CHAPTER 6

Gossypol-A Polyphenolic Compound from Cotton Plant

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Abstract Gossypol ($C_{30}H_{30}O_8$) is a polyphenolic compound derived from the cotton plant (genus *Gossypium*, family Malvaceae). The presence of six phenolic hydroxyl groups and two aldehydic groups makes gossypol chemically reactive. Gossypol can undergo Schiff base formation, ozonolysis, oxidation, and methylation to form gossypol derivatives. Gossypol and its derivatives have been the target of much research due to their multifaceted biological activities including antifertility, antivirus, anticancer, antioxidant, antitrypanosomal, antimicrobial, and antimalarial activities. Because of restricted rotation of the internaphthyl bond, gossypol is a chiral compound, which has two atropisomers (i.e., (+)- and (−)-gossypol) that exhibit different levels of biological activities. This chapter covers the physiochemical properties, analyses, biological properties, and agricultural and clinical implications of gossypol.

I. OVERVIEW OF COTTON AND COTTONSEED PRODUCTS

Cotton has long been known as nature's unique food and fiber plant. It is produced worldwide in tropical and subtropical regions. During the 2003–2004 marketing year, the world production of cotton was approximately 88 million bales, for which the People's Republic of China was the largest producer, followed by the United States, India, and Pakistan, which accounted for approximately three-quarters of the world output (USDA, 2003/2004) (Fig. 6.1). If Brazil and Turkey were added, six countries would account for 83% of world cotton production. Regardless of a continuous declining trend of the share of cotton fibers compared to that of the chemical textile fibers since 1970s, world demand and consumption of cotton fiber has been steadily growing along with the worldwide economic growth. However, much of the growth of cotton production since the end of the Second World War was due to improved yield (output from 0.2 t/ha in 1945/1946 to 0.8 t/ha in 2006/2007 according to the International Cotton Advisory Committee—ICAC), rather than to expanded area (cultivated land increased by only 35% over the 1945/1946–2006/2007 period, expanding from 22.3 to 34.8 million hectares). Meanwhile, the cottonseed production has increased in the same trend. Still, cottonseed ranks third behind soybean and rapeseed in terms of world's oilseed production (USDA, 2007) (Table 6.1).

The composition of cottonseed, which includes oil, protein, carbohydrates, phosphorous compounds, and minerals, varies considerably depending on plant species, variety, and plantation environment. Besides the cotton fiber, the cottonseed oil, and cottonseed protein are other two major products of cotton plants. The former is embedded as droplets (oil bodies) in the tissue of cottonseed (Markman, 1968), which consists

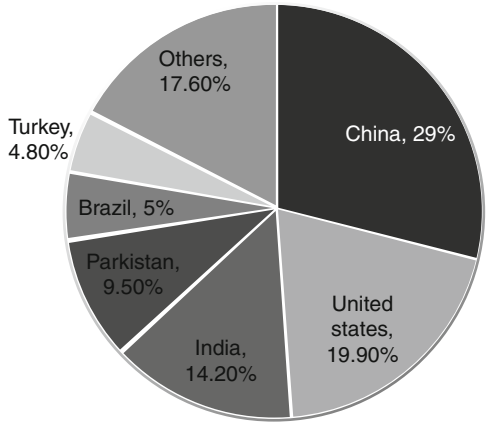


FIGURE 6.1 Cotton production by main countries.

TABLE 6.1 World oilseed production 2007

	Million short tons	Million metric tons
Soybeans	242.3	219.9
Rapeseed	52.5	47.6
Cottonseed	50	45.4
Peanut	36.5	33.1
Sunflower seed	30.5	27.7
Palm kernel	12.2	11.1
Copra	5.9	5.4
Total	430.8	390

of about 45% hull and linters, and 55% kernel in average. The cottonseed kernel is a pointed ovoid body approximately 8–12 mm in length, in which there are innumerable dark spots. These dark spots are pigment glands and unique to the cottonseed. The major component of the pigment gland is free gossypol (FG). Gossypol is a polyphenolic chemical once having a notorious reputation but now considered a promising biological phytochemical. The amount of FG in the cottonseed oil can be significantly affected by the oilseed processing, which can also remarkably influence the protein value of the cottonseed meal (CSM). For example, in screw-pressing (also known as expeller- or cold-pressing), most of the FG binds to amino acids of proteins, which lowers the nutritional value of the protein and the CSM. Although prepress solvent extraction and direct solvent extraction can produce the CSM with high-quality protein, they considerably increase the content of FG in the oil.

