscibility of L-ascorbic acid with α-tocopherol or with any oil-based formula, it is necessary to use ascorbyl fatty acid derivatives instead of vitamin C. It has been demonstrated that the addition of one or more hydrocarbon chains to the ascorbic acid ring, preferably in the 6-position, retains or even enhances the physiological and antioxidant activity performed by the vitamin C [28,29]. Thus, esters of L-ascorbic acid and long-chain fatty acids (Fig. **3**) (esp. palmitic or stearic, E-304) are employed as additives for the stabilization of fats, oils and fatty products, as they retard the autooxidation of unsaturated fatty acids.

Although they are more soluble in fats than the L-ascorbic acid itself, its still poor solubility in edible fats and oils and its insolubility in cold water, limit their use [30]. There are other kinds of ascorbic acid fatty acid esters, such as oleate or linoleate, which present different characteristics and could be used also as foods additives (E-304). These unsaturated fatty acids increase the miscibility with α-tocopherol, oils or fatty products.

Nowadays, palmitate (and stearate) ascorbyl esters are produced synthetically by reacting ascorbic acid with sulphuric acid followed by re-esterification with palmitic acid (or stearic acid), and subsequently purified by recrystallization [30]. This chemical process has some clearly shortcomings such as the use of strong acids and the formation of products mixtures that need complex purification protocols [31,32].

As an alternative, the enzyme-catalysed synthesis of acyl L-ascorbates offers some advantages, such as its high regioselectivity and the moderate reaction conditions [33]. Lipases, esp. that from *C. antarctica*, have been successfully used to catalyse the enzymatic synthesis of ascorbyl derivatives employing saturated and unsaturated free fatty acids, alkyl or vinyl esters as acyl donors (Table **2**) [34]. Tertiary alcohols and acetone are commonly employed as reaction media. The latter is especially appropriate as it is inexpensive, volatile and permitted for use in the manufacture of food products (directive 84-344-CEE) [35]. The use of ionic liquids as greener media for the acylation of L-ascorbic acid has been also described [36].

Fig. (**4**) shows the acylation progress of L-ascorbic acid with vinyl palmitate using lipase B immobilised in two different supports: the ion-exchange resin Lewatit (Novozym 435) and polypropylene (Accurel EP100) [37,38]. Experimental conditions were: 20 mM ascorbic acid, 100 mM vinyl palmitate, 2.5 mg/ml biocatalyst, 2-methyl-2-butanol/DMSO 95:5 (v/v), 40°C, 150 rpm. As shown, the reaction is very fast with both biocatalysts, giving approx. 90% conversion in 8 h. Interestingly, when assaying ethyl palmitate as acyl donor under similar conditions the reaction is significantly

