REVIEW

Clinical Applications of C_{18} and C_{20} Chain Length Polyunsaturated Fatty Acids and Their Biotechnological Production in Plants

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ABSTRACT: A potential revolution in FA therapies is on the horizon. In recent years, the full magnitude of various FA treatments and their overall importance to health has become increasingly apparent. Fetal and infant nutrition studies have clearly shown that FA status at birth can have life-long health implications affecting eye and brain function, insulin resistance, and blood pressure control. As well, nutrition studies have identified dietary imbalances and deficiencies that have the potential to alter the health of future generations severely and to promote progression of age-related degenerative disorders.

Mixtures of naturally occurring FA have shown promise as therapeutic agents for a diverse range of health conditions including atopic eczema, rheumatoid arthritis, cardiovascular disease, and neurological problems. Through the 1990s, the creation of technologies to concentrate and formulate pharmacologically active individual FA components as well as tailored combinations propelled development of this new drug category. However, high production costs and government regulatory encumbrance limited the expansion of this emerging pharmaceutical sector. Fortunately, many countries are now creating regulatory frameworks that are better suited for product evaluation and control of the manufacturing FA products than historical drug models, and hence expansion in this area is now anticipated.

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Underpinning the pharmaceutical/nutraceutical applications of FA is the requirement for high-quality fats and oils from natural sources. The end use and hence the value of an oil depend largely on its FA composition and content. In the Plant Kingdom, well over 800 different FA have been identified; many of these are uncommon and are found in only a few species. This enormous genetic resource is of commercial value as it becomes increasingly feasible to transfer the capacity to synthesize unusual and desirable FA to oil-crop species such as oilseed rape, sunflower, and soybeans. In parallel, improvements in the agronomy and yields of oils from specialty crops such as evening primrose, borage, and *Echium* through plant breeding are also creating a market niche. Oilseed crops generally produce unsaturated FA having chain lengths up to C18, with linoleic acid (LA) and α-linolenic acid (ALA) being the most abundant. However, although there is a dietary requirement for these FA (hence the term EFA), growing clinical evidence indicates that intervention with more highly unsaturated and longer-chain FA is important in optimizing health benefits. Thus, the challenge facing the industry is to supply sufficient high-quality, renewable sources of long-chain PUFA (LCPUFA) in a cost-effective manner. Here we highlight the current clinical findings in LCPUFA research and discuss the biotechnological aspects of oil seed engineering to satisfy potential pharmaceutical end use.

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ESSENTIALITY OF FA IN HUMAN HEALTH

Fat constitutes approximately 15% of the body weight of an average healthy human male and about 22% of a similar female. Historically, the purpose of this fat was thought to be mainly energy storage, with little consideration being given to the possibility of its greater functional role in critical metabolic processes. We now know that FA are of crucial importance to body function. They are structural components of the phospholipids of cell membranes and can affect their fluidity and flexibility, thereby modulating the behavior of membrane-bound proteins including receptors, enzymes, and ion channels that dictate cell function (1,2). They are involved in gene regulation (3) and the transport and disposal of cholesterol (4) and are responsible for the impermeability of the skin to water and for regulation of permeability in the gut and other tissues (5,6). In addition, they are precursors for hormone-like substances that regulate a broad spectrum of functions including blood pressure control, inflammation, and immunity (7–10).

There is a vast array of naturally occurring FA, and the potential to uncover hidden medical applications is enormous. However, we will be restricting our discussion of therapeutic uses of FA primarily to the EFA and their metabolites, given that most of the clinical research has centered on them.

At least 30% of the caloric intake in a typical Western diet is derived from FA. The two dietary EFA, linoleic acid (LA) and α-linolenic acid (ALA), are found mostly in TG oils derived from plant sources including soybean, sunflower, corn, and oilseed rape. These polyunsaturated oils (whose constituent FA have more than one double bond) are typically processed to improve shelf life by modifying their natural *cis*

FIG 1. The configuration of *cis* and *trans* double bonds.

configuration to a *trans* form (Fig. 1). However, once ingested, this change in molecular configuration has a detrimental impact on metabolic function resulting in altered membrane integrity and reduced production of biologically active EFA metabolites (11,12).

PUFA are polyenoic acids having more than one double bond, generally in a *cis* configuration and methylene-interrupted. By convention, PUFA are described as being n-3 or n-6 FA depending on whether the first double bond is three or six carbon atoms away from the methyl (ω carbon), respectively. Additional double bonds are separated from each other by a methylene group. Such FA are also termed ω-3 and ω-6 acids. The n-3 series are derived from ALA (18:3n-3) by chain elongation and further desaturation and include EPA (20:5n-3) and DHA (22:6n-3). On the other hand, n-6 acids are derived from LA (18:2n-6) and include γ-linolenic acid (GLA, 18:3n-6) and arachidonic acid (AA, 20:4n-6). A further unsaturated FA terminology, of particular value to biochemists, defines the position of the double bonds from carbon atom 1 (i.e., the Δ end of the molecule). The Delta system is convenient for denoting all double bonds, their position in the carbon chain, and the specific desaturase enzymes that catalyze their introduction into the molecule. Hence, LA and ALA are represented as $C18:2^{\Delta 9,12}$ and $C18:3^{\Delta 9,12,15}$, respectively. Desaturases, which abstract hydrogen atoms from adjacent CH_2 groups to produce double bonds, can be specifically defined. For example, a Δ12 desaturase or a Δ5 desaturase would introduce double bonds at the Δ12 or Δ5 carbon atoms in the acyl chain, respectively.

The *cis* configurations of LA and ALA give rise to the n-6 and n-3 families of metabolites, respectively, through a series of alternating desaturations (removal of two hydrogen atoms and insertion of a double bond) and elongations (addition of two carbon atoms) (Fig. 2). These FA are precursors for a wide range of short-lived eicosanoids that are generated through the cyclo-oxygenase and lipoxygenase pathways with diverse and significant physiological ramifications depending on their relative concentrations (13) (Table 1).

An n-6 FA remains as n-6 throughout the sequence of reactions; and in mammals, there can be no interconversion between the two families. Even so, these FA are metabolized by the same enzyme sequence, with the higher affinity being for the n-3 series. This resulting competition can permit increased production of eicosanoids derived from one series relative to the other, depending on the relative concentration of the substrates. Interactions between the two series such as inhibition of AA production by EPA enable manipulation of membrane FA distribution and metabolite production through dietary intervention (14).