Since gossypol has a certain degree of toxicity, its presence will significantly influence and limit the applications of the cottonseed oil and CSM as food and animal feed, respectively. Therefore, the US Food and Drug Administration (FDA, 1974) set up mandatory regulations that any cottonseed protein products intended for human use must contain no more than 450 ppm FG. Likewise, the Protein Advisory Group of the United Nations Food and Agriculture Organization and World Health Organization (FAO/WHO) has set limits of 600 ppm of FG and 12,000 ppm total gossypol (TG) for human consumption. Moreover, some additional caution was suggested concerning the presence of gossypol in both CSM and whole cottonseed when used in dairy rations (Poore and Rogers, 1998).

II. OCCURRENCE OF GOSSYPOL

Gossypol is mainly embedded in the cottonseed pigment glands. It constitutes 20–40% of the gland weight and accounts for 0.4–1.7% of the whole kernel. Besides the cottonseed, gossypol glands are also found in some other parts of the cotton plant, such as the bark of plant roots, leaves, seed hulls, and flowers. On the other hand, gossypol content varies largely, depending on the species and variety of cotton plant, climatic and soil conditions of the region, water supply, agrotechnical treatment, and in particular, on the amount and composition of fertilizers used (Markman, 1968; Stansbury *et al.*, 1954). Changes of gossypol content throughout different stages of the cotton maturity have also been reported (Caskey and Gallup, 1931; Gallup, 1927, 1928). Though gossypol only accounts for 0.4–1.7% of whole cottonseed kernel, its production could exceed 40,000 tons annually in the United States alone. Therefore, the gossypol content of cotton plants has become a big issue, both scientifically and commercially, due to its unique biological characteristics (such as antiproliferative activity, antivirus activity, etc.), and influence of its toxicity on use of some commercial cottonseed products such as the CSM as an animal feed, the cottonseed oil in food products, and the cottonseed flour for human consumption. In this context, it is of great significance to review the physiochemical and biological properties of gossypol and its derivatives in the following sections.

III. PHYSIOCHEMICAL PROPERTIES OF GOSSYPOL

Gossypol was first discovered and isolated as a crude pigment by Longmore (1886) from cottonseed oil foot, a mixture of precipitated soaps and gums produced in the refining of crude cottonseed oil with

sodium hydroxide. Forty one years later, its chemical formula $C_{30}H_{30}O_8$ was established by Clark (1927). After another 30 years, its complete structure was verified as 1,1′, 6,6′, 7,7′-hexahydroxy-3,3′-dimethyl-5,5′-diisopropyl-2,2′-binaphthyl-8,8′-dialdehyde (Fig. 6.2) by Edwards (1958) when gossypol was completely synthesized. As shown in Fig. 6.2, gossypol contains polar groups (six hydroxyl and two aldehydic groups) making it soluble in most organic solvents, such as methanol, ethanol, isopropanol, butanol, ethylene glycol, dioxane, diethyl ether, acetone, ethyl acetate, chloroform, carbon tetrachloride, ethylene dichloride, phenol, pyridine, melted naphthalene, and heated vegetable oil. It is less soluble in glycerine, cyclohexane, benzene, gasoline, and petroleum ether. However, the presence of two heavy dialkylnaphthalene groups makes it insoluble in water (Markman, 1968). Using diethyl ether, chloroform, and ligroin, Campbell *et al.* (1937) obtained three gossypol crystals with different melting points of 184, 199, and 214 °C, respectively. Although Adams *et al.* (1960) suggested that these samples were various polymorphic forms of gossypol, it was demonstrated by Ibragimov *et al.* (1995) that only the last sample with the melting point at 214 °C was a nonsolvated compound, while the other two crystals with the melting points of 184 and 199 °C, were really the complexes with the solvents of diethyl ether and chloroform, respectively. A comprehensive review of the discovery, determination of structure, and chemistry of gossypol was published by Adams *et al.* (1960) and Markman (1968).