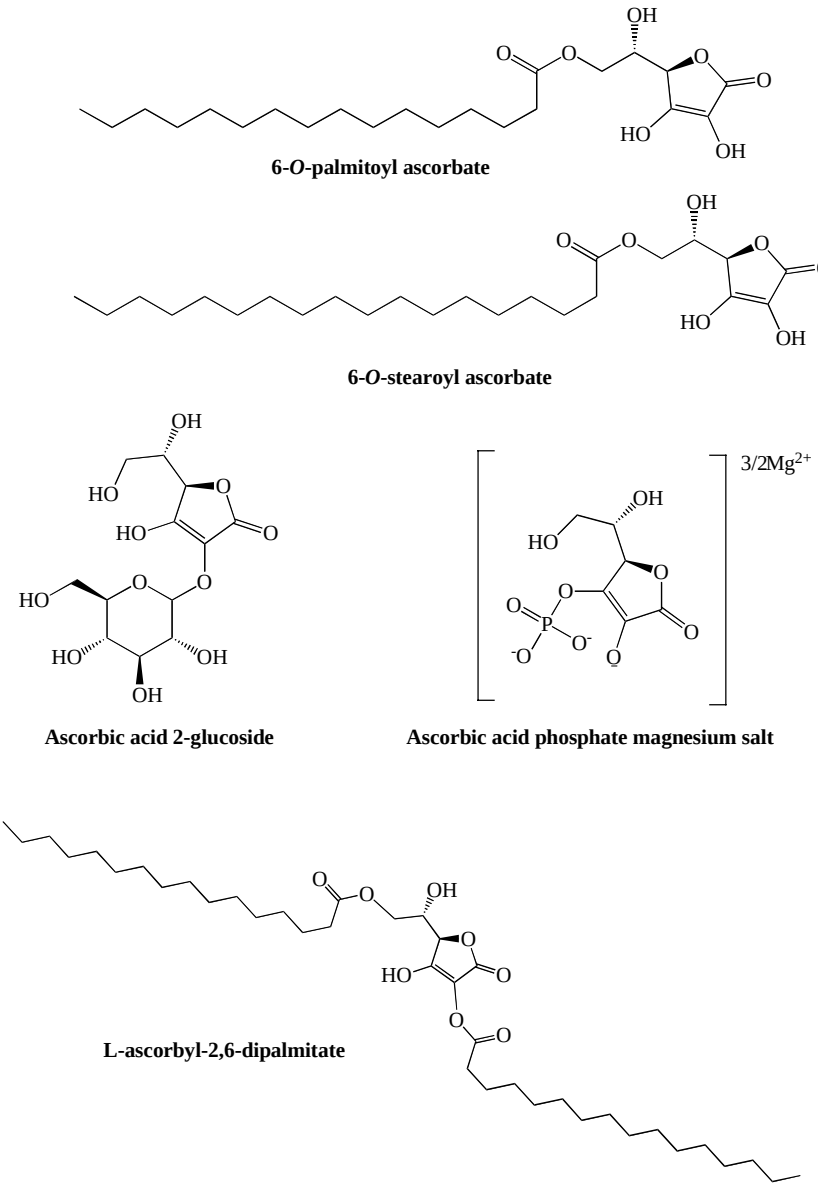

Fig. (3). Commercial derivatives of L-ascorbic acid.

slower (Fig. **4**). When using vinyl palmitate, the reaction yields vinyl alcohol as leaving group, which rapidly tautomerises to acetaldehyde and can be removed by evaporation as its boiling point is only 21 °C, thus displacing the equilibrium and making the reaction completely irreversible [39]. Although good yields and conversions have been achieved using the lipase B from *C. antarctica*, these processes have not been adopted by the industry.

Other vitamin C derivatives are commercially available (Fig. **3**). The novel vitamin C derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (ascorbic acid 2-glucoside) is synthesised enzymatically [33,50] and its biological activity has been evaluated *in vitro* and *in vivo* [51]. The stability of the other transglycosylated ascorbic acid derivatives, such as 6-*O*-alpha-D-glucosyl- and 6-*O*-alpha-D-maltosyl-ascorbic acid, was greatly enhanced against *in vitro* oxidation. The antioxidant effects of glycosyl-derivatives of ascorbic acid on the lipid oxidation in cooked chicken breast meat patties indicated that they had antioxidant activities similar to that of ascorbic acid [52]. It was suggested that the transglycosylated ascorbic acids can possibly be applied as effective antioxidants with improved stability in food, cosmetic, and other applications [53,54]. L-ascorbyl phosphate (magnesium salt) is very stable in creams and lotions, and is readily absorbed through the skin (Fig. **3**). After absorption, it is quickly hydrolysed to vitamin C, which suppresses the skin pigmentation action by melanin. Ascorbyl 2,6-palmitate (Fig. **3**) is also marketed [55]; it is an stable agent that is applied as anti-stress agent for animals in feed compositions [56].

STABILIZATION OF TOCOPHEROLS (VITAMIN E)

Among the natural antioxidants, the term vitamin E describes the beneficial biological activity on humans of a group of structurally related compounds, in particular α, β, γ and δ-tocopherol, and α, β, γ and δ-tocotrienol [30]. Vitamin E enhances the resistance to oxidation of the organisms, owing to its ability to protect polyunsaturated fatty acids

Table 2. Enzymatic Synthesis of Fatty Acid Derivatives of L-Ascorbic Acid

Acyl donor	Solvent	Biocatalyst	Yield (%)	Ref
Methyl palmitate	2M2B[a]	Novozym 435[b]	68	[31]
Phenylbutyric acid	*t*-butanol	Novozym 435	22	[40]
Methyl palmitate, EPA and DHA ethyl esters [d]	2M2B	Novozym 435	≤ 40	[41]
Saturated fatty acids	2M2P[c]	Novozym 435	≤ 65	[42]
Vinyl esters (C8-C16)	*t*-butanol	Chyrazyme L-2 [b]	≤ 91	[32]
Palmitic acid	Hexane	Lipase from *Bacillus stearo-thermophilus*	≤ 97	[43]
EPA[d]	Acetone	Chyrazyme L-2	47	[44]
L-Methyl lactate	2M2B	Novozym 435	80	[45]
Oleic acid, Palmitic acid	2M2B	Novozym 435	71-87	[46]
Saturated fatty acids (C10-C14)	Acetone	Chyrazyme L-2	≤ 60	[47]
Oleic acid, Linoleic acid, Linolenic acid	Acetone	Chyrazyme L-2	60	[48]
Oleic acid	Ionic liquid	Novozym 435	61	[49]
Oleic acid, Linoleic acid	2M2B	Novozym 435	≤ 45	[29]

