Hydrophilic Carotenoid Amphiphiles: Methods of Synthesis and Biological Applications

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Abstract: Carotenoid bioactivity, namely the quenching of reactive oxygen species (ROS) and other excited-state oxidants, suggests significant clinical potential for these natural products. However, a thorough therapeutic evaluation of the carotenoids has been hampered by limited water-solubility and/or water-dispersibility ("hydrophilicity") as well as poor bioavailability. Hydrophilic carotenoid derivatives have been designed and prepared for parenteral administration. Synthetic methods, selected physical characteristics, and potential biological applications of these novel therapeutics will be discussed.

Key Words: Aggregation, antioxidants, carotenoids, hydrophilic carotenoids, reactive oxygen species (ROS), retrometabolic drug design, soft drugs, tissue tropism.

1. INTRODUCTION

Carotenoids are a group of about 750 pigments ranging in color from yellow to red [1]. Widespread in nature, they are responsible for the colors of many fruits, vegetables, and flowers, as well as bird plumage, fish, and crustaceans. Carotenoids are produced by bacteria, algae, and some plants, whereas animals including humans cannot synthesize them, and must assimilate carotenoids from the diet [2, 3]. A typical human diet consists of about 40 carotenoids; some of the most prevalent are shown in Fig. (**1**) [4, 5]. All carotenoids are based on the C40 acyclic, hydrocarbon chain of lycopene, and possess great structural diversity, including cyclic end groups, allenic or acetylenic moieties, and a variety of oxygen-containing functional groups [6]. Based on their chemical composition, carotenoids are divided into carotenes, which are the non-oxygenated hydrocarbons, and xanthophylls, which contain at least one oxygen atom [7]. The high degree of unsaturation gives rise to multiple possible *cis/trans* (*Z/E*) isomers. Although carotenoids tend to isomerize in chemical solutions, the all-*trans* form is the predominant isomer in nature [8]. Of the many naturally-occurring carotenoids, only a few are amphiphilic compounds, possessing hydrophilic head groups attached to the hydrophobic polyene; therefore, few carotenoids are water soluble or water dispersible. The C20 carboxylic acids crocetin and norbixin (annatto and saffron pigments, respectively), and the C20 glycosylester crocin (saffron pigment) are the most water soluble natural carotenoids [9].

Carotenoids contain a highly unsaturated, conjugated chromophore responsible for their physicochemical and biological properties. Carotenoids act as supplemental light receptors in photosynthesis by absorbing light of wavelengths only weakly absorbed by chlorophyll. They protect the tissues in green plants, algae, and photosynthetic bacteria from light damage [7], especially singlet oxygen. Plant extracts containing carotenoids (*e.g.* saffron, anatto, and palm oil) have been used as food colorants and in folk medicine for centuries [8, 10]. Today, a number of carotenoids are commercially important in the food and feed industries where they are used as colorants (*e.g.* in poultry and swine; aquaculture of salmon; and egg production) [8, 11]. Many of the fragrances used in modern perfumes are carotenoid-derived aroma compounds (*e.g.* β-ionone and dihydro-β-ionone) extracted from plants and flowers, formed by *de novo* enzymatic cleavage [12].

In humans, about 50 carotenoids posessing a β-ionone ring are bioconverted to retinoids ("provitamin A carotenoids"), compounds essential for vision, cell growth, and differentiation [7, 13]. The major scientific focus of the carotenoids has historically been their antioxidant mechanism: quenching of reactive oxygen species (ROS) and radical chain-breaking. ROS are generated in the human body through normal metabolic activity, and can be also introduced exogenously through diet and lifestyle. Their potentially harmful reactivity with cellular components such as lipids, proteins, and DNA are directly related to aging and chronic diseases such as atherosclerosis, age-related macular degeneration (ARMD), and some cancers. A number of epidemiological studies have revealed a decreased incidence of such disease states in those populations with diets rich in carotenoids, suggesting a significant clinical potential for this class of polyenic compounds [14, 15]. Carotenoids protect human cells and tissues additionally by other mechanisms, including the regulation of the expression of genes that protect against carcinogenesis and inflammation, as well as stimulation of the immune system (*e.g.* increase in natural killer (NK) cell activity) [4, 5, 16]. Specific carotenoids appear to possess specific functions *in vivo*, in that certain carotenoids exhibit protective effects against targeted disease states such as ARMD and prostate cancer [17]. Intervention trials with β-carotene supplements have shown mixed results; some studies have shown increased cancer incidence in high risk groups (*e.g.* smokers). One potential explanation for the negative results obtained in clinical studies of β-

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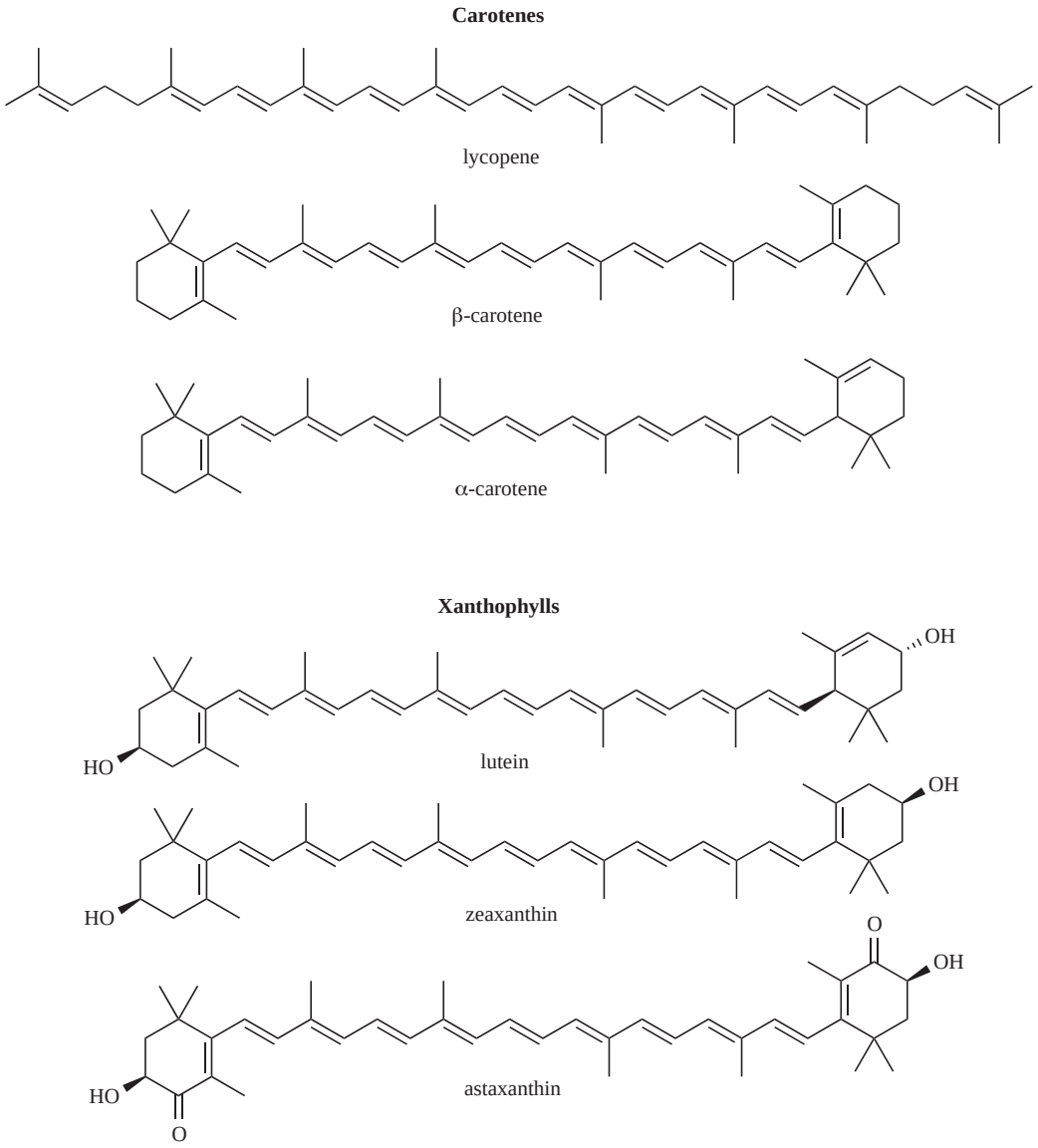


Fig. (1). Some of the most abundant carotenoids in the human diet.

carotene involves the pro-oxidant activity of β-carotene and other carotenoids observed at high partial oxygen pressures and concentrations *in vitro* [16, 18]. Whether carotenoids can act as pro-oxidants in the human body, however, is a matter of dispute [19].

Carotenoids are absorbed together with dietary fat in mammals through passive diffusion into intestinal cells. They are initially carried in blood by chylomicrons, then transported to different organs by lipoproteins [13, 17]. The amount of carotenoids absorbed from food intake varies from a few percent for raw vegetables (in the intact food matrix), to 50% or more after grinding and thermal processing into juice or paste. Increased absorption can also be achieved using micellar solutions, commercial beadlets, or oil-based suspensions [4, 13, 20]. Carotenoids are found in almost all tissues and tend to accumulate in the liver and adipose tissues [13, 17]. However, slight differences in chemical structure can greatly affect organ preference [21], with distinct tissue tropism observed for lutein, zeaxanthin, and lycopene in particular. All carotenoids are metabolized *in vivo*, resulting in cleaved and oxidized products (*e.g.* apo-carotenoids) that can be physiologically active as well [19, 22]. High supplemental intakes of β-carotene (more than 20 mg per day) and canthaxanthin have been shown to cause a yellowing of the skin ("carotenodermia"), and in the case of canthaxanthin, formation of carotenoid crystals in the mammalian retina. However, there have been few other reports of potentially toxic effects of carotenoids that do no possess pro-vitamin A activity [4].

In clinical medicine, the therapeutic use of carotenoids remains largely untapped. Some carotenoids are common ingredients in tanning preparations and sun screens because of their capacity to reduce photo-induced damage of the skin [4]. Derivatives of retinoic acid (RA) have been used to treat psoriasis and eczema, while β-carotene is effective in the treatment of erythopoietic protoporphyrea [23, 24]. Until

recently, the preclinical evaluation and clinical application of carotenoids have been hampered by their limited water solubility and/or water dispersibility (hereafter "hydrophilicity") and poor bioavailability in many test animals (*e.g.* mice, rats, rabbits, dogs, and pigs—the so-called "white fat" animals) [25]. Previously, pharmaceutical administration was mainly limited to oral methods, and has recently been facilitated by imparting hydrophilicity to the lipophilic scaffold. To this end, co-solvent formulations (*e.g.* aqueous THF) [26], liposomal systems [27], microemulsions [28], and complexation with macromolecules such as cyclodextrins [29] have been effectively used. In addition, the use of additives such as penetration enhancers [28] and stabilizers [30] have been explored. Overall, limited increases in bioavailability and purported toxicity have limited the *in vivo* utility of such formulations. Until recently, chemical derivatization of carotenoids had scarcely been reported in the literature [31-35]. One characteristic shared by many of these novel carotenoid derivatives is their propensity to form supramolecular assemblies (aggregates) in aqueous solutions [31], a physical property at once imparting shelf-life stability to aqueous formulations, yet potentially limiting their capacity as antioxidants in the aqueous phase [36]. Aggregate size and type (*J*- and/or *H*-form) appear to be features that can be modulated with appropriate medicinal chemistry.

Using retrometabolic drug design techniques [37], several classes of hydrophilic carotenoid derivatives have been prepared for parenteral (as well as oral) administration. The novel compounds were designed to be effective antioxidants in the derivatized state ("soft drugs"), contrasting with inactive "pro-drugs" of other antioxidants such as the vitamin E esters. These derivatives are readily soluble or dispersible in water, without the need for addition of heat, detergents, cosolvents, or other additives. In several cases, the point at which molecular solutions of the novel compound(s) become dispersions of aggregates has been elucidated. The compounds prepared and investigated thus far have demonstrated potent aqueous-phase radical scavenging abilities *in vitro*, as well as efficacy in model systems of cardiovascular disease, chronic liver injury, and cancer chemoprevention. They will likely find utility in those clinical applications where rapid parenteral delivery of potent antioxidant, anti-inflammatory compounds is necessary to achieve desired therapeutic effects. In this review, we will describe the derivatization of symmetric and asymmetric, cyclic and acyclic carotenoids using principles of retrometabolic medicinal chemistry. The methods of synthesis, achieved hydrophilicity, aggregation behavior, and potential biological applications of several classes of novel, amphiphilic derivatives will be discussed.