In the biosynthesis of LCPUFA (i.e., PUFA having carbon chain lengths >18), the rate-limiting enzyme in the pathway is the Δ6-desaturase (Δ^6 desaturase) converting LA to GLA (15). Various vitamins and minerals are required as cofactors, including zinc and magnesium for Δ^6 desaturase and vitamin B_6 for the elongase that converts GLA to dihomo-γ-linolenic acid (DGLA; 20:3n-6). Additional rate-limiting factors for Δ^6 desaturase include excessive dietary consumption of saturated and *trans* FA, cholesterol and alcohol; increased cortisol and adrenaline production; smoking; viral infections; low insulin production; and genetic predisposition, particularly in people with atopic disorders (16) and in diabetics (17), in females with premenstrual syndrome (18,19), in persons with multiple scle-

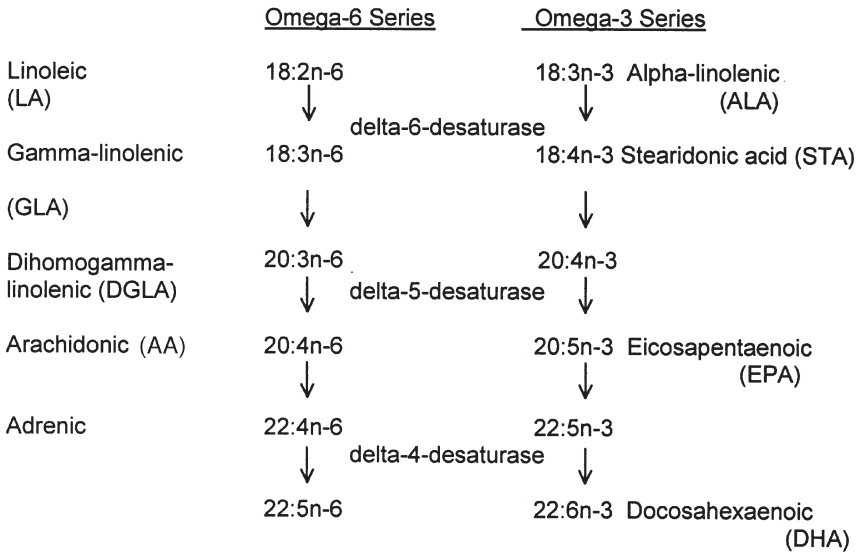

FIG 2. Metabolic pathway for conversion of LA and ALA to their corresponding metabolites.

TABLE 1
Some Eicosanoids and Other Biologically Active Molecules Derived from Various Fatty Acids[a]

FA precursor	Cyclo-oxygenase products	Lipoxygenase products
DGLA	PGE1	LTA3
	PGF1	LTC3
	TXA1	LTD3
		15-OH-DGLA
AA	PGG2	LTA4
	PGH2	LTB4
	PGD2	LTC4
	PGE2	LTD4
	PGF2	LTE4
	PGI2	5-HPETE
	TXA2	
	TXB2	
	6-Keto-PGF1	
	HHT + MDA	
EPA	PGE3	LTA5
	PGF3	LTB5
	Prostacyclin	LTC5
	TXA3	LTD5
		LTE5
		5-HPEPE

[a]PG = prostaglandins; TX = thromboxanes; LT = leuktrienes; HHT = 12-hydroxy-5,8,10-heptadecatrienoic acid; MDA = malondialdehyde; HPETE = hydroperoxy-6,8,11,14-tetraenoic acid; HPEPE = hydroperoxy-6,8,11,14,17-pentaenoic acid.

rosis (MS) (20) and rheumatoid arthritis (21), and perhaps in people having a variety of learning disorders including attention deficit hyperactivity disorder, dyspraxia, and dyslexia (22).

Evidence for the rate-limiting capacities and defects in this metabolic process become apparent by comparing the relative amounts of various FA in body tissues and through selective feeding of specific FA (14). For example, if the blood levels of LA and ALA are normal or elevated and the levels of all their metabolites are significantly below normal, this implies an inability to convert the dietary EFA to their corresponding metabolites. Abnormalities of FA metabolism or intake can sufficiently alter physiology to orchestrate massive abnormalities in structure and function of all body systems through their effects on eicosanoid metabolism, membrane fluidity, and cell signaling pathways. It is these pathophysiological consequences and their FA treatments that will be discussed along with ideas for improved therapies.

FA THERAPIES FOR SPECIFIC HEALTH CONCERNS

Inflammatory disorders. One of the first therapeutic uses of a naturally occurring FA-containing oil was for treatment of atopic eczema. The product, called Epogam®, was Efamol® evening primrose oil (EPO), available as a prescription drug in over a dozen countries including the United Kingdom, Germany, Italy, Ireland, Denmark, South Africa, Australia, and New Zealand. This TG oil contained approximately 72% LA and 9% GLA, with GLA being the active component.

Atopic eczema is associated with an inherent abnormality in FA metabolism resulting in normal or slightly elevated LA levels in blood, breast milk, and adipose tissue along with correspondingly reduced concentrations of LA metabolites (23). These abnormalities are likely representative of a causal rather than effectual manifestation because they also are observed in cord blood and serum phospholipids from infants with high familial risk and no apparent disease (24). Correction of this FA imbalance, subsequent incorporation of adequate FA metabolites into membrane phospholipids to improve the physical structure of skin cells, and resulting alterations in inflammatory response and immune function may be responsible for the measured effectiveness of EPO treatment in most of the 22 clinical studies reported (25). The limitations of EPO treatment for atopic eczema include the low GLA content relative to other oil components resulting in dosages of up to twelve 500 mg capsules daily to achieve a desired effect, and the lack of AA, which may be required and is only partially corrected by GLA supplementation. High doses of the EPO prescription drug Efamast® are also required to achieve effective response for severe breast pain associated with premenstrual syndrome (26). A concentrated TG mixture containing GLA and AA (in the case of atopic eczema) or a diol incorporating both FA may be more efficacious and easier to administer.