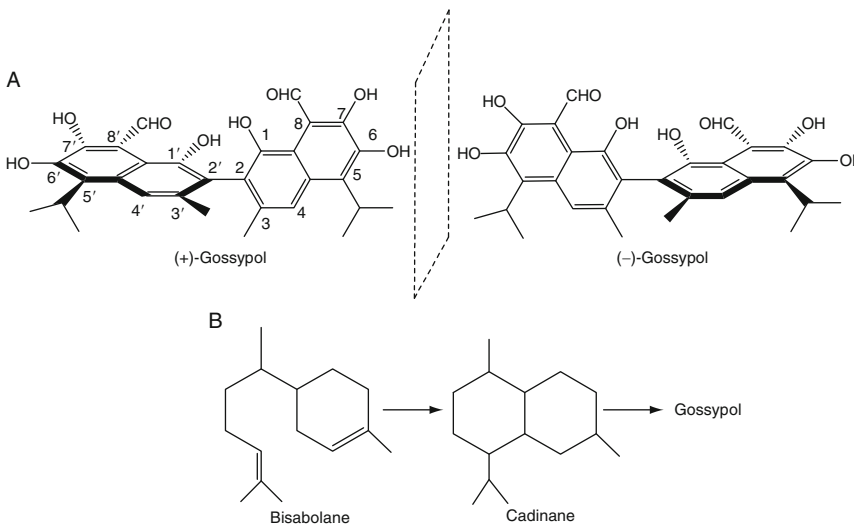


FIGURE 6.2 (A) Atropisomers of gossypol and (B) formation of naphthalene ring in gossypol.

Gossypol exists in two atropisomers due to restricted rotation of its internaphthyl bond (Fig. 6.2). The naphthalene rings of gossypol are derived from sesquiterpenes of the cadinane family, while the cadinanes are formed in the biogenetic cascade from the bisabolane intermediate by a series of putative 1, 2-shifts and cyclization (Fig. 6.2B). An enantiomeric excess of (+)-gossypol was usually found in plant species of *Gossypium arboreum*, *G. herbaceum*, *G. hirsutum*, *G. aboreum*, *G. mustelimum*, and *Thespesia populnea*, while (−)-gossypol was found in excess in *G. barbadens* (Cass *et al.*, 1991; Zhou and Lin, 1988).

Adams *et al.* (1960) proposed that gossypol existed in three tautomeric forms: (a) aldehyde, (b) ketol, and (c) hemiacetal (Fig. 6.3) in different solvents. Use of nuclear magnetic resonance (NMR), mass spectral analysis, and UV spectrometry has confirmed its structural changes in various solutions. In basic solvent systems, gossypol existed mainly as ketol, but in ordinary inert solvents, such as chloroform, benzene, acetone, or dioxane, and in acidic conditions, gossypol existed mainly in the aldehyde form (Stipanovic *et al.*, 1973). While in polar solvents such as dimethyl sulfoxide (DMSO) with alkali condition, the hemiacetal form occurred in dynamic equilibrium with the aldehyde form as well as the ketol form (Abdullaev *et al.*, 1990). Since gossypol is commonly dissolved in DMSO as a stock solution for biological studies, several tautomeric forms of gossypol may simultaneously contribute their biological activities.

Gossypol is chemically reactive due to the reactivity of carbonyl and phenolic hydroxyl groups as well as its bulky binaphthalene structure. Gossypol can react with other compounds to form bound gossypol (BG). In order to better describe the chemical status (forms) of gossypol in cottonseed products, three terms (i.e., FG, BG, and TG) are frequently used. Based on the AOCS (American Oil Chemists Society) official

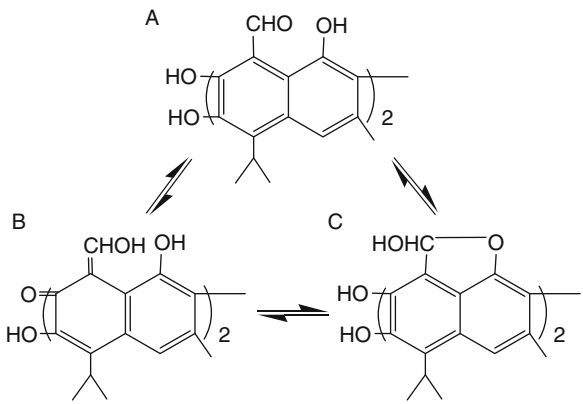


FIGURE 6.3 Tautomeric forms of gossypol: (A) aldehyde, (B) ketol, and (C) hemiacetal.

definition, FG is defined as gossypol and gossypol derivatives in cottonseed products that can be extracted by 70% aqueous acetone (AOCS, Ba 7-58). BG, formed during cottonseed processing by reaction of gossypol with other compounds, is not soluble in aqueous acetone. TG is defined as gossypol and gossypol derivatives, both FG and BG, which can react with 3-amino-1-propanol in dimethylformamide solution (AOCS, Ba 8-78). However, since the AOCS method (AOCS, Ba 8-78) only measures gossypol, gossypol analogs, and gossypol derivatives that have an available aldehyde moiety for the derivatization reaction, a fraction of gossypol derivatives that are unable to undergo this reaction will be excluded from the TG value. Regardless of the aforementioned shortcomings, FG and TG are still determined empirically, and BG is determined mathematically by the equation BG = TG − FG.