2. DESIGN AND SYNTHESIS OF CAROTENOID AMPHIPHILES

Hawaii Biotech, Inc. (HBI) has successfully derivatized several C40 xanthophylls to yield "bolaform" amphiphiles—possessing one hydrophilic head group for each end of the hydrophobic chromophore, the rigid internal spacer of the molecule. Significant increases in hydrophilicity over the natural carotenoids were achieved, sufficient for parenteral administration of aqueous formulations [38-41] into test animals. The carotenoids astaxanthin, lutein, zeaxanthin, and lycophyll were used as scaffolds, their hydroxyls used as handles for derivatization. In specific model systems, these carotenoids have shown both superior ROS quenching ability [42] and lipid antioxidant capacity compared to completely hydrocarbon carotenoids in previous studies [43]. As stated earlier, the oxygen-substituted β-ionone ring does not possess pro-vitamin A activity, a feature also shared by the lycophyll parent compound. These polar carotenoids can be more efficiently absorbed, and metabolized more rapidly in humans, when compared to the carotenes [44, 45]. Additionally, the selective accumulation of particular carotenoids in specific tissues [21] suggests a protective evolutionary role in humans against ROS and associated disease states, including ARMD, certain cancers, as well as non-specific chronic inflammation.

The various carotenoid scaffolds were derivatized with naturally-occurring compounds including short chain organic acids, sugars, amino acids, and other antioxidants, and conjugated using ester, glycoside, carbamate, and carbonate linkages (Schemes **1**-**7**). The derivatizing moieties were selected to impart distinct physical and chemical characteristics to the resulting soft drug. In nature, many oxygen-substituted carotenoids are stabilized by esterification with one or two medium- to long-chain fatty acids [4, 46]. Esterified carotenoids are effectively hydrolyzed before gastrointestinal absorption in the human body [21]; esterification does not appear to impair bioavailability of the free carotenoids, and may in fact improve overall fractional absorption [47]. Glycosidic (anomeric ether) linkages are present in carotenoid saccharides extracted from various microorganisms and algae [48]. Carotenoids esterified with small end groups may exhibit higher antioxidant activity due to favorable steric and/or electronic considerations [49]. Natural linkages (*e.g.* esters, glycosides) are enzymatically cleaved to free carotenoids *in vivo*, after both oral and parenteral administration. Derivatization of natural scaffolds with naturally-occurring conjugating moieties has so far retained the inherent antioxidant activity, as well as the safety, of the parent compounds.

Astaxanthin-Based Derivatives

Astaxanthin, one of the most abundant carotenoids in nature, is the main pigment in marine animals and seafood (*e.g.* salmon, shrimp, lobster, and fish eggs) [46]. For decades, astaxanthin has been used as a feed additive in aquaculture [11]. More recently, it has become evident that astaxanthin possesses many beneficial biological properties for humans [46]. Consequently, astaxanthin is now commercially available as a health supplement in several countries including the United States [50]. Astaxanthin has demonstrated potent antioxidant activity in many studies, superior to _-carotene and the major antioxidant found in lipids, vitamin E. In particular, astaxanthin has shown to be very efficient in protection against lipid peroxidation in cell membranes [51-53]. Among several natural and synthetic carotenoids tested in a model system, astaxanthin was termed a "class III" antioxidant, that is, demonstrating strong antioxidant activity—perfectly quenching excited molecular states—without evidence of pro-oxidant activity [49]. Its favorable antioxidant properties have been attributed to its polarity and unique structure: α-hydroxy keto groups conjugated to the polyene chromophore [51]. Astaxanthin is believed to play a key therapeutic role in several disease states including in-

flammation, liver and heart diseases, and some cancers [46]. Astaxanthin is readily absorbed and incorporated into human plasma lipoproteins, relatively evenly between HDL and LDL, with the balance found in VLDL [54]. Recently, short-chain oxidative metabolites of astaxanthin were identified in human plasma [55]. The favorable bioavailability, potent antioxidant and anti-inflammatory properties, and desirable safety profile of astaxanthin supported its use as a suitable candidate scaffold in retrometabolic therapeutic (soft drug) design.

Synthesis of Astaxanthin Derivatives

Through esterification of astaxanthin (1:2:1 statistical mixture of *3S,3´S*, *meso*, and *3R,3´R* isomers of astaxanthin) with succinic acid, Cardax (**3**) was synthesized in multi-gram scale, according to Scheme (**1**) [38]. The esterification was performed under thermodynamic conditions (at room temperature), keeping the amount of *cis*-isomerization to a minimum (< 3%). Salt formation was afforded by treating the disuccinic acid with sodium isopropoxide. After work-up, Cardax was obtained in 59% overall yield from astaxanthin. The synthesis was also successfully applied to astaxanthin's individual stereoisomers, which had been first separated by preparative chromatography.

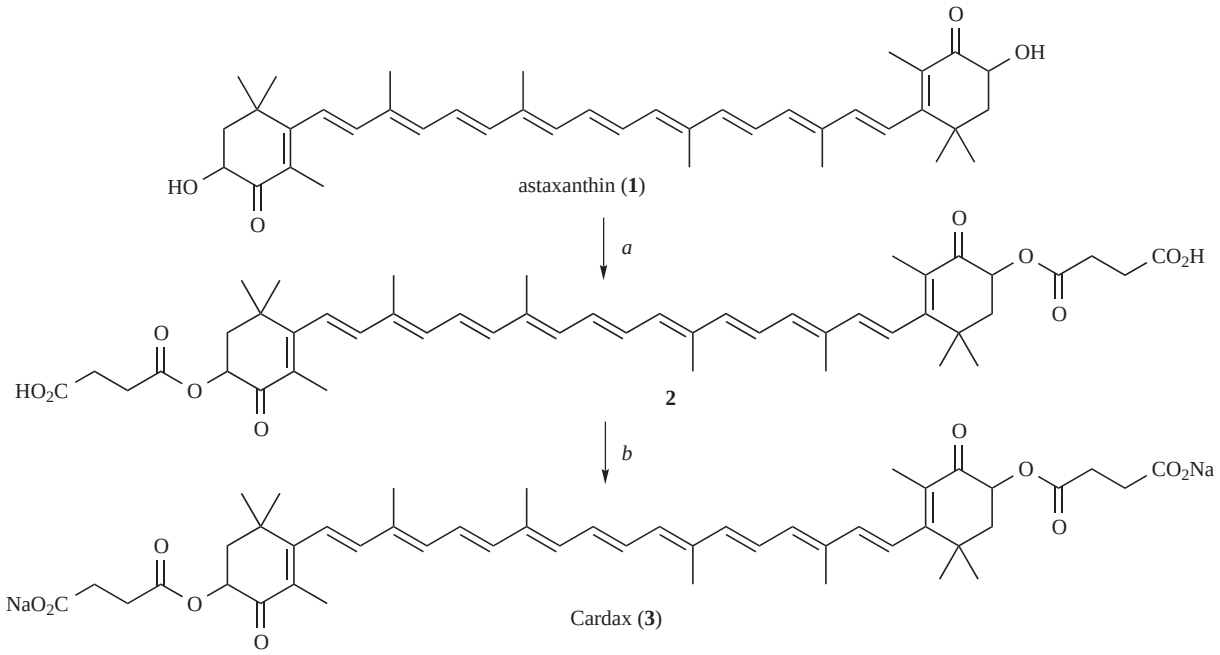
The highly hydrophilic dilysinate conjugate of astaxanthin (lys_2AST (**5**)) was prepared using standard carbodiimide methods and a suitably protected form of lysine, as illustrated in Scheme (**2**) [39]. Astaxanthin was first esterified, then lysine's amine protecting groups were removed with dry HCl to afford lys_2AST in 64% yield over two steps.

Astaxanthin diphosphate (**7**)—pAST—was synthesized as illustrated in Scheme (**3**) [40]. Astaxanthin was diphosphorylated to yield a bis-(2-cyanoethyl)-protected diphosphate. Basic removal of the phosphate protecting groups, followed by sodium ion exchange afforded pAST in 28% yield.

As seen in Schemes (**4**) and (**5**), a focused chemical library of astaxanthin conjugates was prepared, utilizing astaxanthin or astaxanthin disuccinate as starting materials [41]. Using standard methods, non-toxic natural materials such as amino acids, sugars, and antioxidants were coupled to astaxanthin's scaffold. Amide, carbonate, carbamate, and ester-containing derivatives were prepared.

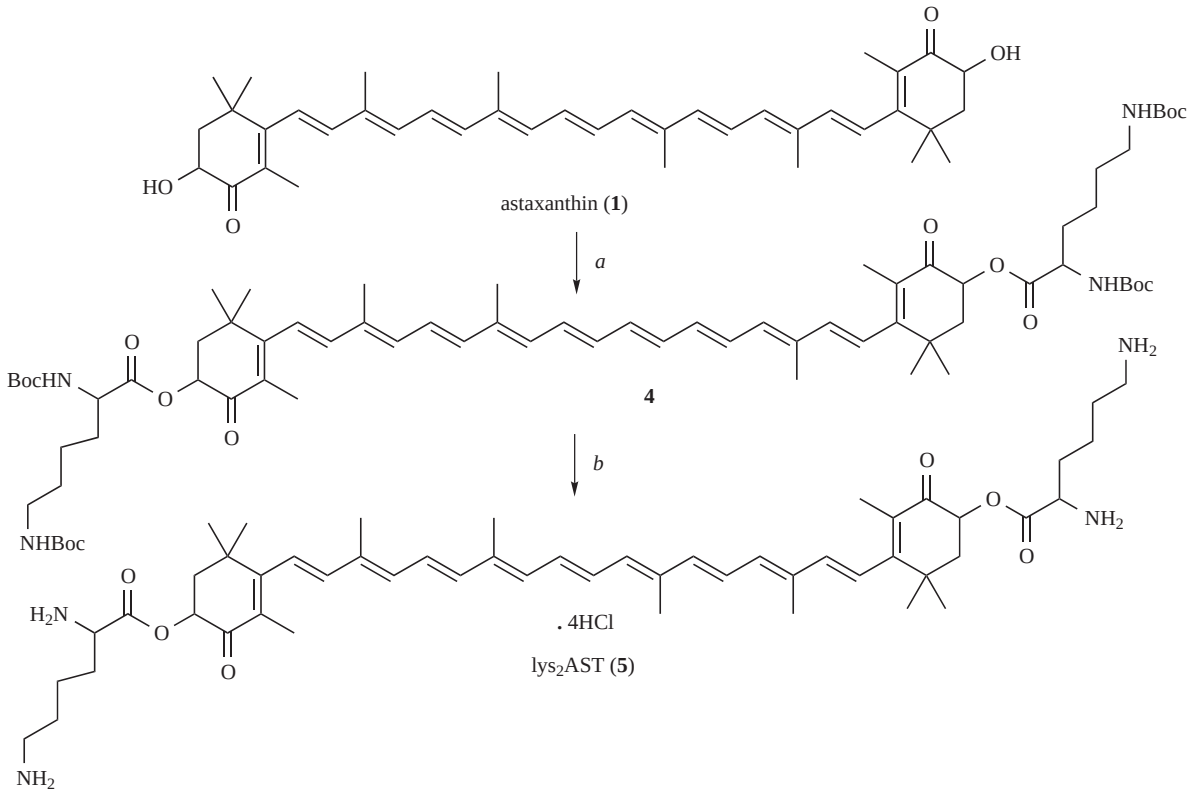
Lutein and Zeaxanthin-Based Derivatives

Lutein and zeaxanthin are oxygenated carotenoids found in green leafy vegetables and fruits, and are therefore among the major carotenoids humans obtain from diet. They exist in free, glycosylated, and esterified forms, *e.g.* their mono- and diesters are present in peaches and squash [21]. Lutein and zeaxanthin have demonstrated comparable singlet oxygen quenching abilities to β-carotene in apolar organic solutions [33]. Zeaxanthin was found to be nearly as effective as α-tocopherol in protecting model membranes from induced oxidative stress [56]. Zeaxanthin has been shown to stabilize biomembrane systems, suggesting a protective role against oxidative damage in cell membranes [57]. Likewise, lutein has been found to correlate with protection against atherosclerosis [58]. An inverse correlation between risk of ARMD and lutein and zeaxanthin intake indicates a probable protective function of both carotenoids in the eye. Interestingly, lutein and zeaxanthin exclusively comprise the macular pigment of the human retina. In fact, the concentration of lutein and zeaxanthin in the center of the *macula lutea* is up to