Combinations of naturally occurring TG oils were the next means of creating therapeutic options based on FA. EPO (or borage oil) and fish oil (FO) combinations (27) have shown promise for treatment of rheumatoid arthritis largely as a result of the combined effects of GLA and EPA on inflammation. EPO supplementation elevates DGLA concentrations, resulting in the production of the anti-inflammatory prostaglandin E_1 (PGE1). As well, DGLA can be converted to 15-hydroxy-DGLA, which inhibits the conversion of AA to pro-inflammatory leukotrienes. Including EPA from FO blocks the conversion of DGLA to AA and further enhances PGE1 levels relative to the concentration of pro-inflammatory PGE2 by allowing more DGLA to be available as its substrate. This therapeutic approach is more advantageous than use of nonsteroidal anti-inflammatory drugs (NSAID) because it lacks the side effects associated with NSAID and a persistent disease state related to continued production of the inflammatory mediator, leuketriene B_4 (28). However, the requirement for high doses of these naturally occurring oils can lead to poor patient compliance and reduced effectiveness of the medication, making the use of designer TG appealing. It is noteworthy that similar mixtures of EPO and FO may play a role in treatment or prevention of osteoporosis owing to their synergistic abilities to increase calcium absorption from the gut, to reduce its urinary excretion, and to promote its deposition in bone rather than arterial walls and kidneys (29).

Cardiovascular disease. Concurrent administration of GLA and EPA to achieve simultaneous increases in both DGLA and EPA is theoretically of value to cardiovascular health (30,31). n-6-Derived PGE1 and PGI2 elicit a wide range of desirable actions on the cardiovascular system including vasodilation, blood pressure reduction, platelet aggregation inhibition, cholesterol synthesis inhibition, and enhancement of cholesterol excretion. The dietary and supplemental n-3 metabolites have an even more established and wider range of benefits (32). However, they have

a tendency to increase LDL-cholesterol, a potent marker of cardiovascular risk. Consequently, combinations of n-6 and n-3 metabolites are more likely to achieve a desired response than either treatment alone. The most recent evidence of this was a study completed in healthy women showing that simultaneous supplementation of 4 g of EPA + DHA and 2 g GLA daily achieved a 43% reduction in the 10-yr risk of myocardial infarction (33). Other investigative uses of similar combinations have been in the treatment of restenosis following angioplasty where purified GLA in a 3:1 ratio with EPA/DHA achieved a 31% greater reduction in restenosis than the placebo (34). Similar investigations with FO and EPA alone have been unsuccessful (35,36). Specifically configured TG or a combination of diols containing GLA, EPA, and DHA may be advantageous alternatives to high-dose mixtures of naturally occurring oils in the treatment and prevention of cardiovascular disease.

Neurological conditions. Diabetes leads to broad damage to both the sensory and autonomic nerves associated with Δ^6 desaturase inhibition, resulting in lack of GLA and further metabolites in the nerves and the blood vessels that supply them. These abnormalities lead to increased blood viscosity, constriction of microvessels, and nerve hypoxia. These effects, combined with the loss of EFA metabolites required for the myelin sheath, lead to the loss of normal nerve structure and function. Clinical trials using EPO to treat diabetic neuropathy have proven to be effective both for slowing the progression of the disease and for repairing some of the associated nerve damage, probably through increased prostacyclin and reduced thromboxane production from AA (37). Again, high doses of the product may have a negative impact on patient compliance.

Continued investigations into the effects of FA treatments for diabetic neuropathy have led to suggestions of combining GLA treatment with aldose reductase inhibitors (37) and free radical scavengers. A novel compound of the latter, produced by conjugation of GLA with α-lipoic acid, has produced improvements in both electrophysiological and neurochemical correlates in diabetic neuropathy animal models (38,39). Another unique compound, ascorbyl-GLA, provided a correction for nerve conduction velocity and blood flow that was 40 times that of EPO and greater than an equimolar mixture of ascorbic acid and GLA (40).

Mixtures of naturally occurring oils containing LCPUFA that are EFA metabolites along with other nutrients, and pure pharmacoactive compounds may prove beneficial for MS. Evidence of n-6 (41) and n-3 (42) LCPUFA deficiency exists in MS, and supplementation with oils containing these FA has shown some benefit (43,44). Combination products containing these plus another promising mixture of lofepramine, L-phenylalanine, and vitamin B_{12} deserve consideration (45).

Recent advances in psychotropic drug development have included an ethyl ester of purified EPA for treatment of depression, unresponsive schizophrenia, and tardive dyskinesia. This product is believed to function through modulation of postreceptor signal transduction and is well tolerated despite modification from its naturally occurring TG configuration, where it normally exists in combination with of a variety of other FA (46). It has also shown promise for treatment of Huntington's disease (47).

FA treatments for other brain disorders have focused on combinations of EPO and high-DHA-containing tuna oil for various learning disorders, including attention deficit hyperactivity disorder (48), dyslexia, and dyspraxia, where the endogenous supply of LCPUFA appears to be compromised. DHA deficiency is also associated with normal aging and Alzheimer's disease (AD), and dietary supplementation with this FA may reduce the risk of incidence of AD (49). Our modern diet, high in *trans* and saturated FA and lacking in certain vitamins and minerals, may be responsible for the inability to convert dietary EFA to LCPUFA, including GLA, AA, and DHA, and may contribute to this imbalance in the diet. However, other evidence for abnormalities in FA and phospholipid metabolism associated with learning disorders as well as autism highlights a need for more research in this area. The likelihood of these conditions being the result of multiple gene expression enhances the potential for creating targeted lipid drug treatments (50).

Cancer. A review of FA research in relation to cancer treatment uncovers the greatest variety of creative FA therapy options. Naturally occurring FO have been recommended as preventive agents for breast, colon, and prostate cancer (51) whereas other TG oils have demonstrated antitumor activity in transplantable mammary tumors (52). There are a number of potential positive effects of exogenous lipids on cancer chemotherapy (53–56), highlighting the possibility of using specific dietary FA as adjunct therapies. It has been suggested that DHA may increase the response of mammary tumors to cytotoxic agents (57); FO supplementation has been found to enhance irinotecan efficacy and ameliorate intestinal side effects in mice with breast carcinoma xenographs (58), and GLA was shown to enhance the effectiveness of tamoxifen in human endocrine-sensitive breast cancer treatment (59) and to improve cytotoxic activity of vinorelbine on human breast carcinoma cell lines (60). Most recently, gene expression studies have confirmed an inverse relationship between Δ^6 desaturase levels and tumor aggressiveness in breast cancer cells, confirming a target for drug development in this area (61).

In addition to using FA to enhance effectiveness of conventional therapies, FA are used to reduce side effects associated with the disease itself (cancer cachexia). In the early 1990s, a combination of EPA and a TG concentrate of GLA was shown to decrease early and late radiation-induced normal tissue damage in pig skin (62,63). This was followed by a study in tumor-bearing mice where EPO supplementation reduced skin sensitivity to radiation-induced moist desquamation and prevented increased blood flow without altering tumor sensitivity to the treatment (64). Recently, GLA was found to reduce risks significantly in conjunction with curative radiosurgery for large arteriovenous malformations in humans (65). In pancreatic cancer patients, FO, concentrated EPA, and EPA diester emulsions were shown to stabilize the rate of weight, adipose, and muscle tissue loss associated with the disease (66–68).