[a]2-methyl-2-butanol (*t*-amyl alcohol).
[b]Different immobilised preparations of lipase B from *C. antarctica*.
[c]2-methyl-2-pentanol.
[d]EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.

from peroxidation and to scavenge free radicals. It is a primary antioxidant as it terminates the free radical chains in lipid oxidation [26]. Vitamin E has an important presence in the animal nutrition market, where high doses of this antioxidant are applied to improve the quality and shelf-life of meat [30].

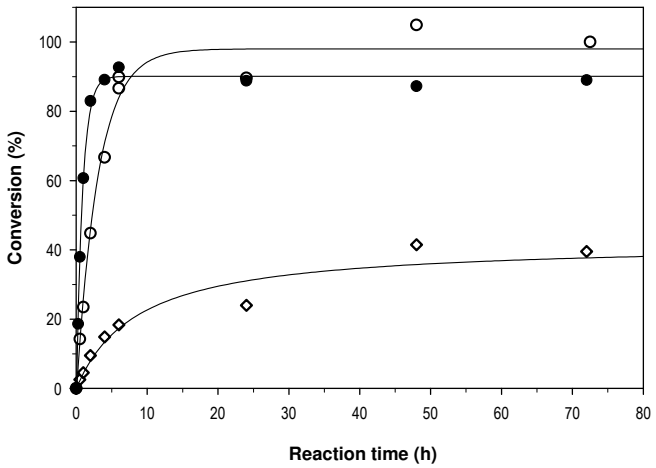

Fig. (4). Time-course of ascorbyl palmitate synthesis by transesterification of vinyl palmitate and L-ascorbic acid catalysed by: (○) Novozym 435; (●) *C. antarctica* lipase B adsorbed on Accurel. (◊) Transesterification of ethyl palmitate and L-ascorbic acid catalysed by Novozym 435.

The tocopherols are characterised by the 6-chromanol ring structure methylated to varying degrees at the 5, 7 and 8 position (Fig. **5**). At the position 2, there is a C16 saturated side chain that has no effect on its antioxidant activity but serves to insert and hold the chemically reactive "head" in biomembranes [57]. Tocotrienols are unsaturated at the 3', 7' and 11' positions of the side chain. Tocopherols have three chiral centers at carbons 2, 4'- and 8'- (Fig. **5**), and the naturally occurring isomers have the RRR-configuration [58]. It seems to exist a structure-function relationship in vitamin E. Birringer *et al*. [44] explained that tocopherols have three different domains: functional one, responsible of the antioxidant activity; signalling one, regulator of the protein kinase C; and the hydrophobic one, analogue in biological membranes and lipoproteins.

Considering the so-called "vitamin E activity", which is related with gene expression and regulation, apoptosis, and signal transduction, there is agreement that the RRR-α-tocopherol is the most bioactive compound [59,60] as it is specifically recognised by membranes [11]. Thus, the isomer with inverted stereochemistry at position 2 has only 30% of the biological activity of the RRR. Attempts are being made to develop an efficient and stereocontrolled synthesis of the natural form of α-tocopherol [61]. In contrast, the antioxidant activity of tocopherols is in the order of $\delta > \gamma > \beta > \alpha$. The antioxidant activity of tocotrienols generally exceeds those of tocopherols.