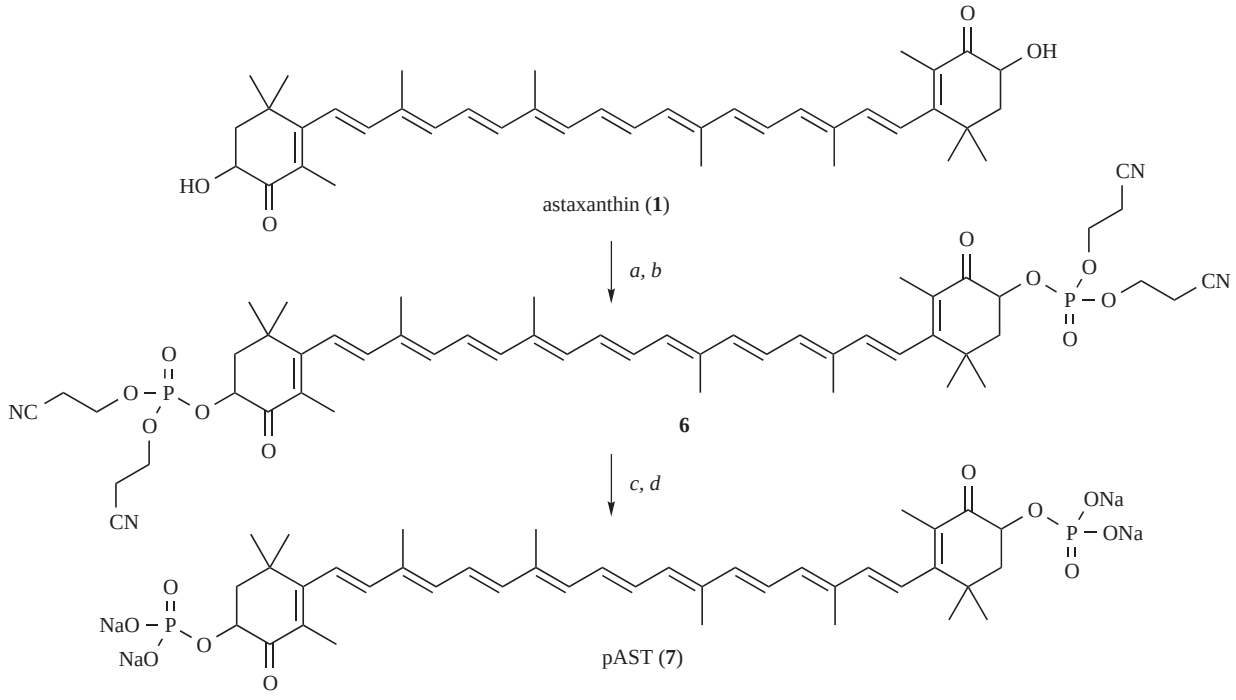
Conditions: *a*. succinic anhydride, DMAP, CH_2Cl_2. *b*. Na^{i}OPr, ^{i}PrOH.

Scheme (1). Synthesis of the sodium disuccinate of astaxanthin, Cardax (**3**).



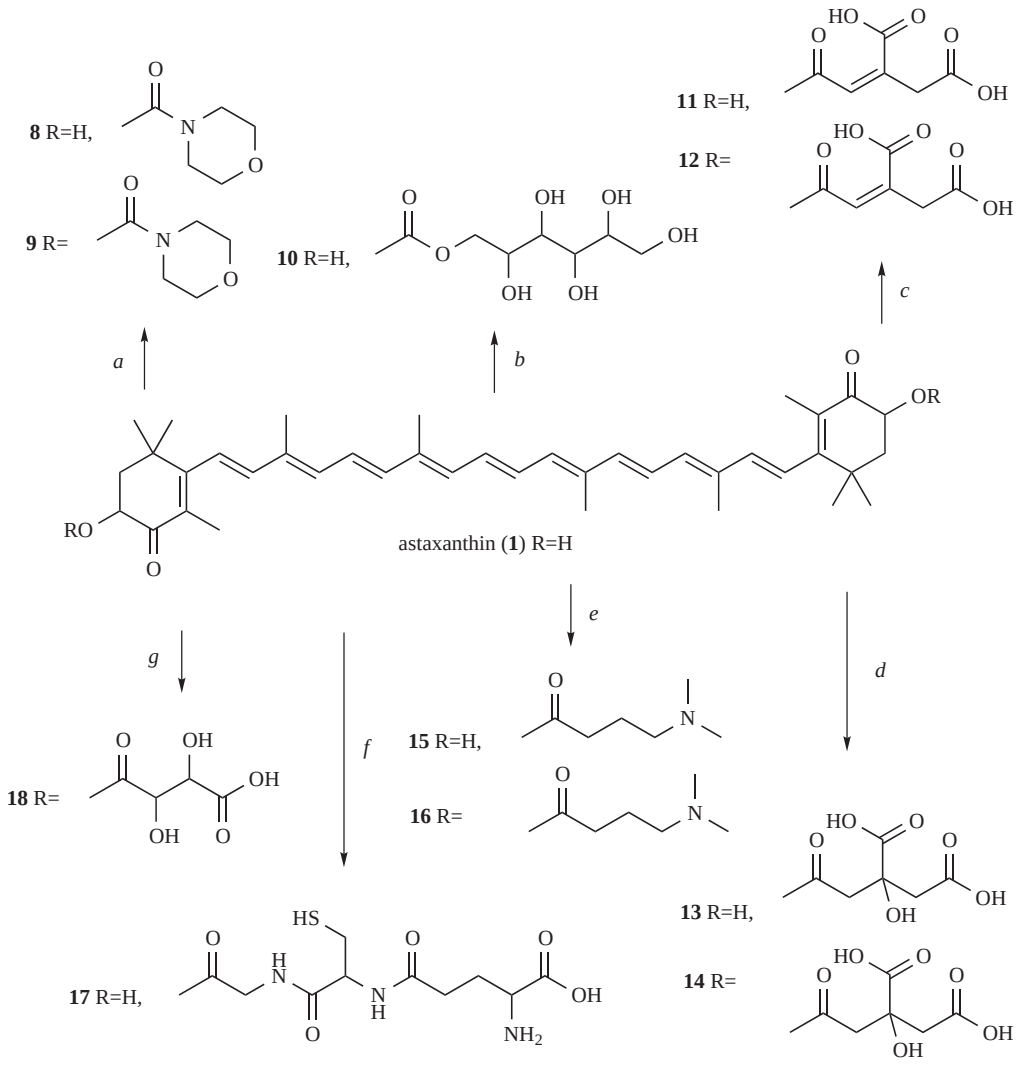
Conditions: *a*. DIC, DMAP, Boclys(Boc)OH, CH_2Cl_2. *b*. HCl, dioxane.

Scheme (2). Synthesis of the dilysinate conjugate of astaxanthin, lys_2AST (**5**).



Conditions: *a*. bis-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite, CH_2Cl_2. *b*. I_2, pyridine/CH_2Cl_2/water (3:1:1). *c*. aq. dimethylamine. *d*. IR-120 resin (Na^+).

Scheme (3). Synthesis of the sodium diphosphate of astaxanthin, pAST (**7**).



Conditions: *a*. DIPEA, DMAP, 4-morpholine carbonyl chloride, CH_2Cl_2/DMF *b*. DIPEA, 1,2,2,2-tetrachloroethyl chloroformate, DMAP, mannitol, CH_2Cl_2/DMF. *c*. DIPEA, aconitic anhydride, DMAP, CH_2Cl_2/DMF. *d*. citric acid, DIPEA, DIC, DMAP, then **1**, CH_2Cl_2. *e*. 4-(dimethylamino)butyric acid hydrochloride, DIPEA, DMAP, HOBt-H_2O, DIC, **1**, CH_2Cl_2/DMF. *f*. glutathione, DIPEA, DMAP, HOBt-H_2O, DIC, **1**, CH_2Cl_2/DMF. *g*. tartaric acid, DIPEA, DMAP, HOBt-H_2O, **1**, CH_2Cl_2/DMF.

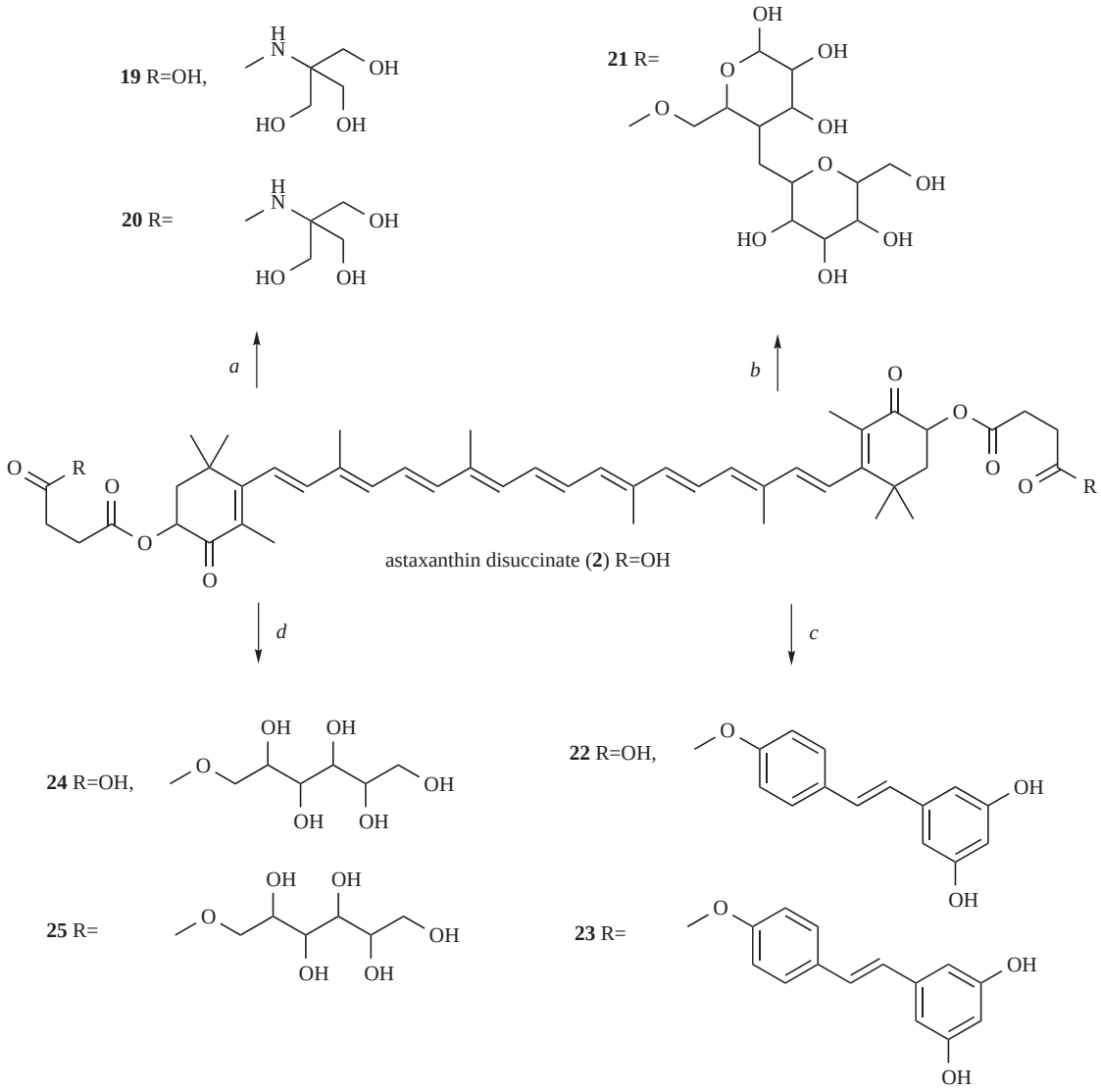
Scheme (4). Synthesis of astaxanthin—natural compound conjugates.

1000-fold greater than in other human tissues including plasma, suggesting a concentrative uptake mechanism in the eye as yet incompletely characterized [59].

Lutein supplements are often high in diesters and must be hydrolyzed before oral absorption for effective bioavailability [21]. However, ester hydrolysis does not appear to be the rate-limiting step, rather the dissolution characteristics of supplement formulations seem to be the most important factors regulating bioavailability [47]. In addition, dose-proportionality appears to be a problem after oral administration of both lutein and zeaxanthin [60, 61]. Parenteral administration—with 100% bioavailability by definition—overcomes these problems associated with oral bioavailability, and may allow effective therapeutic concentrations and the subsequent salutatory clinical effects to be achieved in selected populations.