A wide assortment of FA and their derivatives has been investigated as anticancer therapies. Low doses of purified GLA

have been safely administered intratumorally in human gliomas and have been recommended for future studies using higher doses (69). Topically applied GLA is an effective agent against superficial bladder carcinoma whereas the novel compound meglumine-GLA synergistically enhanced the cytotoxicity of epirubicin toward human urothelial carcinoma cell lines (70). Encouraging data were also obtained with the lithium salt of GLA in a phase III study on pancreatic cancer patients (71,72). A propane diol GLA/EPA emulsion provided a 640-fold higher dose of both GLA and EPA as compared with free PUFA. Subsequent metabolism of this emulsion created a potentially desirable cytotoxic PUFA content in tumors without causing adverse effects in host mice bearing human pancreatic carcinomas (73). Similarly, low-toxicity maintenance therapy of mantle cell lymphoma and other tumors that overexpress cyclin D1 has been demonstrated using concentrated EPA in combination with a variety of other specific drugs (74).

Fetal and infant nutrition. By far the most well-researched area of LCPUFA impact on health has been in fetal and infant nutrition (75–77). The likelihood of developing lipid drug treatments for use during pregnancy and breast-feeding is small given the risks normally associated with experimental investigations in this patient population. However, the topic still warrants discussion within the context of this paper, as there is overwhelming evidence of significant deficiencies in the FA composition of our modern diet with ramifications for fetal and infant development affecting health throughout life. As well, FA treatments aimed at preventing developmental impairment in genetically compromised infants including phenylketonurics could be a possibility in the future (78).

The 2003 Congress of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) addressed dietary choices within populations that impact tissue FA and eicosanoid content and the direct impact of these choices on health (79). In essence, typical Westernized diets contain too many saturated and *trans* fats relative to *cis* PUFA, and the ratio of LA to ALA is too high, as is the ratio of EFA to their corresponding LCPUFA metabolites. These imbalances in FA intake contribute to the development of a broad spectrum of health conditions ranging from cardiovascular disease to cognitive decline (80,81).

Compounding this problem is a misunderstanding in the North American general population that these imbalances can be corrected simply by eating a low-fat diet and/or supplementing one's diet with ALA-rich flaxseed oil. The former, aimed at reducing the saturated and *trans* FA content of the diet, simultaneously and detrimentally reduces the *cis* EFA and *cis* LCPUFA intake. The latter attempts to correct the n-6 vs. n-3 imbalance but instead further contributes to the already excessive intake of EFA relative to LCPUFA. Neither of these approaches corrects the ratio of EFA to their corresponding LCPUFA metabolites.

The problem is amplified further in pregnant and breast-feeding women where the developing fetus is dependent on the maternal supply of DHA and AA. DHA is an important structural component of nerve membranes and is particularly prevalent in the retina and brain. AA is an important component of cell membranes, in particular in the brain, and is a precursor to PG that regulate cell growth. These dietary FA are needed for normal development of an infant's brain, eyes, and nervous system. During the last trimester when the fetal brain is growing rapidly, and continuing during the first 12 wk after birth, the DHA content of the brain increases three- to fivefold (82,83). Studies have linked DHA deficiency in the mother's diet to the incidence of low birth-weight babies, low head circumference (83), and low placental weight (84). As well, studies indicate that DHA status at birth may have long-term effects on the child. A number of studies have demonstrated better visual and intellectual function in children supplied with adequate DHA and AA during early development (85). Evidence indicates that adequate AA concentrations in fetal cell membranes dictate proper functioning of membrane-bound proteins that can prevent the development of insulin resistance (Type II diabetes) and high blood pressure later in life (86,87).

DHA and AA are also important during lactation. DHA levels in human milk have dropped by 30% in the last 14 yr in some developed countries (88). In China and Japan, the levels are four times higher than those found in westernized countries. During lactation, 70–80 mg of DHA/d is preferentially transferred from mother to baby through the milk (89). This places huge demands on a mother who may not be eating enough DHA to satisfy even her own requirements. Reduced DHA levels in the mother also have been implicated in the development of postnatal depression (90,91). Particularly low DHA levels are found in women vegetarians, following multiple births (i.e., twins or triplets), and during subsequent pregnancies that are close together.

ISSFAL recommends an Adequate Daily Intake of DHA for pregnant and lactating women to be 300 mg. A recent survey of pregnant Canadians found this intake to be significantly less at 160 ± 20 mg, indicating a need for dietary supplementation (92). These reduced dietary intakes agree with surveys in other countries including Australia, where the average woman eats only 60–70 mg of DHA per day, and the United States, where DHA intake is even less at 54 mg/d. The same study found AA intake in pregnant Canadians to be 121 ± 8 mg/d. During the third trimester of gestation the fetus accumulates approximately 250–400 mg of AA per day. The low reported intake of AA, evidence demonstrating dietary effects on maternal endogenous synthesis of AA, and the negative impact of low AA levels on fetal AA status and birth weight all suggest dietary supplementation with AA and its precursors during pregnancy is also a necessity (92). For this purpose, natural TG oil combinations of EPO and high-DHA tuna oil have recently been formulated and are available in a number of countries for use during pregnancy and breast-feeding. More tailored combinations may be possible in the future.

SOURCES OF PUFA AND LCPUFA IN NATURE

The LCPUFA—AA, EPA, and DHA—are currently the subject of much interest because of their important roles in human

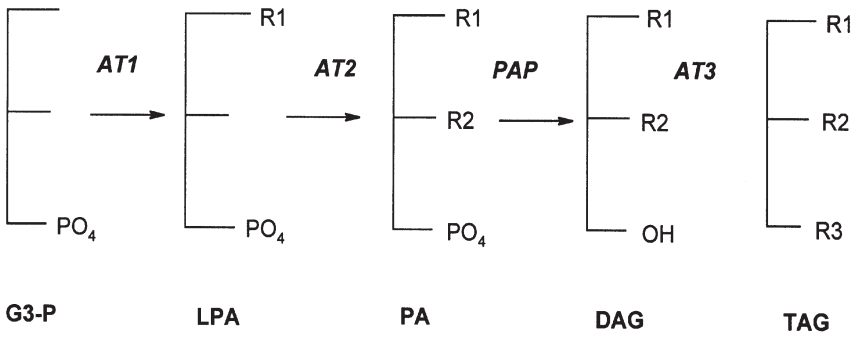

FIG 3. Biosynthesis of TAG. Glycerol-3-phosphate (G3-P) undergoes acylation to yield lysophosphatidic acid (LPA) and phosphatidic acid (PA) through the action of glycerol phosphate acyltransferase (AT1) and lysophosphatidic acid acyltransferase (AT2), respectively. A phosphatidic acid phosphohydrolase (PAP) activity yields diacylglycerol (DAG) which undergoes acylation, catalyzed by diacylglycerol acyltransferase (AT3) to form triacylglycerol (TAG).