Some evidence indicates that the majority of gossypol exists as Schiff bases formed by the condensation between the aldehydic groups of gossypol and amino groups of proteins during cottonseed processing (Cater, 1968; Lyman *et al.*, 1959). However, this chemical complex alone cannot fully account for gossypol's complex behavior. In fact, gossypol could be chelated by iron in cottonseed products to form insoluble metal complexes (Muzaffaruddin and Saxena, 1966), subject to be oxidized (Haas and Shirley, 1965; Scheiffele and Shirley, 1964), or may form gossypol polymers (Anderson *et al.*, 1984). Additionally, its phenolic groups may react to form esters and ethers with other carboxylic compounds and phenols in cottonseed plants.

In the past several decades, much work has been done on the Schiff base reaction between gossypol and amino groups (Fig. 6.4). One investigation (Lyman *et al.*, 1959) on gossypol–protein complexes revealed that ε-amino group of lysine in crystalline BSA (bovine serum albumin) or cotton protein was involved in the gossypol–protein interaction. Furthermore, it was shown that the molar ratio of free ε-amino groups to gossypol varied according to the experimental conditions, but averaged about 1.5 mol of lysine bound per mol of gossypol. At low concentrations of gossypol, the molar ratio of lysine to gossypol was 2:1, indicating that both of the reactive aldehyde groups of gossypol were linked to lysine. On the other hand, sedimentation velocity studies of these gossypol–protein complexes suggested the presence of one to four different

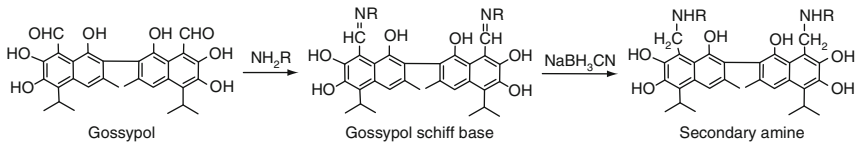


FIGURE 6.4 Gossypol Schiff base formation.

compounds, thus indicating that gossypol probably formed a cross-link between two or more protein molecules. Besides, involvement of arginine in the reactions has also been reported (Baliga, 1956; Martinez *et al.*, 1961). Damaty and Hudson (1979) concluded that lysine, serine, methionine, along with some other hydrophobic amino acids in cottonseed protein, were easily able to participate in the formation of insoluble material after their study on the chemical nature of the interactions between gossypol and proteins using selective proteolysis, gel filtration, and amino acid analysis. Reddy and Rao (1987) studied the interactions between gossypol and proteins (i.e., gossypin, congossypin, and glycinin) using a difference spectral method. They found that the interactions were entirely reversible, and suggested that both hydrophobic and ionic interactions were involved in the reaction of gossypol with proteins. Later, Strøm-Hansen *et al.* (1989) studied the interaction of gossypol with amino acids and peptides using circular dichroism (CD) and NMR, which gave evidence that hydrophobic interaction might be responsible for a significant proportion of the interaction between gossypol and proteins. This opinion was also supported by the finding that gossypol was bound competitively at the bilirubin-binding site on albumin (Royer and Vander Jagt, 1983) because this site was known to be linked with many hydrophobic residues, with only one or two positively charged amino acids being present.

Later, more model complexes of gossypol with amino acids (lysine, asparagine, glutamine, and glycine), peptides (hippuryl-L-lysine, L-alanyl-L-lysine, glycyl-L-lysine, L-histidyl-L-lysine), and purified proteins (glandless cottonseed protein, cottonseed globulin, insulin) were investigated and partially characterized (Cater, 1968). It was concluded that the rate of reaction of gossypol with amino acids increased with an increase of pH value (5.7–7.5), and was shown to be related to the distance of the amino group from the carboxyl group within the molecule. After using CD to study the reaction of (+)-gossypol with BSA, human serum albumin, lactate dehydrogenase (LDH), malate dehydrogenase, alkaline phosphatase, lysozyme, protamine, and poly-L-lysine, Whaley *et al.* (1984a,b) found that (+)-gossypol bound to albumin with the same affinity as (+/−)-gossypol.