Synthesis of Lutein and Zeaxanthin Derivatives

Amphiphilic lutein and zeaxanthin derivatives, including disuccinate, diphosphate, and diglycosyl esters have been designed and prepared (Scheme **6**) [62, 63]. Standard synthetic methods were employed to effect esterifications, deblocking of protecting groups, and salt formation. The preparation of these compounds is highlighted by work-up procedures that effectively maintain the sensitive chromophores, yielding target derivatives in good purity. To date, disuccinate salts of both carotenoids (dZEA(**28**), dLUT(**29**)), lutein disuccinate diglucosyl ester (gluLUT(**31**)), and lutein diphosphate salt (pLUT(**35**)) have been prepared. Due to the prohibitive cost of commercial, neat zeaxanthin, synthesis of glycosyl ester and diphosphate derivatives of this scaffold await acquisition of adequate quantities of sufficient purity. However, given its chemical similarities with lutein, syn-



Conditions: *a*. DIPEA, HOBt-H_2O, DIC, tris(hydroxymethyl)aminomethane, DMAP, CH_2Cl_2/DMF. *b*. DIPEA, HOBt-H_2O, DIC, maltose-H_2O, DMAP, CH_2Cl_2/DMF. *c*. DIPEA, HOBt-H_2O, DIC, resveratrol, DMAP, CH_2Cl_2/DMF. *d*. DIPEA, HOBt-H_2O, DIC, sorbitol, DMAP, CH_2Cl_2/DMF.

Scheme (5). Synthesis of astaxanthin disuccinate—natural compound conjugates.

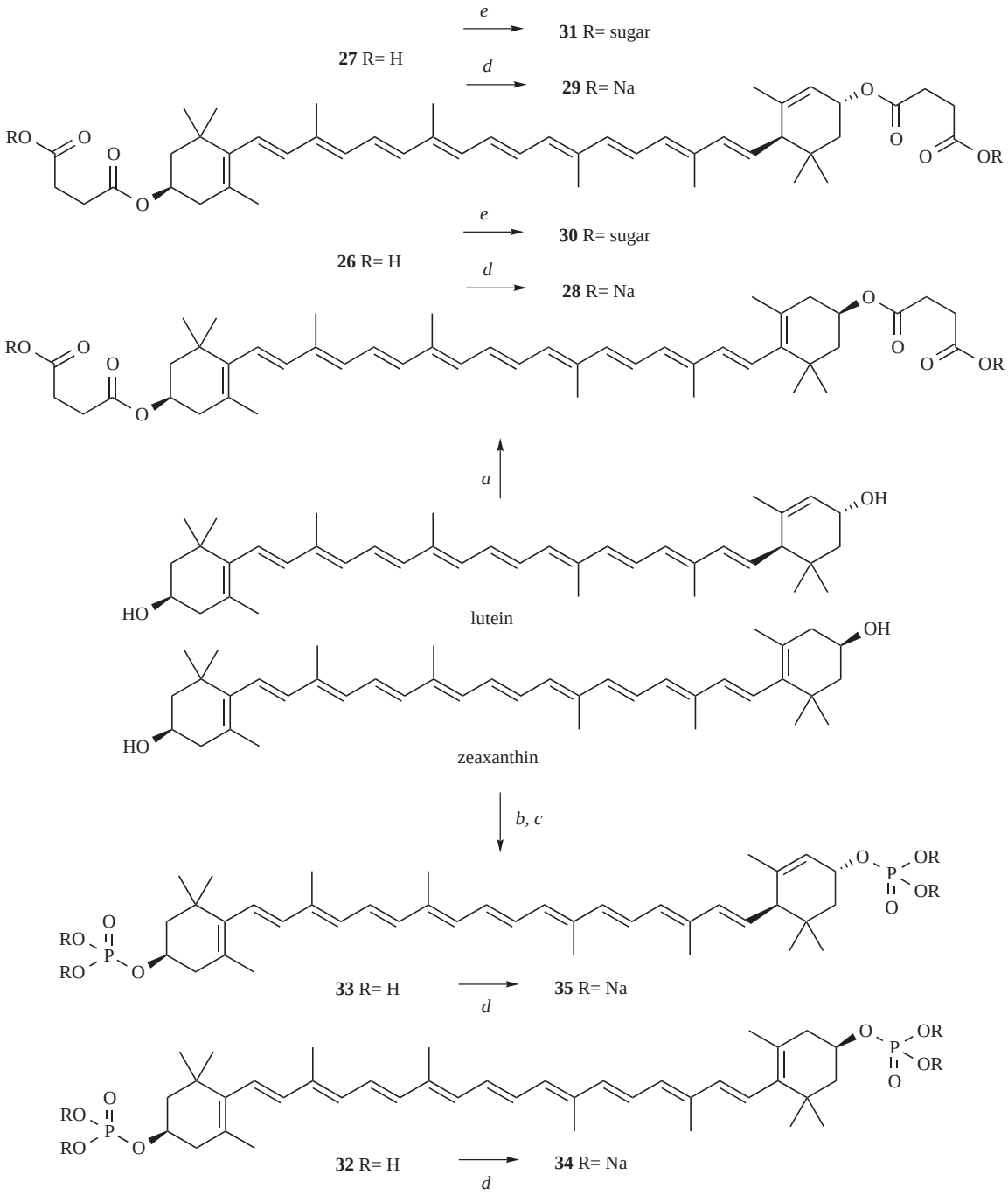
thetic methods as elaborated for lutein (Scheme **6**) should be easily amenable to this medicinally-relevant carotenoid.

Lycophyll-Based Derivatives

Lycopene, the primary carotenoid in natural tomato products, has shown promising protective effects against prostate cancer [64]. Lycopene also prevents oxidation of low-density lipoprotein (LDL) cholesterol, and may reduce the risk of developing atherosclerosis and coronary heart disease [65]. These latter effects may be associated with lycopene's potent antioxidant activity and singlet oxygen quenching ability. Commercially-available lycopene is utilized currently in nutritional supplements [11]. Lycopene is also the predominant carotenoid in human plasma, and tends to selectively accumulate in tissues such as testes, adrenal glands, and prostate. A number of oxygenated metabolites with possible physiological roles *per se* have been found in plasma and tissues [16]. Whether lycopene is the primary efficacious substance, or if other natural constituents in tomatoes (*e.g.* lycoxanthin and lycophyll) show bioactivity as well, is currently under investigation. Lycophyll, also known as lycopene-16,16'-diol [66], is an analog of lycopene, and therefore belongs to the same carotenoid class as astaxanthin, lutein, and zeaxanthin. Its efficient total synthesis, and facile purification of selected geometric isomers, have recently been reported [67, 68]. Lycophyll possesses the same chromophore as lycopene and most likely exhibits comparable antioxidant activity. Hence, it was also chosen as a suitable scaffold for designing potential chemopreventive/chemotherapeutic agents with retrometabolic synthesis, in particular targeting prostate cancer.

Synthesis of Lycophyll Derivatives

As seen in Scheme (**7**), amphiphilic lycophyll disuccinate salt (dLYC(**37**)), disuccinate diglucosyl ester (gluLYC(**38**)), and diphosphate salt (pLYC(**40**)) have been prepared [69].



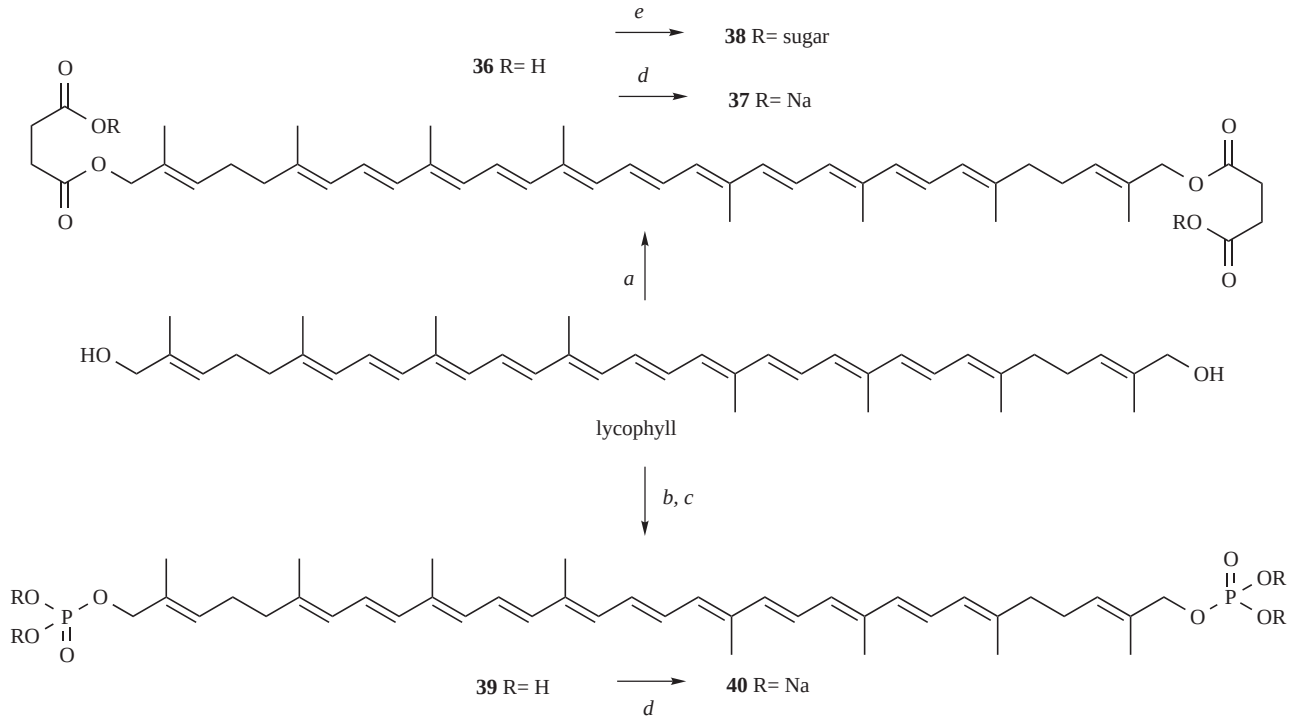
Conditions: *a*. succinic anhydride, DMF, CH_2Cl_2. *b*. dibenzyl phosphoroiodidate, pyridine, CH_2Cl_2. *c*. bromotrimethylsilane, pyridine, CH_2Cl_2. *d*. sodium methoxide, methanol. *e. (i).* carbonyldiimidazole, DMF. *(ii).* sugar, sodium hydride, pyridine.

Scheme (6). Synthesis of lutein and zeaxanthin diesters.

The propensity of the lycopene chromophore to easily isomerize and/or decompose under mild chemical conditions make the preparation of sufficiently pure lycophyll derivatives a challenging endeavor. Recently, synthetic methods originally developed for lutein derivatives have been successfully applied to lycophyll's scaffold [69]. The medicinal investigation of novel, hydrophilic analogs of lycopene is currently ongoing.

3. AQUEOUS AGGREGATION AND DISPERSIBILITY

Of the novel amphiphilic carotenoids we have investigated, all form clear, orange- to red-colored dispersions in water. As seen in Table (**1**), the reported maximum dispersibilites of the novel analogs range from 2.6 to 181.6 mg/mL [38-40, 62, 70], demonstrating that hydrophilicity of carotenoids can be improved by varying the conjugated group (for in-depth discussions of critical aggregate concentration



Conditions: *a*. succinic anhydride, DIPEA, CH_2Cl_2. *b*. dibenzyl phosphoroiodidate, pyridine, CH_2Cl_2. *c*. bromotrimethylsilane, pyridine, CH_2Cl_2. *d.* sodium methoxide, methanol. *e. (i).* carbonyldiimidazole, DMF. *(ii).* sugar, sodium hydride, pyridine.

Scheme (7). Synthesis of lycophyll diesters.

and other surface- and aggregation parameters, the reader is directed to the primary published articles). The diphosphate esters of lutein and astaxanthin demonstrated comparable hydrophilicity, while a small difference was observed for the disuccinate ester series, perhaps attributable to astaxanthin's keto groups. Of particular note is the impressive hydrophilicity of lys_2AST (186.6 mg/mL), largely attributed to the lysine esters containing two ammonium moieties each [39].