health and nutrition, as just discussed. Unlike animal tissues, which are generally rich sources of C_{20} PUFA, higher plant oil seeds tend to be dominated by C_{18} chain length FA containing typically one to three double bonds (93). Some plants, such as evening primrose and borage, accumulate significant levels of GLA in their seed oils and have been used for therapeutic purposes. When long-chain FA are present in the oil, such as in the older varieties of oilseed rape, they are generally monounsaturated, e.g., erucic acid (22:1) (94). However, organisms often referred to as "lower" organisms such as algae and fungi are also often rich in C_{20} PUFA, and the possibility of culturing these species in bioreactors to provide valuable LCPUFA has been the subject of much research (95). However, the cost of running such bioreactors (carbon source provision, heating, and lighting in the case of photosynthetic organisms) may prove inhibitory to large-scale commercial production. DHA and EPA are usually obtained from FO, but fish stocks are in decline, and the derived oils are often contaminated with pollutants and toxins such as dioxins and heavy metals (96). The production of fish (and FO) *via* aquaculture also requires the supplementation of fish feeds with EPA/DHA, putting additional strain on this diminishing resource (97). Alternative sources of LCPUFA are therefore clearly desirable, and the concept of obtaining them from higher plants in commercial and sustainable quantities is particularly attractive. Here we summarize the recent advances in C_{18} PUFA synthesis and the subsequent bioengineering of C_{20} PUFA for potential nutraceutical/pharmaceutical end use.

Biosynthesis of plant oils containing C_{18} PUFA. To engineer oils for specific purposes, one must have a sound understanding of the biosynthetic pathways by which TAG and their constituent PUFA are produced. Here we summarize the recent advances in the area and the biotechnological achievements specifically aimed at producing FO substitutes in plants. To our knowledge, the production of GLA-rich oils can be satisfied from existing species without recourse to genetic modification.

The synthesis of vegetable fats and oils takes place in the endoplasmic reticulum of oilseeds and is accomplished by a series of membrane-bound enzymes. The deposition of oil reserves usually takes place over a narrow window of time following pollination. Initially, studies using active membrane preparations and radiolabeled substrates indicated that a major route of TAG synthesis is the glycerol-3-phosphate acylation pathway in which glycerol-3-phosphate is first acylated at the *sn*-1 position by glycerol-3-phosphate acyltransferase (AT1) to yield 1-MAG-3-phosphate (98) (see Fig. 3). This then serves as a substrate for a second acyltransferase, acyl-CoA:1-MAG-3-phosphate acyltransferase (AT2), which forms an ester with the *sn*-2 carbon hydroxyl group of the glycerol backbone with an acyl group from the acyl-CoA pool (99,100). In general, AT1 has a preference for saturated FA, and AT2 for monunsaturates and polyunsaturates. AT1 and AT2 genes have been cloned from several sources (101).

The phosphatidic acid so formed is then dephosphorylated by hydrolysis by phosphatidate phosphohydrolase (102) to yield DAG, which can then be acylated at its *sn*-3 carbon hydroxyl to yield TAG by a third acylating enzyme (AT3) referred to as acyl-CoA:DAG acyltransferase (Fig. 3). The genes encoding these enzymes have been reported from several sources and are classified into two gene families: diacylglycerol acyltransferase 1 (DGAT1), which is related to the acyl-CoA:cholesterol acyltransferase gene family, and a new gene family, DGAT2, found in fungi, plants, and animals (103). Overexpression of DGAT in wild-type *Arabidopsis* enhances oil deposition, which suggests that it plays a significant role in the quantity of seed TAG produced (104).

In recent years a number of other reactions have been demonstrated in addition to the pathway just outlined. These reactions are related to the synthesis of PUFA, which takes place primarily on the phospholipid, PC.

Synthesis of C_{18} PUFA on phospholipids. Oleate (18:1 Δ9; OL), the principal FA exported from the plastid, is ligated to CoA on the outer envelope membrane and serves as a substrate for a number of competing reactions. In tissues that accumulate C_{18} PUFA, a major route of metabolism is *via* PC, the most abundant phospholipid component of the endoplasmic reticu-

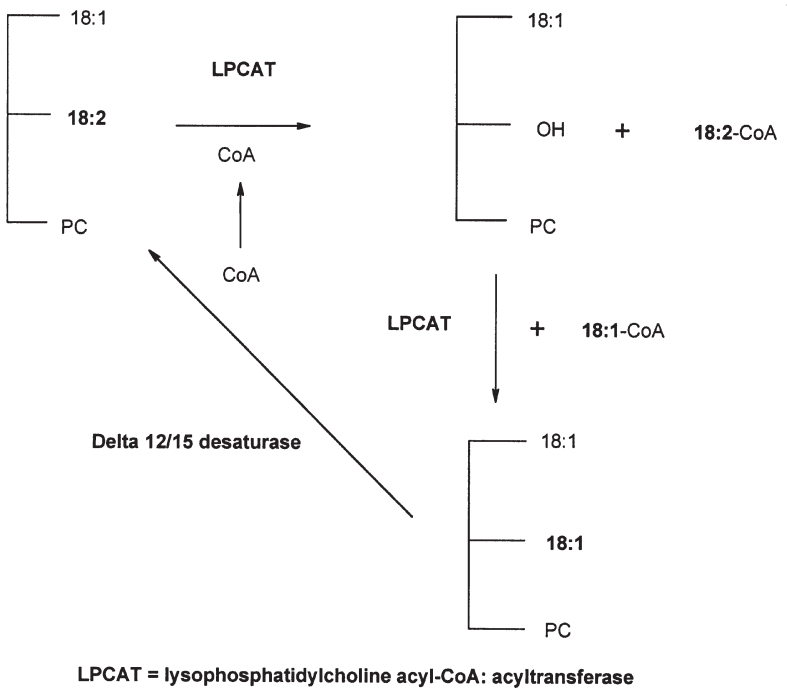

FIG 4. Acyl exchange between acyl groups at position *sn*-2 of PC with acyl groups in the acyl-CoA pool. Oleate at position *sn*-1 of PC can also be desaturated to linoleate (not shown) and introduction of a further double bond into the acyl chain by Δ15 desaturase is possible on the PC substrate.

lum. An equilibration between acyl groups in PC and the acyl-CoA pool occurs, and by this reaction OL enters the *sn*-2 position of PC for desaturation to LA and, in those species that accumulate them, to ALA and GLA. Little synthesis of GLA takes place at the *sn*-1 position of PC in borage, and this has been identified as a rate-limiting step in the synthesis of this component in these plants (100). Concomitantly, the products from that position enter the acyl-CoA pool and are made available for the acylation of the glycerol backbone. This acyl exchange mechanism is considered to be catalyzed by the enzyme acyl-CoA:lysophosphatidylcholine acyltransferase working in both forward and reverse directions (105) (Fig. 4).