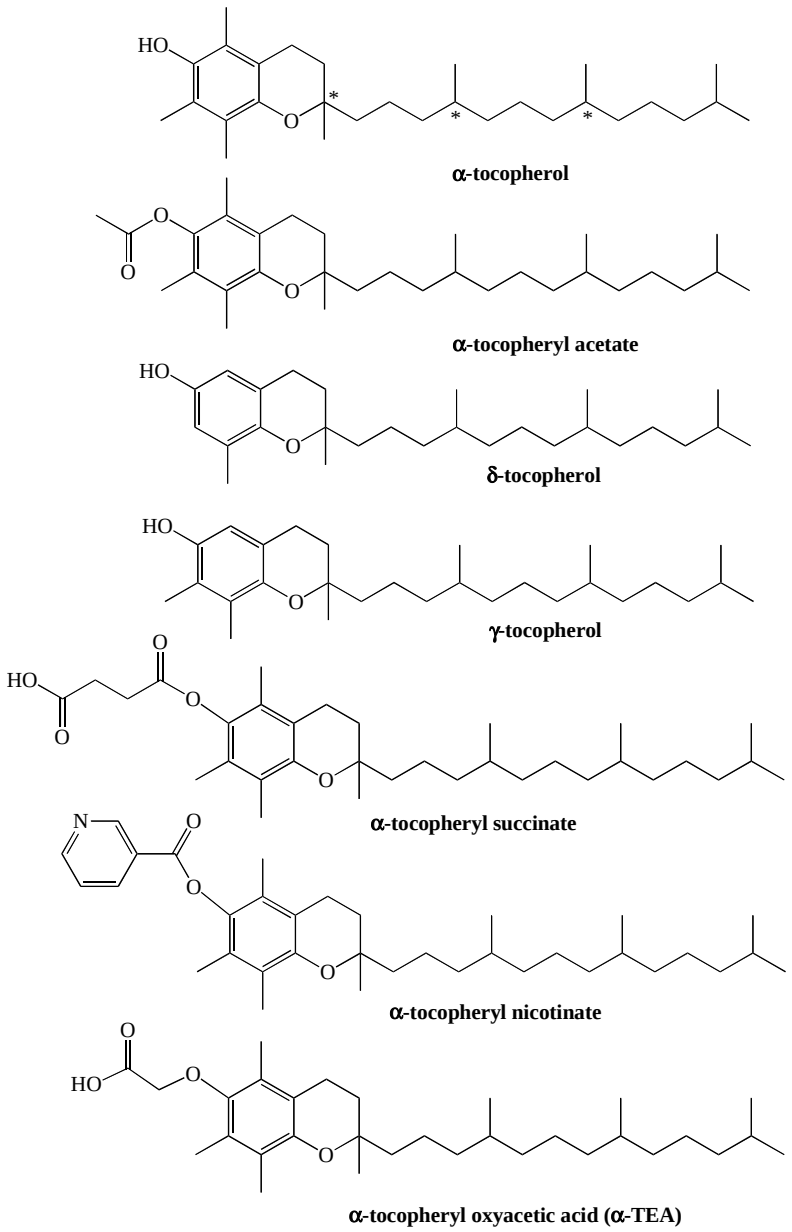

Fig. (5). Structure of α-tocopherol (the location of the three chiral centers is marked with an asterisk) and its commercial derivatives.

Different forms of vitamin E are marketed: RRR-α-tocopherol, pure (all-*rac)*-α-tocopherol, mixed tocopherols having various contents of α-, γ- and δ-derivatives, vitamin E esters (acetate, succinate or nicotinate) and ethers (α-TEA) (Fig. **5**). Commercially available RRR-α-tocopherol is derived from deodorizer distillate, a by-product of soybean oil refining process. The synthetic vitamin E (α-tocopherol) is obtained by reaction of trimethylhydroquinone with isophytol [62], without any control of stereochemistry, and consists of eight stereoisomers in equal proportions (RRR, RRS, RSR, RSS, SSS, SRR, SRS, SSR) designated all racemic (all-*rac*)- α -tocopherol [60]. Although (all *rac*)-α-tocopherol is not as biologically active as the natural RRR stereoisomer [63], the production volume is above 1000 t per annum [64].

In order to develop novel antioxidants better than vitamin E, several attempts to synthesize vitamin E analogs have been reported [57]. Vitamin E is generally administered as a prodrug in the form of all-*rac*-α-tocopheryl acetate (vitamin E acetate) or all-*rac*-α-tocopheryl succinate (Fig. **5**). Vitamin E acetate carries an acetyl moiety at the C-6 phenolic group to increase its stability in the presence of light and oxygen, but this modification blocks the antioxidant properties [65]. For this reason, vitamin E acetate is not suitable for use as antioxidant in lipids and lipid-containing foods. However, vitamin E acetate is added to foods to increase vitamin E content because the shelf life of vitamin E acetate is greater than that of the unesterified tocopherol [66]. *In vivo*, unspecific esterases rapidly cleave the ester bond and release the active α-tocopherol. Tocopheryl acetate is the major sales form of vitamin E, both in the human [67] and animal [68] nutrition markets. Vitamin E acetate is so versatile that has even been patented for use in liquid dish cleaning compositions [69].