In collaboration with others, it has been shown that the prepared novel carotenoid amphiphiles exhibit self-assembly properties similar to natural carotenoids [39, 71-74]. In polar organic solutions (*e.g.* acetone, ethanol, and methanol) most carotenoids display a fine 3-peak absorption curve in the visible spectral region (420-500 nm). Upon addition of water, self-assembly leads to pronounced changes in the UV-VIS spectrum. Aggregation of carotenoids generates the appearance of a blue-shifted peak (from 370 to 410 nm) which is believed to arise from "card-pack" or "*H*-type" orientation of the polyene chains. Sometimes a red-shifted absorption band near 510 nm is also observed, the result of "head-to-tail" or "*J*-type" polyene orientation. Aggregates in aqueous solutions are held together by weak non-covalent forces, and can be disrupted by an increase in temperature or by addition of co-solvent. Both chemical structure and experimental conditions seem to dictate the type of carotenoid aggregation observed. While the parent compounds are readily destroyed by irradiation or oxidation, aggregation serves to protect and preserve the sensitive chromophore [75-78].

The hydrophilic lys_2AST forms non-spontaneous, blue-shifted aggregates in water (22 nm), increasing in size with increasing ionic strength (47 nm in Ringer buffer). Astaxanthin dilysinate exhibits card-pack or *H*-type aggregates in aqueous dispersions, investigated using circular dichroism (CD) spectroscopy [39, 74]. UV-VIS spectra of Cardax in water revealed blue-shifted spectra (50 nm), suggesting card-pack (*H*-type) aggregate formation; no evidence was obtained for the presence of head-to-tail aggregation. Preliminary investigation of disodium disuccinate lutein (dLUT) by UV-VIS spectroscopy indicated head-to-tail (*J*-type) aggregation in aqueous solution, while evidence of card-pack aggregation (18 nm blue shift) was observed for the diphosphate ester (pLUT) [62, 79].

Table 1. Hydrophilicities of Novel Carotenoid Amphiphiles

	dLUT (C40)	***Cardax (C40)***	***pAST (C40)***	***pLUT (C40)***	***lys_2AST (C40)***
Water dispersibility (mg/mL)	2.9	8.6	25.2	29.3	181.6

In collaboration with others, the physicochemical properties of aqueous dispersions of Cardax have been comprehensively studied by determination of critical micelle concentration (cmc), surface activity, surface concentration, molecule area, free energy of adsorption and micellation, adsorption-aggregate energy relationship, and equilibrium constants [71, 72]. Dynamic light scattering revealed that Cardax formed large aggregates (1.3 μm at 0.06 mg/mL). The charge of the polar end groups and the rigid spacer of Cardax was thought to prevent the formation of small, curved aggregates; rather, self-assembly of Cardax was believed to create large, extended monolayer structures. Larger aggregates were observed in solutions of increasing ionic strength. Cardax also formed aggregates below the critical micelle concentration (cmc). Due to the long hydrophobic chromophore, it was hypothesized that the derivatives existed as monomers and smaller aggregates below cmc, and formed larger aggregates over cmc, as was suggested for lysophophocholines with long fatty acids [80]. In contrast, the neutral and relatively short-chain (C20) glycoside crocin only formed aggregates over its cmc [81]. By adding a co-solvent to the aqueous dispersions, the self-assemblies of these derivatives were disrupted into monomeric solutions; 41% aqueous ethanol was sufficient to disaggregate Cardax [71, 73]. Monomeric aqueous solutions of carotenoids are favored therapeutic vehicles for use in certain model system efficacy studies.

4. BIOLOGICAL APPLICATIONS OF NOVEL HYDROPHILIC CAROTENOID AMPHIPHILES

Activity Against Aqueous-Phase Reactive Oxygen Species (ROS)

Free radicals and reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$) and superoxide anion ($O_2^{\bullet-}$), are major contributors to the process of oxidative stress in the human body [5]. In developing potential therapeutic "soft drug" antioxidants, a first critical step involves investigating aqueous-phase singlet oxygen quenching or superoxide anion-scavenging abilities. The aggregation behavior of most carotenoids at even low water concentrations inhibits their interaction with aqueous-phase radicals [36]. Antioxidant activity has traditionally been measured in organic solvents or micellar systems; the water-soluble carotenoids crocin and crocetin being the two exceptions [82, 83]. Radical quenching capacity of antioxidants is related to chemical structure, specifically the degree of conjugated unsaturation and the presence of heteroatom functional groups [51, 84].

The direct superoxide anion scavenging abilities of a number of prepared carotenoid amphiphiles have been evaluated in an *in vitro* human neutrophil assay utilizing spin-trap (DEPMPO) electron paramagnetic resonance (EPR) spectroscopy. Each novel derivative was tested in either neat water or in an ethanolic formulation, summarized in Table (**2**) [39, 62, 73, 85]. The scavenging ability of each derivative was dose-dependent, and near complete quenching of the induced superoxide anion signal was achieved at millimolar concentrations for all derivatives. Over 90% of the superoxide anion was scavenged by an aqueous dispersion of lys_2AST at 100 μM. A 3 mM dispersion of Cardax and a 5mM dispersion of pLUT were required to achieve comparable superoxide anion quenching. Superior scavenging ability was observed using the aqueous ethanolic solutions. Such findings indicate that supramolecular assembly reduces the interaction of carotenoid polyenes with aqueous-phase radicals. Interestingly, studies with β-carotene indicated no interaction between aqueous-phase radicals and the hydrophobic carotene [36].

Table 2. Mean Percent (%) Inhibition of Aqueous Superoxide Anion ($O_2^{\bullet-}$) by Hydrophilic Carotenoids

Carotenoid Derivative	*Solvent*	*Concentration (mM)*	*Mean Inhibition (%)*
Astaxanthin (C40)	DMSO	0.1	28.0
Cardax (C40) (3S, 3´S, meso, and 3R,3´R / 1:2:1)	Water EtOH (33%) EtOH (33%) EtOH (33%) EtOH (33%)	0.1 0.1 0.5 1.0 3.0	19.3 38.0 60.1 78.0 95.0
lys_2AST (C40)	water water water water	0.001 0.01 0.05 0.1	10.3 46.7 86.0 95.7
dLUT (C40)	water water water EtOH (40%)	1.0 3.0 5.0 0.1	13.0 61.7 74.5 5.0
pLUT (C40)	water water water EtOH (40%)	1.0 3.0 5.0 0.1	9.3 72.3 91.0 18.0

Chromophore length and achieved hydrophilicity are directly related to aqueous-phase radical scavenging, as evidenced when comparing the mean scavenging abilities of Cardax and lys_2AST. The highly hydrophilic lys_2AST (~182 mg/mL) demonstrated the most effective superoxide anion scavenging activity in aqueous solutions. Near complete scavenging (95.7%) was achieved with only a 100 μM water solution without the addition of a co-solvent [39]; Cardax, contrastingly, required mM concentrations and addition of EtOH to completely suppress the measured superoxide anion signal. These results demonstrated that the prepared novel, hydrophilic carotenoid amphiphiles exhibit acceptable aqueous-phase, direct scavenging ability of superoxide anion ($O_2^{\bullet-}$) produced from isolated human neutrophils, and may be potent scavengers of other ROS and free radicals in biological systems. The hydrophilic derivatives demonstrated increased scavenging ability over the parent carotenoids, advancing the potential utility of carotenoids in various *in vitro*, *in vivo*, and clinical applications. Enzymatic cleavage of Cardax and lys_2AST *in vivo* yields free astaxanthin, maintaining the antioxidant activity of the parent scaffolds in the lipid layers of cell membranes. A dual-phase protective mode of action of such novel amphiphilic carotenoid derivatives lends precedence to broad clinical therapeutic applications in both acute and chronic conditions mediated by oxidative and inflammatory processes.

Plasma Protein Binding Studies

Supramolecular assembly, a form of aqueous self-formulation, may stabilize aqueous formulations from oxidation and enhance storage capability. However, large aggregates (> μm size) may complicate parenteral administration *in vivo* and delivery to *in vitro* model systems. The latter was overcome by using the co-solvent ethanol, which totally disaggregated the assemblies into monomers and was non-toxic at the concentrations used in cell-based systems [85]. Prior to parenteral administration in relevant animal models, it was necessary to investigate possible aggregate lability of potential clinical carotenoid therapeutics, facilitated by plasma protein binding. Human serum albumin (HSA) is the major plasma protein in humans, and was previously shown to bind the water-soluble natural carotenoid, crocetin [86]. The *in vitro* plasma protein binding with fatty acid-free human serum albumin (HSA) was investigated for both *meso*-Cardax and lys_2AST using circular dicroism (CD), UV-VIS spectroscopy, and fluorescence quenching [74, 87]. Induced CD bands obtained in buffered solutions demonstrated that both derivatives preferentially bound to albumin in monomeric form at low ligand per protein (L/P) ratios (1:1 up to 1:5). A greater HSA affinity for the dilysinate astaxanthin compound than for the disuccinate astaxanthin compound was believed to be a result of their differences in hydrogen donor and acceptor abilities. At higher L/P ratios, a tendency toward HSA-induced chiral aggregation was observed. Fluorescence quenching of HSA upon addition of the individual derivatives showed that *meso*-Cardax and lys_2AST both bound to sites distinct from the more common small molecule binding sites of HSA. These results suggested that the bonds that allow supramolecular assemblies to form (van der Waals and hydrogen bonds) would be readily overcome in the complex, dynamic biochemical conditions of the *in vivo* biological environment. Based on these findings, prospective parenteral use of such hydrophilic derivatives was postulated to result in carotenoid-plasma protein associations and binding at sites that would not appreciably compete with substances possessing affinities for HSA, such as fatty acids or other therapeutics. Further, possible interactions with circulating blood cells or other lipoproteins might produce disaggregated, monomeric, highly efficacious blood-borne reservoirs of carotenoids.

Cardiovascular Applications

Coronary heart disease is the major cause of morbidity and mortality in Western countries. Epidemological and clinical data indicate that dietary antioxidants such as carotenoids might protect against cardiovascular disease [5, 16]. Oxidative damage of low-density lipoprotein (LDL) is one of the main factors implicated in the initiation and progression of atherosclerosis [88]. Astaxanthin has demonstrated protective effects for LDL against oxidation in humans [89], as well as increasing blood levels of animal high-density lipoprotein (HDL), the form of blood cholesterol inversely correlated with coronary heart disease [46]. Studies in rodents have shown an accumulation of free astaxanthin in the heart after chronic administration of esterified, natural source astaxanthin, suggesting tissue-specific distribution [90]. Therefore, therapeutic administration of astaxanthin or astaxanthin-based soft drugs might represent an approach for the treatment and/or prevention of cardiovascular disease in humans.

Ischemia/reperfusion (I/R) injury is damage in ischemic tissue caused by restoration of blood flow, and may occur after myocardial infarction (MI) or during medical interventions. Such damage is the result of the release of reactive oxygen and nitrogen species from mitochondria, neutrophils, and other leukocytes that are found in the ischemic tissue or the reperfusate. Radical scavenging and lipid peroxidation chain-breaking antioxidants have been suggested as potential preventive agents for I/R injury [91]. Administration of effective concentrations of antioxidants such as carotenoids during the reperfusion period, or as a pre-treatment to at-risk patients, may prevent or inhibit I/R injury. Studies in rats treated with an amphiphilic α-tocopherol derivative have shown promising protective effects [92]; vitamin E shares the dual antioxidant role with carotenoids, but may not be effective at low oxygen tension or low physiologic concentration [93].

Cardax's demonstrated potent aqueous ROS scavenging ability [85] and association with the human plasma protein HSA [87] supported the examination of its cardioprotective efficacy in animal experimental infarction studies. In the seminal study, Cardax was given intravenously (i.v.) as an aqueous formulation to male Sprague-Dawley rats at one of three doses (25, 50, or 75 mg/kg) each day for four days, prior to the induced infarct carried out on day 5. On the fifth day, the rats underwent thirty minutes of left anterior descending coronary artery occlusion, followed by two hours of reperfusion. All doses of Cardax resulted in mean reductions of infarct size, with significant mean reductions at the 2 higher doses translating to 41% salvage and 56% salvage, respectively. In addition, a significant dose-dependent cor-

relation was observed between plasma concentrations of free, non-esterified astaxanthin and myocardial salvage at the end of reperfusion. The linear correlation between plasma levels of non-esterified astaxanthin and measured infarct size allowed extrapolation of therapeutic target levels to larger, more physiologically relevant animals. The results are summarized in Table (**3**) [94].