DAG may be used for PC synthesis by a CDP-choline:DAG choline-phosphotransferase-catalyzed reaction (106) (Fig. 5). In oilseeds, this reaction also operates in the reverse direction: The head group is removed and the DAG moiety is regenerated. Oleate moieties entering PC at positions *sn*-1 and *sn*-2 of DAG *via* the glycerol-3-phosphate pathway are thus made available to the desaturases responsible for LA and ALA synthesis as already described. However, it appears that only acyl groups esterified at the *sn*-2 position of PC are rapidly turned over and exchanged with the acyl-CoA pool .

In addition to the direct acylation of DAG, it has been demonstrated that a transacylase activity can generate TAG and MAG from two molecules of DAG (107) (Fig. 6). It is also apparent that microsomal membranes catalyze the production of lysophosphatidylcholine from MAG. This appears to occur *via* a phosphocholine transfer between PC and MAG, the products being DAG and lysophosphatidylcholine. The lyso-derivative can be rapidly reacylated from acyl-CoA. Such a combination of reactions could yield TAG without the participation of diacylglycerol acyltransferase (AT3) and without any accumulation of MAG. Recently, DAG production from MAG and acyl-CoA has been demonstrated by an acyl-CoA:MAG acyltransferase (MAGAT) (108). This pathway plays an important role in dietary fat absorption by catalyzing a rate-limiting step in the resynthesis of DAG in enterocytes (109), although its role in the overall production of TAG in oilseeds awaits further evaluation. Direct acylation of glycerol has not been established for oilseeds but has been demonstrated in hyperglycerolemic mammalian tissues produced in diabetes or other pathological conditions (110).

A new acyl-CoA independent pathway for TAG biosynthesis involving a phospholipid:DAG acyltransferase (PDAT) has also recently been described (111). This reaction is catalyzed by an enzyme called phospholipid:diacylglycerol acyltransferase, or PDAT, and has been demonstrated in a number of oilseed tissues as well as in yeast endomembranes (Fig. 7). The yeast and *Arabidopsis* genes have been cloned and shown to have homology with animal lecithin:cholesterol acyltransferase, which catalyzes the acyl-CoA-independent synthesis of cholesterol esters. The AtPDAT used different phospholipids as acyl donor and accepted acyl groups ranging from C_{10} to C_{22}. The rate of activity was highly dependent on acyl composition with highest activities for acyl groups containing several double bonds, epoxy, or hydroxy groups. The enzyme used both *sn*-positions of PC but had a threefold preference for the *sn*-2 position (112). Overexpression of PDAT in *Arabidopsis*, however, had little effect on

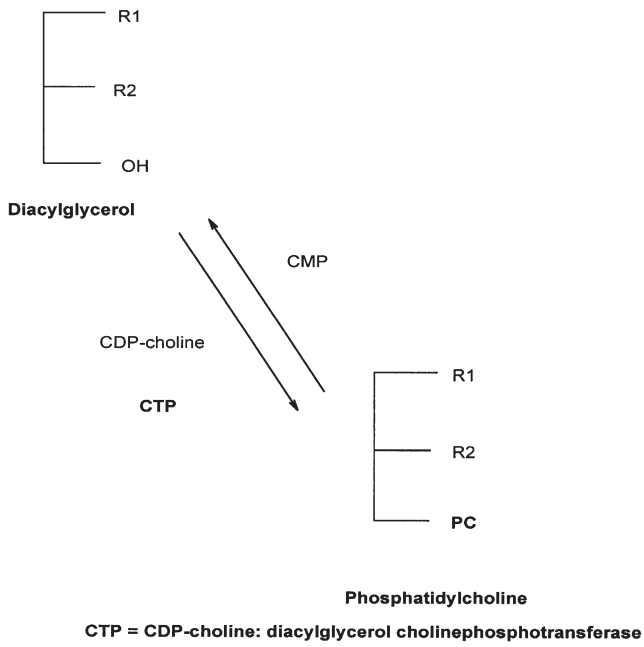

FIG 5. Interconversion of DAG with PC. This reaction offers opportunities for enrichement of PUFA in the DAG pool by desaturation when converted to PC. CMP, cytidine monophosphate; CDP, cytidine diphosphate.

the lipid composition, and in plants with impaired PDAT activity (a T-DNA insertion mutant) TAG synthesis was unimpeded and was performed by transacylation of DAG.

Formation of LCPUFA. Oilseeds are rich sources of the C_{18} PUFA, principally 18:2n-6, 18:3n-3, and 18:3n-6. Generation of oils rich in C_{20} and C_{22} PUFA requires the insertion of additional elongases and desaturases from exogenous sources. Until recently, one of the major obstacles to generating these LCPUFA was the availability of "elongases" specifically capable of elongating C_{18} PUFA. The first reported isolation of PUFA-specific elongase was described from the microalga, *Isochrysis* (113). The functional analysis of *IgASE1*, by expression in *Saccharomyces cerevisiae*, revealed that the C_{18}-Δ9 PUFA LA (18:2n-6, Δ9,12) and ALA (18:3n-3 Δ9,12,15) were elongated to eicosadienoic acid (EDA; 20:2n-6, Δ11,14) and eicosatrienoic acid (ETA; 20:3n-3, Δ11,14,17), respectively. Constitutive expression of *IgASE1* in *Arabidopsis* resulted in the accumulation of EDA and ETA in all tissues examined, with no visible effects on plant morphology (114). Positional analysis of the various lipid classes indicated that these novel FA were largely excluded from the *sn*-2 position of seed TAG, whereas they were enriched in the same position in PC. EDA and ETA are precursors of AA (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), synthesized *via* the so-called omega6Δ^8 desaturase and omega3Δ^8 desaturase biosynthetic pathways, respectively. The synthesis of significant quantities of EDA and ETA in a higher plant is a key step in the production of LCPUFA in oilseed species (see following paragraphs).