The vitamin E acetate is synthesised by chemical acylation with acetic acid or acetic anhydride using different acid or base catalysts such as sulphuric acid or pyridine [70,71].

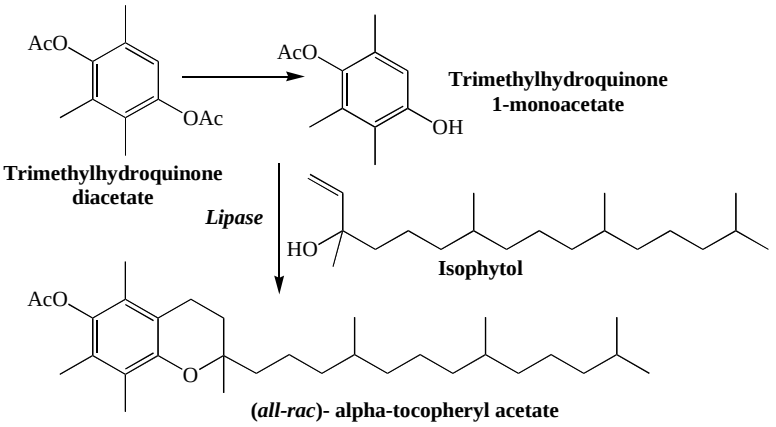

Fig. (6). Chemoenzymatic process for the synthesis of vitamin E acetate.

Several continuous or discontinuous processes have been described [64]. We recently described for the first time the enzymatic acetylation of vitamin E [37,72]. Out of 15 lipases, esterases and proteases screened, only the lipase B from *C. antarctica* catalysed the acylation. The acetylation of δ-tocopherol was faster than that of α-tocopherol, probably due to its lower methylation degree. By computational conformation studies, it has been demonstrated that the acceptor binding site of lipase B from *C. antarctica* is deep (compared with other lipases, e.g. that from *Thermomyces lanuginosus)* [73], which partly explains the broader specificity of *C. antarctica* lipase B [74,75].

An alternative route to *all-rac*-α-tocopheryl acetate is the reaction of trimethyl-hydroquinone-1-monoacetate with isophytol (Fig. **6**) [76]. The diacetate is easy to obtain in large amounts from cheap α-isophorone; however, the selective hydrolysis to 1-monoacetate is difficult to achieve by standard chemical methods. As an alternative, the lipase-catalysed hydrolysis has been reported [76]. Lipase from *T. lanuginosus* (formerly *Humicola lanuginosa*) hydrolysed regioselectively the diacetate in water-saturated *t*-butyl methyl ether [76]. This lipase is inexpensive as it is used as an additive for detergents. Its immobilization on polypropylene enhanced activity without affecting regioselectivity. Neither monoacetate isomer nor hydroquinone (the product of total hydrolysis) were detected making this process superior to chemical deacetylation [77].

With the aim to improve the stability to light and the solubility in water, other analogues have been synthesised using amino acids such as glycine, alanine and pyroglutamic acid. These compounds have the advantage that the hydrolysis produces not only α-tocopherol, but also nutritional components, to obtain a synergic effect [60]. In addition, α-tocopheryl nicotinate (Fig. **5**) has shown antiarrhythmic activity and is used in Europe and Japan as a lipid-lowering agent. Other new application of vitamin E is as antiproliferative agent in different kinds of cancer. In this context, a novel vitamin E analogue was synthesised by Lawson *et al*. [61] that contains a nonhydrolyzable ether linked acetic acid (α-TEA).

In this context, a glucosyl derivative of vitamin E has also been synthesised enzymatically from 2-hydroxymethyl-2,5,7,8-tetramethylchroman-6-ol and maltose by transglycosylation with α-glucosidase from *Saccharomyces* species [78].

CONCLUSIONS

Antioxidants are increasingly important additives in food processing. As well as their traditional role in inhibiting the development of oxidative rancidity in fat-based foods, particularly meat and dairy products and fried foods, more recent research has suggested a new role in inhibiting cardiovascular disease and cancer. Stabilization of antioxidant vitamins for food and feed applications is a critical step to expand their range of applications.

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