Such promising initial results facilitated similar studies performed with dogs and rabbits [95, 96]. In both studies, one chronic dose (50 mg/kg, i.v.) was given for four days prior to the experimental infarction performed on day 5, affording a mean myocardial salvage of 51% for rabbits and 68% for dogs. The degree of protection was again directly correlated with the serum concentrations of free astaxanthin, which reached approximately 200 nM in rabbits and 600 nM in dogs. These results parallel the antioxidant and radical scavenging ED_{50} of astaxanthin, reported to be 200 nM in a model system [52]. One hundred percent (100%) cardioprotection was achieved in a chronic treatment group of dogs (2 out of 3 animals) having plasma concentrations of non-esterfied astaxanthin above 1.0 μM. The observed mean myocardial tissue concentration in rabbits was several-fold higher than in plasma, suggesting a highly favorable myocardium to serum ratio after subchronic i.v. administration of Cardax in these animals. In canines, acute cardioprotection by Cardax was also tested by single dose i.v. administration two hours prior to occlusion. Single dose i.v. treatment at 50 mg/kg significantly reduced the infarct size, resulting in mean salvage of 47%. As summarized in Table (**4**), as animal size increased, so too did mean myocardial salvage, demonstrating appropriate pharmacokinetic scaling across the species. Taken together, these results show that protective levels of non-esterified, free astaxanthin can be achieved in both plasma and heart after i.v. delivery of aqueous formulations of Cardax. These studies indicate significant acute and chronic cardioprotection from damage due to myocardial infarction in the I/R injury setting.

Most recently, oral administration of Cardax in the rat model of experimental infarction showed protective effects of 2 supplementary concentrations of drug administered *ad libitum* in feed for 7 days prior to experimental infarction [97]. Dose-dependent increases in non-esterified astaxanthin were achieved in heart tissue, and reflected dose-dependent mean myocardial salvage after infarction. Plasma markers of oxidative stress (molecular fingerprints of the oxidative modification of arachidonic and lineleic acid, respectively) were also significantly reduced at the higher dose of Cardax tested. These results effectively extend the pharmacologic profile of Cardax in this setting to oral pre-treatment clinical paradigms, for which the statin medications are currently utilized.

Tissue damage resulting from myocardial ischemia and reperfusion is not only caused by ROS, but also promoted by myocardial inflammation mediated by activation of the inflammatory complement system. Selective inhibitors of the complement system have shown great potential in limiting reperfusion injury [98-100]. Astaxanthin itself has shown potent anti-inflammatory activity in rodents, including inhibition of the expression of various inflammatory mediators (*e.g.* nitric oxide, prostaglandin E2, and cyclooxygenase 2) [101]. To evaluate the anti-inflammatory effects of Cardax, complement activity was assessed at the end of reperfusion in a rabbit experimental infarction model using a red blood cell lysis assay [96]. An immunofluorescence method was used to determine the presence of tissue-bound C-reactive protein (CRP) and membrane attack complex (MAC), known participants in the inflammation cascade [98, 102]. Cardax was shown to significantly reduce tissue deposition of CRP and MAC in the infarcted area [96]. Such findings indicate that the mechanism of action by which Cardax reduces the tissue damage associated with I/R injury includes both antioxidant and anti-inflammatory (anti-complement) components.

Cardioprotection using a carotenoid soft drug such as Cardax may be extended to other carotenoid scaffolds. Lutein has shown protective effects against the development of early atherosclerosis in epidemiological, *in vitro*, and mouse model studies [58]. In a recent pharmacokinetic and I/R injury study, lutein was administered (i.v. and orally) at a single 0.5 mg/kg dose to male Wistar rats six hours before induced intestinal I/R. Protective effects against oxidative stress caused by intestinal I/R were observed, as evidenced by the recovered deciduation of enterocytes, lessened damage of villi, and suppressed lipid peroxidation [103]. Future infarct studies with hydrophilic lutein derivatives could potentially validate lutein-based compounds as alternative cardioprotective agents.

Hepatitis

Of the four major types of hepatitis (A-D), hepatitis C is the most prevalent liver disease in the world, considered an epidemic by The World Health Organization. Patients with

Table 3. Correlation Between Dose and Mean Infarct Size (IS/AAR,%), Myocardial Salvage, and Plasma Concentrations of Non-Esterified Astaxanthin in Sprague-Dawley Rats After i.v. Administration of Cardax (Disodium Disuccinate Astaxanthin)

Dose (mg/kg)	***0***	***25***	***50***	***75***
IS/AAR (%)	59.0	47	35	26
Myocardial salvage (%)	0	20	41	56
Mean plasma concentration of free astaxanthin (nM)	-	107	333	612

Table 4. Mean Infarct Size (IS/AAR,%), Myocardial Salvage, and Plasma Concentrations of Non-Esterified Astaxanthin in Three Different Animal Experimental Infarction Models After i.v. Administration (50 mg/kg) of Cardax (Disodium Disuccinate Astaxanthin)

Species	***Rats***	***Rabbits***	***Dogs***
Weight (kg)	0.2	2.5	9.0
IS/AAR (%)	35 ± 3	25.8 ± 4.7	6.6 ± 2.8
Myocardial salvage (%)	41	52	68
Mean plasma concentration of free astaxanthin (nM)	335 ± 56	222 ± 51	632 ± 333

chronic hepatitis C demonstrate evidence of increased oxidative stress and elevated lipid peroxidation products in liver tissue, in turn leading to fibrosis and irregular regeneration of hepatocytes, primary causes of serious liver damage in these patients. ROS release can cause increased DNA damage, and therefore chronic hepatitis C infection is considered a major progenitor of liver cancer (hepatocellular carcinoma, or HCC). Unfortunately, there are no vaccines or cures for chronic hepatitis C infection [104, 105]. A promising treatment would be to protect and support the liver against the smoldering, recurrent oxidative damage in chronic infection. Hepatitis C patients supplemented with both lycopene (10 mg/day) and vitamin E for a year exhibited a significant reduction in the incidence of hepatocellular carcinoma [106], promising initial data on the protective effects of liver-specific antioxidants.

To achieve efficacious levels in target tissues, preferential tissue accumulation of a therapeutic is essential. Tissue tropism, oral bioavailability, and pharmacokinetics of astaxanthin disuccinate were evaluated in C57BL/6 mice [107]. Both single and multiple oral doses (500 mg/kg) of a lipophilic emulsion of astaxanthin disuccinate (Heptax) were administered, where an apparent solubility of greater than 50 mg/mL was achieved. Free plasma and tissue levels of astaxanthin were monitored over 72 hours. Single oral administration of the test compound resulted in maximum concentration after six hours. Free astaxanthin was measured in heart (694 nM), liver (1735 nM), and plasma (381 nM). A slight increase in mean peak levels of astaxanthin in both liver and plasma after multiple dosing (11-day regimen) was observed in comparison to the concentrations achieved after single dosing. These results suggest that protective levels (> 200 nM) can be obtained with once-daily dosing. Additionally, it was noted that therapeutic levels of carotenoid persisted for at least 24 h after multiple dosing, indicating a favorable accumulation effect.

Animal infarct and pharmacokinetic studies discussed above show that protective levels of non-esterified, free astaxanthin can be achieved in both plasma and target tissues (heart and liver), after oral and/or i.v. delivery of the sodium salt of astaxanthin disuccinate. An effective organ tissue tropism and desirable plasma clearance of non-esterified, free astaxanthin was achieved; hepatic and cardioprotection against induced oxidative stress, as manifested during infarct or hepatitis, can be achieved using a subchronic dosing schedule.

Cancer Chemoprevention

Carotenoids have been shown to prevent oxidative damage to DNA, a condition known to promote carcinogenesis. Additionally, a number of studies have shown that carotenoids may exert cancer chemoprevention by other mechanisms. Experimental animal and cell culture carcinogenesis studies have demonstrated that carotenoid cancer chemopreventive activity is strongly correlated with an ability to increase gap junctional intercellular communication (GJIC) through the up-regulation of connexin 43 (Cx43) gene expression. This chemopreventive activity is independent of any provitamin A or lipid-phase antioxidant properties. Approximately 20 different connexins are expressed in mammals, and form aqueous gap junction channels (connexons). Connexons assemble into large clusters (or gap junction plaques) in the adjacent membranes of communicating cells, enabling direct transfer of cellular signals, nutrients, and waste products between contacting cells. Connexons are believed to be important for normal cell development and growth regulation. Connexin 43 is the most widely expressed member of the gap junction family of genes and is strongly down-regulated in human cancers and in several premalignant conditions. Carotenoids have been shown to stimulate GJIC in a dose-dependent manner. In contrast, α-tocopherol does not upregulate Cx43 expression [26, 108].

Until recently, experimental animal and cell culture studies of carotenoid carcinogenesis were problematic due to poor carotenoid bioavailability and a lack of suitable *in vitro* carotenoid delivery vehicles. As well, carotenoid availability was largely limited to only those obtainable in beadlet form (*e.g.* lycopene, canthaxanthin, and β-carotene). Investigation of other carotenoids was made possible by using tetrahydrofuran (THF) as a co-solvent, however THF is not considered optimal for animal and clinical studies [26, 108]. Alternatively, the use of hydrophilic carotenoids can be used to achieve desired clinical experimental conditions. As an example, cells treated with aqueous ethanolic formulations of Cardax assembled Cx43 into immunoreactive plaques in regions of cell-cell contact, consistent with the formation of gap junctions [108, 109]. Cardax was shown to both upregulate the expression of the tumor suppressor gene Cx43

and increase GJIC. After treatment with a micromolar Cardax aqueous ethanolic formulation, the induction levels of Cx43 were higher than with cells treated with a neat water formulation of Cardax. Interestingly, a higher potency in the up-regulation of Cx43 was observed for neat water formulations of Cardax than for free carotenoids in organic vehicles. Aqueous ethanolic formulations of pAST likewise demonstrated enhanced upregulation of Cx43 protein expression and induced functional GJIC, compared to astaxanthin delivered in THF. Astaxanthin diphosphate (pAST) completely inhibited carcinogen-induced neoplastic transformation in 10T1/2 cells at micromolar concentrations, while Cardax demonstrated only 40% inhibition at this concentration range [40].

Upregulation of GJIC has been hypothesized to be an important indicator of chemopreventive potential, supported by studies in both Cx32 or Cx43-deficient mice more susceptible to liver and lung cancer, respectively [110, 111]. The ability of carotenoids to upregulate Cx43 as well as gap junctional intercellular communication (GJIC) is well documented [26]. Cardax and pAST share these abilities with the parent carotenoid astaxanthin and may prove to be more potent than astaxanthin alone. Astaxanthin diphosphate (pAST) demonstrated 100% efficacy in preventing carcinogen-induced neoplastic transformation. Following such promising *in vivo* results, the chemopreventive activity of several hydrophilic carotenoid derivatives is currently being explored.

Macular Degeneration

Age-related macular degeneration (ARMD) is the major cause of irreversible blindness in the elderly. A significant prevalence is found after the age of 55, with heightened risk in patients with Type I and II diabetes (42 and 53%, respectively) [47]. Elevated dietary intake and serum levels of lutein and zeaxanthin have been correlated with a decreased risk of ARMD. However, because of variable bioavailability from different food sources, the correlation between lutein and zeaxanthin intake and incidence of ARMD remains problematic. In post-mortem retinas diagnosed with ARMD, the amount of macular pigment was less than in control donor groups. The role and function of lutein and zeaxanthin in the macular pigment was reviewed recently. These carotenoids are believed to filter high energy, short wavelength blue light, protecting polyunsaturated lipids in the photoreceptor membranes against associated oxidative damage [112]. Uptake, stabilization, and effective antioxidant activity of lutein and zeaxanthin in the retina is thought to be achieved and mediated by specific xanthophyll-binding proteins [59]. A normal western diet consists of 1.3-3 mg/day combined lutein and zeaxanthin. Average serum values for lutein and zeaxanthin were reported to be approximately 250 nM and 90 nM, respectively. A combined intake of supplements of more than 5.8 mg/day, affording serum level concentrations of over 670 nM, corresponded to the lowest risk of ARMD [112]. Lutein and zeaxanthin antioxidant and radical scavenging ED_{50} values were calculated at 700 and 400 nM, respectively [52]. The administration of hydrophilic lutein and zeaxanthin derivatives in aqueous formulation will most likely provide desirable therapeutic levels in human plasma, and overcome the problems with oral bioavailability of lutein and zeaxanthin mentioned previously. These plasma concentrations may then translate into increased macular pigment in affected individuals, with the potential to ameliorate progression of disease and impact visual acuity in the short term in these patients.