LCPUFA synthesis in mammals proceeds predominantly by a Δ^6 desaturation pathway, in which the characteristic first step is the Δ^6 desaturation of LA and ALA to yield GLA (18:3 $\Delta^{6,9,12}$) and stearidonic acid (18:4 $\Delta^{6,9,12,15}$), respectively. Further FA elongation and desaturation steps give rise to AA and EPA (Fig. 2). Accordingly, genes encoding Δ^6 desaturases (115–120), Δ^6 elongase components (121–123), and Δ^5 desaturases (124–127) have been cloned from a variety of organisms including higher plants, algae, mosses, fungi, nematodes, and humans. Both Δ^6 and Δ^5 desaturases are "front end" desaturases and are produced as fusion proteins containing an N-terminal cytochrome b_5, the electron transport component. These membrane-bound microsomal FA desaturases are known to have three conserved histidine boxes, comprising a total of up to eight histidine residues that are involved in iron chelation at the active site of the enzyme.

An alternative pathway for the biosynthesis of AA and EPA operates in some organisms. Here, LA and ALA are first elongated specifically to EDA (20:2 $\Delta^{11,14}$) and ETrA (20:3 $\Delta^{11,14,17}$), respectively. Subsequent Δ^8 and Δ^5 desaturation of these products yields AA and EPA. The Δ^8 desaturase, isolated from the protist *Euglena gracilis* (128), is highly homologous to the Δ^6 and Δ^5 desaturases from the *Caenorhabditis elegans* and introduces a double bond at the Δ^8 position in C_{20} FA that have an existing Δ^{11} unsaturation. Recently, *A. thaliana* was transformed sequentially with genes encoding a Δ^9-specific elongating activity from *I. galbana*, a Δ^8 desaturase from *E. gracilis,* and a Δ^5 desaturase from *Mortierella alpina* (129). Instrumental in the successful reconstitution of these C_{20} PUFA biosynthetic pathways was the *Isochrysis* C_{18}-Δ^9-elongating activity, which may bypass rate-limiting steps present in the conventional Δ^6 desaturase/elongase pathways. Recent attempts to reconstitute the conventional n-6 and n-3 Δ^6 desaturation pathways in yeast, using genes encoding Δ^6 desaturase, Δ^6 elongating activity, and Δ^5 desaturase, met with little success and may reflect inherent differences between plant and yeast systems since the latter do not naturally synthesize PUFA (130). Whether expression of these conventional n-6 and n-3 Δ^6 desaturation pathways in *Arabidopsis* would generate AA and EPA remains to be determined. The leaves of the triply transformed plants using the Δ9 elongase and Δ^8 and Δ^5 desaturases accumulated around 22% of FA as C_{20} chain-length products with AA and EPA accounting for some 7 and 3%, respectively (129).

ENGINEERING FO IN PLANTS: FUTURE PERSPECTIVES

The demonstration of AA and EPA production in *Arabidopsis* suggests that it may be possible to engineer pathways to produce these components in other tissues of agronomic importance such as oilseeds. This will require the seed-specific expression of these genes in oilseeds rich in starter substrates for Δ^9 elongase, which appears to be the rate-limiting step in the overall production of LCPUFA. However, FO are also rich in DHA (22:6 $\Delta^{4,7,10,13,16,19}$), and its production in plants will require an additional elongation step and a further desaturation

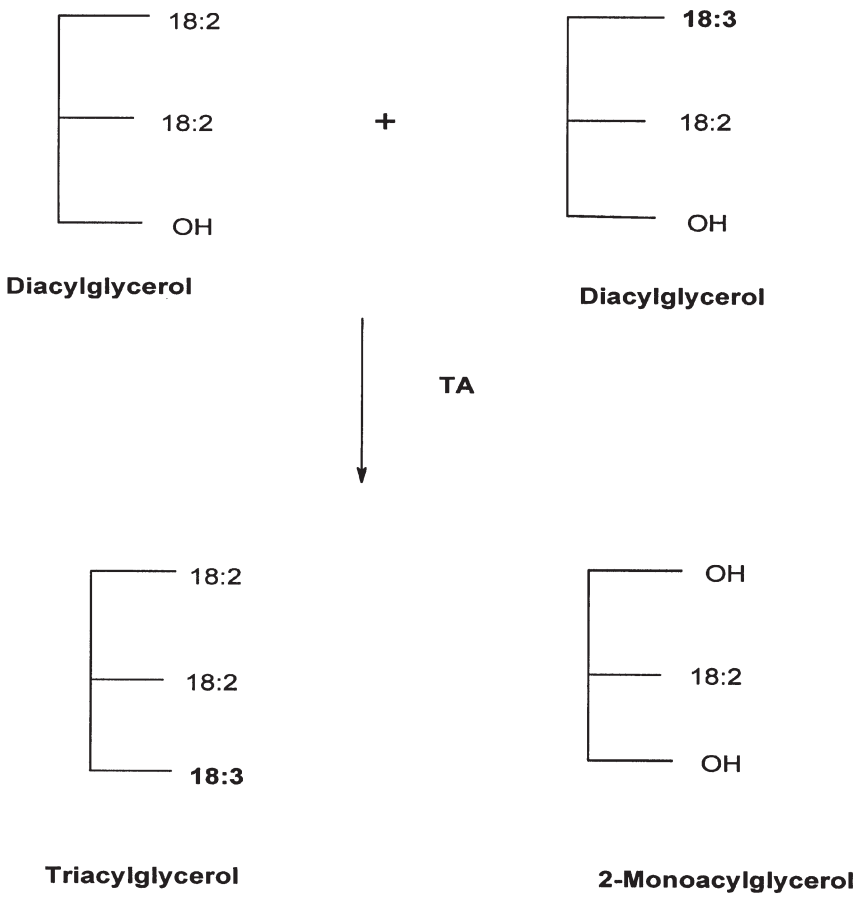

FIG 6. Transacylation reactions result in TAG synthesis without the requirement for AT3 activity. For abbreviation see Figure 3.

involving the insertion of a double bond in the Δ^4 position. In this regard, expression of *Thraustochytrium* Δ^4 desaturase in *Brassica juncea* demonstrated the production of DHA in the leaves supplied with exogenous 22:5n-3 (131). Δ^4 Desaturases appear to be involved in Δ^4 desaturation of sphingolipids involved in eukaryotic cell signaling (132) and have recently been cloned from several sources including the marine microalga *Pavlova lutheri* (133), the yeast *Schizosaccharomyces pombe*, humans, *Drosophila*, and *Candida*. However, the conversion of EPA (20:5n-3) to 22:5n-3 will require the expression of an elongase with significant activity toward EPA. To produce commercial levels of oils containing significant quantities of EPA and DHA will require the seed-specific expression of five genes (Δ^9 elongase; Δ^8, Δ^5, and Δ^4 desaturases; and an elongase for EPA) to produce these components from 18:2- and 18:3-rich oilseed stocks. The feasibility of producing FO substitutes from plants is likely achievable; however, whether such commodities will be commercially viable in the future will require consumer acceptance and the favorable resolution of current regulatory issues relating to genetically modified products.