Prostate Cancer

Prostate cancer is one of the most prevalent diseases associated with mortality among men [113]. High intake of tomato products is associated with low incidence of prostate cancer [64], and lycopene has therefore been suggested as a chemopreventive candidate. A randomized clinical trial of lycopene supplementation (15 mg twice a day) three weeks before radical prostatectomy resulted in decreased growth of prostate tumor and reduced indices of proliferation in treated individuals—thus suggesting a chemotherapeutic role for lycopene as well. Upregulation of tumor suppressor protein Cx43 and increased GJIC are some of the mechanisms by which lycopene is believed to protect against prostate cancer beyond strict antioxidant function. In the prostatectomy trial, carotene levels in prostatic tissue increased by 47% compared to the control group. Additionally, no significant change in plasma concentrations was observed, indicating a marked tissue-specific differentiation [114]. Although lycopene in tomatoes can be absorbed more efficiently if processed into tomato juice, sauce, paste or ketchup, the overall bioavailability is low; the absorption of lycopene is less efficient than the polar carotenoid astaxanthin [115]. Delivery of a highly potent radical scavenger to prostatic tissue, aimed at restoring or augmenting endogenous antioxidant levels, could prove to be a promising area of prostate cancer research [116]. Further, hydrophilic lycopene derivatives should facilitate parenteral delivery of lycopene analogs to prostatic tissues, again overcoming the dose-proportionality issues inherent with oral delivery of many carotenoids. As demonstrated for Cardax, therapeutic levels of astaxanthin are readily achieved after either oral delivery in a lipid-based vehicle or in feed, as well as i.v. administration of hydrophilic formulations. Recently, the lycopene analog lycophyll was succesfully prepared [67], and novel, amphiphilic derivatives of lycophyll are currently being developed.

CONCLUSIONS

Until recently, chemical derivatization of carotenoids to produce novel hydrophilic derivatives have been sparingly reported. By utilizing retrometabolic drug design, Hawaii Biotech, Inc. has succesfully prepared several classes of bolaform carotenoid amphiphiles that are effective aqueous-phase antioxidants in the derivatized state (soft drugs). These novel derivatives are readily formulated in water without need of heat, detergents, co-solvents, or other additives. They exhibit hydrophilicity of up to 181.6 mg/mL, and are sufficient for parenteral administration in aqueous formulations. The self-assembly behavior and biophysical behavior(s) in aqueous solutions were studied for a number of the novel derivatives. Total disruption of aggregates is afforded by addition of less polar solvents. Aqueous monomeric solutions of several of the bolaform amphiphiles have been characterized as potent direct scavengers of aqueous-phase superoxide anion. *In vitro* interaction of the sodium disuccinate of astaxanthin (Cardax) and the dilysinate conjugate of astaxanthin (lys_2AST) with human serum albumin (HSA), us-

ing circular dichroism spectroscopy, demonstrated immediate and preferential binding to HSA at molar ratios of ligand to HSA of 1:1 and 1:5, respectively, suggesting a desirable *in vivo* distribution of both soft drugs. Cardax demonstrated cardioprotective efficacy after both i.v. and oral administration in three different animal experimental infarction models. In dogs, nearly 70% mean myocardial salvage was achieved with subchronic dosing, with 100% cardioprotection observed when therapeutic serum thresholds of drug metabolite are achieved. Tissue accumulation and plasma clearance of non-esterified, free astaxanthin after parenteral administration suggests effective target organ tissue delivery to heart and liver using the prepared astaxanthin derivative. Protective levels of non-esterified, free astaxanthin were achieved in both plasma and target tissues (heart and liver) based on the reported ED_{50} value of astaxanthin (200 nM). Cell studies of Cardax and astaxanthin diphosphate (pAST) demonstrated upregulation of the expression of connexin 43 (Cx43), increase in the size and number of Cx43 immunoreactive gap junctional plaques, and functional increase in gap junctional intercellular communication (GJIC)—and for pAST, 100% suppression of neoplastic transformation in the model system study—strongly suggesting potential chemopreventive properties *in vivo.* After administration of Cardax and pAST, detection of free astaxanthin in plasma, cells, and target tissues demonstrates the success in the design and intended application of these novel carotenoids. The work summarized in the current review validates present and future chemical, biological, and medicinal investigations of novel, hydrophilic carotenoid derivatives utilizing astaxanthin, lutein, zeaxanthin, lycophyll, and other carotenoid scaffolds conjugated to natural, non-toxic substances such as amino acids, sugars, antioxidants, and other nutritive substances. It is hoped that such efforts will serve to effectively combat those diseases and disease states associated with oxidative stress and inflammation.

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ABBREVIATIONS

ARMD = Age-related macular degeneration

Boc = Benzyloxy carbonyl

Cardax = Disodium disuccinate astaxanthin

CD = Circular dichroism

CRP = C-reactive protein

Cx43 = Connexin 43

DEPMPO = 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide

DIC = 1,3-diisopropylcarbodiimide

DIPEA = *N*,*N*-diisopropylethylamine

dLUT = Disodium disuccinate lutein

dLYC = Disodium disuccinate lycophyll

dZEA = Disodium disuccinate zeaxanthin

DMF = *N*,*N*-dimethylformamide

EPR = Electron paramagnetic resonance (spectroscopy)

GJIC = Gap junctional intercellular communication

gluLUT = Disuccinate diglucosyl ester of lutein

gluLYC = Disuccinate diglucosyl ester of lycophyll

HDL = High density lipoprotein

HOBt = 1-hydroxybenzotriazole

HSA = Human serum albumin

LDL = Low density lipoprotein

lys_2AST = Dilysinate astaxanthin

MAC = Membrane attack complex

NMR = Nuclear magnetic resonance (spectroscopy)

pAST = Tetrasodium diphosphate astaxanthin

pLUT = Tetrasodium diphosphate lutein

pLYC = Tetrasodium diphosphate lycophyll

pLUT = Tetrasodium diphosphate lutein

ROS = Reactive oxygen species

THF = Tetrahydrofuran

UV-VIS = Ultraviolet-visible spectroscopy

REFERENCES

[1] Britton, G.; Liaaen-Jensen, S.; Pfander, H.; Mercadante, A.Z.; Egeland, E.S., Eds. *Carotenoids Handbook*, Birkhäuser: Basel, **2004.**

[2] Isler, O. In *Carotenoids;* Isler, O., Ed.; Birkhäuser: Basel, **1971**; pp. 11-28.

[3] Weedon, B.C.L. In *Carotenoids;* Isler, O., Ed.; Birkhäuser: Basel, **1971**; pp. 29-59.

[4] Faulks, R.M. *Educ. Chem.*, **2004**, *41*, 21.

[5] Kiokias, S.; Gordon, M.H. *Food Rev. Int.*, **2004**, *20*, 99.

[6] Weedon, B.C.L.; Moss, G.P. In *Carotenoids, Vol. 1A: Isolation and Analysis;* Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser: Basel, **1995**; pp. 27-34.

[7] Krinsky, N.I. In *NATO ASI Series, Sub-Series A (Life Sciences): Free Radicals, Oxidative Stress and Antioxidants: Pathological*

and Physiological Significance; Özben, T., Ed.; Plenum: New York, **1998**; Vol. *296*, pp. 323-332.
[8] Bauernfeind, J.C. *J. Agric. Food Chem.*, **1972**, *20*, 456.
[9] Bauernfeind, J.C.; Brubacher, G.B.; Kläui, H.A.; Marusich, W.L. In *Carotenoids;* Isler, O., Ed.; Birkhäuser: Basel, **1971**; pp. 743-764.
[10] Abdullaev, F.I.; Espinosa-Aguirre, J.J. *Cancer Detect. Prev.*, **2004**, *28*, 426.
[11] Ernst, H. *Pure Appl. Chem.*, **2002**, *74*, 2213–2226.
[12] Kaiser, R. *An interdisciplinary journal of research on carotenoids (Abstracts presented at the 14th International Symposium on Carotenoids, 17-22 July, Edinburgh)*, **2005**, *9*, 22.
[13] Olson, J.A. *Pure Appl. Chem.*, **1994**, *66*, 1011.
[14] Kirsh, V.A.; Mayne, S.T.; Peters, U.; Chatterjee, N.; Leitzmann, M.F.; Dixon, L.B.; Urban, D.A.; Crawford, E.D.; Hayes, R.B. *Cancer Epidemiol. Biomarkers Prev.*, **2006**, *15*, 92.
[15] Ruano-Ravina, A.; Figueiras, A.; Freire-Garabal, M.; Barros-Dios, J.M. *Curr. Pharm. Des.*, **2006**, *12*, 599.
[16] Tapiero, H.; Townsend, D.M.; Tew, K.D. *Biomed. Pharmacother.*, **2004**, *58*, 100.
[17] Furr, H.C.; Clark, R.M. *J. Nutr. Biochem.*, **1997**, *8*, 364.
[18] Russell, R.M. *Int. J. Vitam. Nutr. Res.*, **1998**, *68*, 349.
[19] Krinsky, N.I. *Ann. N. Y. Acad. Sci.*, **1998**, *854*, 443.
[20] Odeberg, J.M.; Lignell, A.; Pettersson, A.; Hoglund, P. *Eur. J. Pharm. Sci.*, **2003**, *19*, 299.
[21] Zaripheh, S.; Erdman, J.W., Jr. *J. Nutr.*, **2002**, *132*, 531S.
[22] Nagao, A. *J. Nutr.*, **2004**, *134*, 237S.
[23] Bensasson, R.V.; Land, E.J.; Truscott, T.G. In *Excited States and Free Radicals in Biology and Medicine: Contributions from Flash Photolysis and Pulsed Radiolysis*; Oxford University Press: Oxford, **1993**; pp. 65, 201-224.
[24] Mathews-Roth, M.M. *Ann. N. Y. Acad. Sci.*, **1993**, *691*, 127.
[25] van Vliet, T. *Eur. J. Clin. Nutr.*, **1996**, *50*, S32.
[26] Bertram, J.S. *Nutr. Rev.*, **1999**, *57*, 182.
[27] Boileau, A.C.; Merchen, N.R.; Wasson, K.; Atkinson, C.A.; Erdman, J.W., Jr. *J. Nutr.*, **1999**, *129*, 1176.
[28] Eugster, C.; Eugster, C.H.; Haldemann, W.; Rivara, G. *US 5,536,504*. Marigen S.A., Switzerland, **1996**.
[29] Lockwood, S.F.; O'Malley, S.; Mosher, G.L. *J. Pharm. Sci.*, **2003**, *92*, 922.
[30] Horn, D.; Rieger, J. *Angew. Chem. Int. Ed.*, **2001**, *40*, 4330.
[31] Bikádi, Z.; Zsila, F.; Deli, J.; Mady, G.; Simonyi, M. *Enantiomer*, **2002**, *7*, 67.
[32] Hertzberg, S.; Liaaen-Jensen, S. *Acta Chem. Scand. B.*, **1985**, *39*, 629.
[33] Oliveros, E.; Braun, A.M.; Aminian-Saghafi, T.; Sliwka, H.R. *New J. Chem.*, **1994**, *18*, 535.
[34] Pfander, H.; Dumont, R.; Läderach, M. *Chimia*, **1980**, *34*, 20.
[35] Karagiannidou, E.; Størseth, T.R.; Sliwka, H.-R.; Partali, V.; Malterud, K.E.; Tsimidou, M. *Eur. J. Lipid Sci. Technol.*, **2003**, *105*, 419.
[36] Lindig, B.A.; Rodgers, M.A.J. *Photochem. Photobiol.*, **1981**, *33*, 627.
[37] Buchwald, P.; Bodor, N. *Pharmazie*, **2002**, *57*, 87.
[38] Frey, D.A.; Kataisto, E.W.; Ekmanis, J.L.; O'Malley, S.; Lockwood, S.F. *Org. Process. Res. Dev.*, **2004**, *8*, 796.
[39] Jackson, H.L.; Cardounel, A.J.; Zweier, J.L.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 3985.
[40] Hix, L.M.; Frey, D.A.; McLaws, M.D.; Østerlie, M.; Lockwood, S.F.; Bertram, J.S. *Carcinogenesis*, **2005**, *26*, 1634.
[41] Lockwood, S.F.; O'Malley, S.; Watumull, D.G.; Hix, L.; Jackson, H.L.; Nadolski, G. *US 2004/0162329*. Hawaii Biotech, Inc., USA, **2004**.
[42] Hirayama, O.; Nakamura, K.; Hamada, S.; Kobayasi, Y. *Lipids*, **1994**, *29*, 149.
[43] Palozza, P.; Krinsky, N.I. *Arch. Biochem. Biophys.*, **1992**, *297*, 291.
[44] Oshima, S.; Sakamoto, H.; Ishiguro, Y.; Terao, J. *J. Nutr.*, **1997**, *127*, 1475.
[45] Gärtner, C.; Stahl, W.; Sies, H. *Int. J. Vitam. Nutr. Res.*, **1996**, *66*, 119.
[46] Guerin, M.; Huntley, M.E.; Olaizola, M. *Trends Biotechnol.*, **2003**, *21*, 210.
[47] Bowen, P.E.; Herbst-Espinosa, S.M.; Hussain, E.A.; Stacewicz-Sapuntzakis, M. *J. Nutr.*, **2002**, *132*, 3668.
[48] Meyer, P.; Riesen, R.; Pfander, H. In *Carotenoids, Vol. 1A: Isolation and Analysis;* Britton, G.; Liaaen-Jensen, S.; Pfander, H., Eds.; Birkhäuser: Basel, **1995**; pp. 277-282.
[49] Martin, H.D.; Jager, C.; Ruck, C.; Schmidt, M.; Walsh, R.; Paust, J. *J. Prakt. Chem.*, **1999**, *341*, 302.
[50] Spiller, G.A.; Dewell, A. *J. Med. Food*, **2003**, *6*, 51.
[51] Goto, S.; Kogure, K.; Abe, K.; Kimata, Y.; Kitahama, K.; Yamashita, E.; Terada, H. *Biochim. Biophys. Acta*, **2001**, *1512*, 251.
[52] Miki, W. *Pure Appl. Chem.*, **1991**, *63*, 141.
[53] Kurashige, M.; Okimasu, E.; Inoue, M.; Utsumi, K. *Physiol. Chem. Phys. Med. NMR*, **1990**, *22*, 27.
[54] Østerlie, M.; Bjerkeng, B.; Liaaen-Jensen, S. *J. Nutr. Biochem.*, **2000**, *11*, 482.
[55] Kistler, A.; Liechti, H.; Pichard, L.; Wolz, E.; Oesterhelt, G.; Hayes, A.; Maurel, P. *Arch. Toxicol.*, **2002**, *75*, 665.
[56] Woodall, A.A.; Britton, G.; Jackson, M.J. *Biochem. Soc. Trans.*, **1995**, *23*, 133S.
[57] Gabrielska, J.; Gruszecki, W.I. *Biochim. Biophys. Acta*, **1996**, *1285*, 167.
[58] Dwyer, J.H.; Navab, M.; Dwyer, K.M.; Hassan, K.; Sun, P.; Shircore, A.; Hama-Levy, S.; Hough, G.; Wang, X.; Drake, T.; Merz, C.N.; Fogelman, A.M. *Circulation*, **2001**, *103*, 2922.
[59] Billsten, H.H.; Bhosale, P.; Yemelyanov, A.; Bernstein, P.S.; Polivka, T. *Photochem. Photobiol.*, **2003**, *78*, 138.
[60] Hartmann, D.; Thürmann, P.A.; Spitzer, V.; Schalch, W.; Manner, B.; Cohn, W. *Am. J. Clin. Nutr.*, **2004**, *79*, 410.
[61] Thürmann, P.A.; Schalch, W.; Aebischer, J.C.; Tenter, U.; Cohn, W. *Am. J. Clin. Nutr.*, **2005**, *82*, 88.
[62] Nadolski, G.; Cardounel, A.J.; Zweier, J.L.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2006**, *16*, 775.
[63] Nadolski, G.; Lockwood, S.F. *The synthesis of water-dispersible zeaxanthin esters, in prep*.
[64] Giovannucci, E. *J. Natl. Cancer Inst.*, **1999**, *91*, 317.
[65] Agarwal, S.; Rao, A.V. *Lipids*, **1998**, *33*, 981.
[66] Cholnoky, L.; Szabolcs, J.; Waight, E.S. *Tetrahedron Lett.*, **1968**, *16*, 1931.
[67] Jackson, H.L.; Nadolski, G.T.; Braun, C.; Lockwood, S.F. *Org. Process. Res. Dev.*, **2005**, *9*, 830.
[68] Braun, C.L.; Jackson, H.L.; Lockwood, S.F.; Nadolski, G. *J. Chromatogr. B*, **2006**, *834*, 208.
[69] Nadolski, G.; Lockwood, S.F. *The synthesis of water-dispersible lycophyll esters, in prep*.
[70] Foss, B.J.; Naess, S.N.; Sliwka, H.R.; Partali, V. *Angew. Chem. Int. Ed.*, **2003**, *42*, 5237.
[71] Foss, B.J.; Sliwka, H.R.; Partali, V.; Naess, S.N.; Elgsaeter, A.; Melø, T.B.; Naqvi, K.R. *Chem. Phys. Lipids*, **2005**, *134*, 85.
[72] Foss, B.J.; Sliwka, H.R.; Partali, V.; Naess, S.N.; Elgsaeter, A.; Melø, T.B.; Naqvi, K.R.; O'Malley, S.; Lockwood, S.F. *Chem. Phys. Lipids*, **2005**, *135*, 157.
[73] Foss, B.J.; Sliwka, H.R.; Partali, V.; Cardounel, A.J.; Zweier, J.L.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 2807.
[74] Zsila, F.; Fitos, I.; Bikádi, Z.; Simonyi, M.; Jackson, H.L.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2004**, *14*, 5357.
[75] Salares, V.R.; Young, N.M.; Carey, P.R.; Bernstein, H.J. *J. Raman Spectrosc.*, **1977**, *6*, 282.
[76] Ruban, A.V.; Horton, P.; Young, A.J. *J. Photochem. Photobiol. B.*, **1993**, *21*, 229.
[77] Buchwald, M.; Jencks, W.P. *Biochemistry*, **1968**, *7*, 834.
[78] Lüddecke, E.; Auweter, H.; Schweikert, L. *EP 930 022*. BASF AG, Germany, **1998**.
[79] Zsila, F.; Nadolski, G.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2006**, in press.
[80] Robinson, N.; Saunders, L. *J. Pharm. Pharmacol.*, **1958**, *10*, 384.
[81] Næss, S.N.; Elgsaeter, A.; Foss, B.J.; Li, B.; Sliwka, H.-R.; Partali, V.; Melø, T.B.; Naqvi, K.R. *Helv. Chim. Acta*, **2006**, *89*, 45.
[82] Manitto, P.; Speranza, G.; Monti, D.; Gramatica, P. *Tetrahedron Lett.*, **1987**, *28*, 4221.
[83] Matheson, I.B.C.; Rodgers, M.A.J. *Photochem. Photobiol.*, **1982**, *36*, 1.
[84] Lee, S.H.; Min, D.B. *J. Agric. Food Chem.*, **1990**, *38*, 1630.
[85] Cardounel, A.J.; Dumitrescu, C.; Zweier, J.L.; Lockwood, S.F. *Biochem. Biophys. Res. Commun.*, **2003**, *307*, 704.
[86] Zsila, F.; Bikádi, Z.; Simonyi, M. *Tetrahedron Asymmetry*, **2001**, *12*, 3125.

[87] Zsila, F.; Simonyi, M.; Lockwood, S.F. *Bioorg. Med. Chem. Lett.*, **2003**, *13*, 4093.
[88] Goulinet, S.; Chapman, M.J. *Arterioscler. Thromb. Vasc. Biol.*, **1997**, *17*, 786.
[89] Iwamoto, T.; Hosoda, K.; Hirano, R.; Kurata, H.; Matsumoto, A.; Miki, W.; Kamiyama, M.; Itakura, H.; Yamamoto, S.; Kondo, K. *J. Atheroscler. Thromb.*, **2000**, *7*, 216.
[90] Aoi, W.; Naito, Y.; Sakuma, K.; Kuchide, M.; Tokuda, H.; Maoka, T.; Toyokuni, S.; Oka, S.; Yasuhara, M.; Yoshikawa, T. *Antioxid. Redox. Signal.*, **2003**, *5*, 139.
[91] Maxwell, S.R.; Lip, G.Y. *Int. J. Cardiol.*, **1997**, *58*, 95.
[92] Petty, M.A.; Lukovic, L.; Grisar, J.M.; Dow, J.; Bolkenius, F.N.; De Jong, W. *Eur. J. Pharmacol.*, **1994**, *255*, 215.
[93] Burton, G.W.; Ingold, K.U. *Science*, **1984**, *224*, 569.
[94] Gross, G.J.; Lockwood, S.F. *Life Sci.*, **2004**, *75*, 215.
[95] Gross, G.J.; Lockwood, S.F. *Mol. Cell. Biochem.*, **2005**, *272*, 221.
[96] Lauver, D.A.; Lockwood, S.F.; Lucchesi, B.R. *J. Pharmacol. Exp. Ther.*, **2005**, *314*, 686.
[97] Gross, G.J.; Hazen, S.L.; Lockwood, S.F. *Mol. Cell. Biochem.*, **2006**, *283*, 23.
[98] Bhole, D.; Stahl, G.L. *Crit. Care Med.*, **2003**, *31*, S97.
[99] Lucchesi, B.R. *Arzneimittelforschung*, **1994**, *44*, 420.
[100] Homeister, J.W.; Lucchesi, B.R. *Annu. Rev. Pharmacol. Toxicol.*, **1994**, *34*, 17.
[101] Ohgami, K.; Shiratori, K.; Kotake, S.; Nishida, T.; Mizuki, N.; Yazawa, K.; Ohno, S. *Invest. Ophthalmol. Vis. Sci.*, **2003**, *44*, 2694.
[102] Volanakis, J.E. *Ann. N. Y. Acad. Sci.*, **1982**, *389*, 235.
[103] Iseki, K.; Ogura, W.; Kurokawa, T.; Itagaki, S.; Hirano, T.; Mizuno, S. *An Interdisciplinary Journal of Research on Carotenoids (Abstracts presented at the 14th International Symposium on Carotenoids, 17-22 July, Edinburgh)*, **2005**, *9*, 123.
[104] Poynard, T.; Yuen, M.F.; Ratziu, V.; Lai, C.L. *Lancet*, **2003**, *362*, 2095.
[105] Shibata, M.; Morizane, T.; Uchida, T.; Yamagami, T.; Onozuka, Y.; Nakano, M.; Mitamura, K.; Ueno, Y. *Lancet*, **1998**, *351*, 1773.
[106] Nishino, H.; Jinno, K. *AACR Conference*, Boston. **2002**, 107.
[107] Showalter, L.A.; Weinman, S.A.; Østerlie, M.; Lockwood, S.F. *Comp. Biochem. Physiol. C. Toxicol. Pharmacol.*, **2004**, *137*, 227.
[108] Hix, L.M.; Lockwood, S.F.; Bertram, J.S. *Redox Rep.*, **2004**, *9*, 181.
[109] Hix, L.M.; Lockwood, S.F.; Bertram, J.S. *Cancer Lett.*, **2004**, *211*, 25.
[110] Temme, A.; Buchmann, A.; Gabriel, H.D.; Nelles, E.; Schwarz, M.; Willecke, K. *Curr. Biol.*, **1997**, *7*, 713.
[111] Avanzo, J.L.; Mesnil, M.; Hernandez-Blazquez, F.J.; Mackowiak, II; Mori, C.M.; da Silva, T.C.; Oloris, S.C.; Garate, A.P.; Massironi, S.M.; Yamasaki, H.; Dagli, M.L. *Carcinogenesis*, **2004**, *25*, 1973.
[112] Landrum, J.T.; Bone, R.A. *Arch. Biochem. Biophys.*, **2001**, *385*, 28.
[113] Simzar, S. *Nutrition Noteworthy*, **2002**, *5*, 1.
[114] Kucuk, O.; Sarkar, F.H.; Sakr, W.; Djuric, Z.; Pollak, M.N.; Khachik, F.; Li, Y.W.; Banerjee, M.; Grignon, D.; Bertram, J.S.; Crissman, J.D.; Pontes, E.J.; Wood, D.P., Jr. *Cancer Epidemiol. Biomarkers Prev.*, **2001**, *10*, 861.
[115] Clark, R.M.; Yao, L.; She, L.; Furr, H.C. *Lipids*, **2000**, *35*, 803.
[116] Petros, J.A.; Baumann, A.K.; Ruiz-Pesini, E.; Amin, M.B.; Sun, C.Q.; Hall, J.; Lim, S.; Issa, M.M.; Flanders, W.D.; Hosseini, S.H.; Marshall, F.F.; Wallace, D.C. *Proc. Natl. Acad. Sci. USA*, **2005**, *102*, 719.

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