GOVERNMENT REGULATIONS

Naturally occurring TG oils are not pure substances, making it difficult to apply standard methods of quality, efficacy, and safety evaluation as per active pharmaceutical agents. As a result, it is often impossible for drug development companies to create the information necessary to satisfy regulatory requirements and achieve product license approvals for such products. As lipid drug development progressed to include FA esters, salts, diols, and designer TG, the purity of the active ingredients improved but regulatory hurdles were still insurmountable in many countries. However, the current changing regulatory environment in certain countries including Canada and Australia is opening opportunities to new product development in this drug category. As a consequence of these new regulations, there is a blurring of the line between what constitutes a "drug"

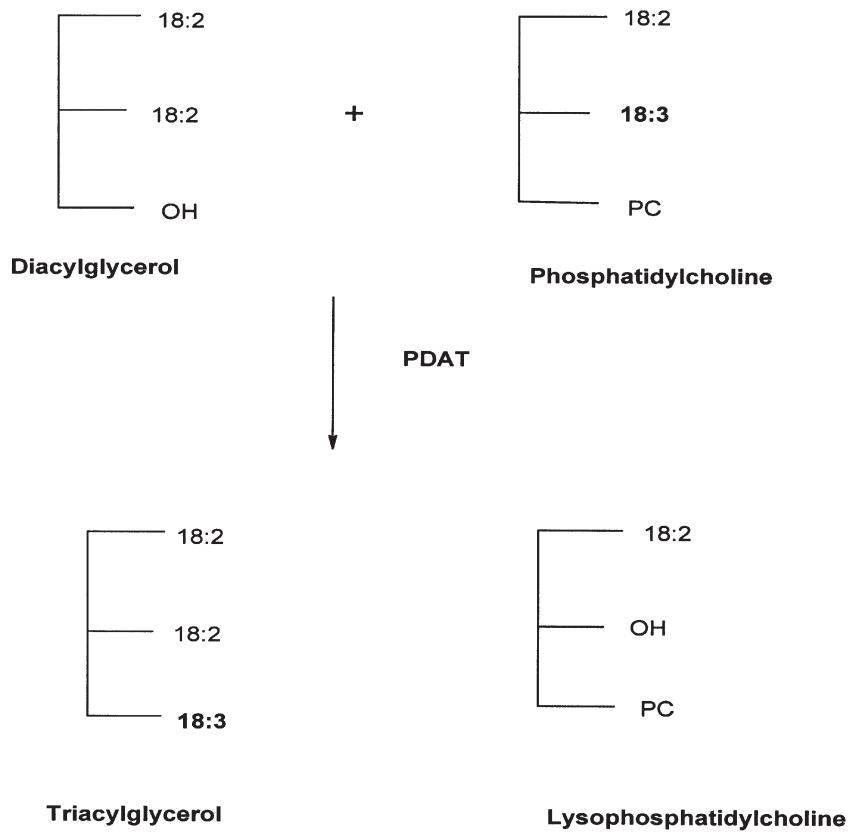

FIG 7. Reaction of DAG with PC results in net TAG synthesis in an independent AT3 manner (see Fig. 3 for details).

and a "nondrug" substance. For example, historically in Canada, if a product was used to diagnose, treat, mitigate, or prevent a disease, disorder, or abnormal physical state then it was classified as a "drug," making it necessary to follow stringent requirements to prove safety, efficacy, and quality of the product. That statement now also applies to "natural health products," which are defined as a separate consumer product category having their own standards of evidence for safety, quality, and efficacy (1). These standards are more appropriate for lipid-based therapeutic agents as they allow for licensing of relatively crude extracts. As well, submissions do not require inclusion of *in vitro* and animal data if human studies provide sufficient evidence to support the health claims and safety of the product, and the reverse is also true. (The situation is similar in Australia where bibliographical submissions are sometimes acceptable on a case-by-case basis if clinical data are not available.) However, the biggest advantage is that these new regulations enable cost-effective development of combinations of two or more pharmacologically active ingredients for therapeutic use—an option that is virtually financially unattainable in most other markets due to the regulatory requirements to confirm safety and efficacy of such combinations. This opens the door for development of a whole range of unique lipid combinations for targeted indications.

Another stumbling block for development of lipid-based drug products in the past has been patent protection, which has been somewhat difficult to navigate owing to the commonality of the components. However, creative strategies to develop novel lipid compounds with highly patentable applications have emerged including one dubbed "combinatorial lipids." These new compounds can be synthesized by using certain FA with known therapeutic value that are linked through a variety of specific chemical bonds to one another, to other bioactive compounds, such as amino acids, peptides, sugars, hormones, or vitamins, or to an existing drug substance. The type of linkage used imparts a specific biological property to the compound. This characteristic and the characteristics for the lipid component and the other bioactive compound are all known. Therefore, one can predict with reasonable certainty the therapeutic value and the toxicology of the compound. In combinatorial lipids created to date, the potentiation of one or both active ingredients with improved therapeutic results has been ob-

served (134). Some examples of combinatorial lipids mentioned previously in this article include meglumine-GLA and ascorbyl-GLA. Combinatorial lipids could become a financially attractive means to extending patent life for a vast array of pure drug substances while simultaneously improving their bioavailability and reducing their toxicity.

In this review we have attempted to summarize our present understanding of the potential uses of GLA and LCPUFA including EPA and DHA as nutraceuticals. In the last decade, enormous advances have been made in lipid molecular biology, with many genes cloned and characterized. These advances have made it possible to manipulate FA profiles and to introduce "foreign" genes into species, leading to the formation of novel products in plants, including the recent production of EPA. The availability of other genes such as Δ4 desaturase makes it highly probable that DHA production in oilseed crops can be achieved in the near future.

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