A Schiff base is a relatively labile bond that is readily reversible by hydrolysis in aqueous solution and can be chemically stabilized by reduction. The formation of a Schiff base is enhanced at alkaline pH values, but is still not entirely stable unless reduced to a secondary or tertiary amine linkage (Hermanson, 1995). The addition of sodium borohydride or sodium cyanoborohydride will result in reduction of the Schiff base intermediate into a relatively stable secondary amine. Both borohydride and cyanoborohydride have been used for reductive amination purposes, but borohydride will simultaneously reduce the reactive aldehyde groups to hydroxyls and convert Schiff bases present to

secondary amines. Cyanoborohydride, by contrast, is a milder reducing agent that is at least five times milder than borohydride in reductive amination processes. Cyanoborohydride does not reduce aldehydes, but it is very effective for Schiff base reduction (Hermanson, 1995; Lane, 1975). Thus, higher yields of conjugate formation can be obtained using cyanoborohydride instead of borohydride for the stabilization of Schiff base products.

Gossypol is unstable and can be readily oxidized under various reaction conditions into different products with slight structural differences (Fig. 6.5). Oxidation of gossypol usually proceeds in alkaline solution. Ismailov *et al.* (1994) reported the preparation of gossindane through gossypol oxidation by pure oxygen in alkaline medium. Later, Talipov and Ibragimov (1999) determined the single crystal structure of gossindane through X-ray diffraction analysis and confirmed that the chemical contained an indane nucleus instead of the gossypol naphthalene nucleus, for which the chemical gossindane was named. In addition, gossypol-*o*-binaphthoquinone was prepared in an early stage of the reaction between gossypol and oxygen via a Dakin-type reaction (Scheiffele and Shirley, 1964). Besides *o*-binaphthoquinone, *p*-binaphthoquinone, also called gossypolone, can be obtained from the reaction of ferric chloride hexahydrate and gossypol in acetic acid or acetone, which displays an orange color (Shirley and Sheehan, 1955). During the oxidation of gossypol by potassium permanganate in NaOH medium, some decomposition compounds (e.g., formic acid, acetic acid, *n*-butyric acid, *iso*-butyric acid) were found by Clark (1928). Gossypol can also be ozonized in acetic acid solution resulting in gossypolic acid in a very low yield, along with large amounts of oxalic acid (Fig. 6.6) (Karrer and Tobler, 1932).

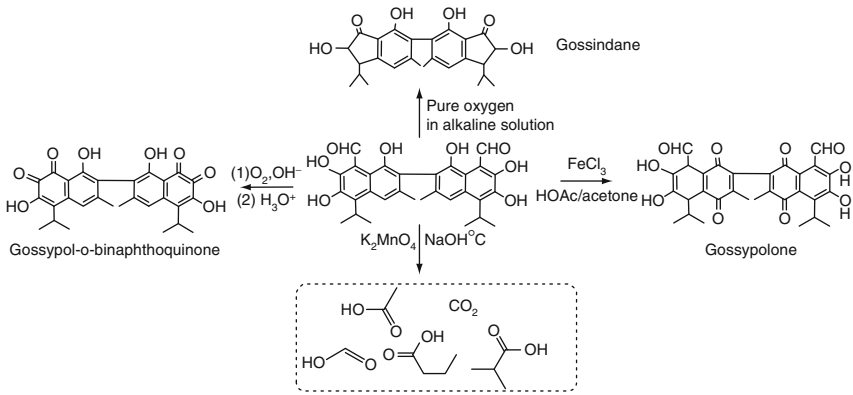


FIGURE 6.5 Oxidation of gossypol.

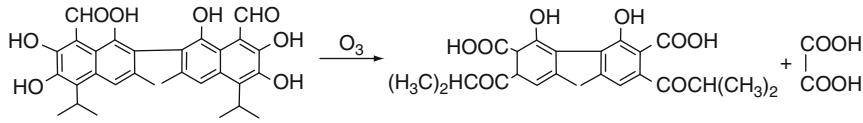


FIGURE 6.6 Ozonolysis of gossypol.

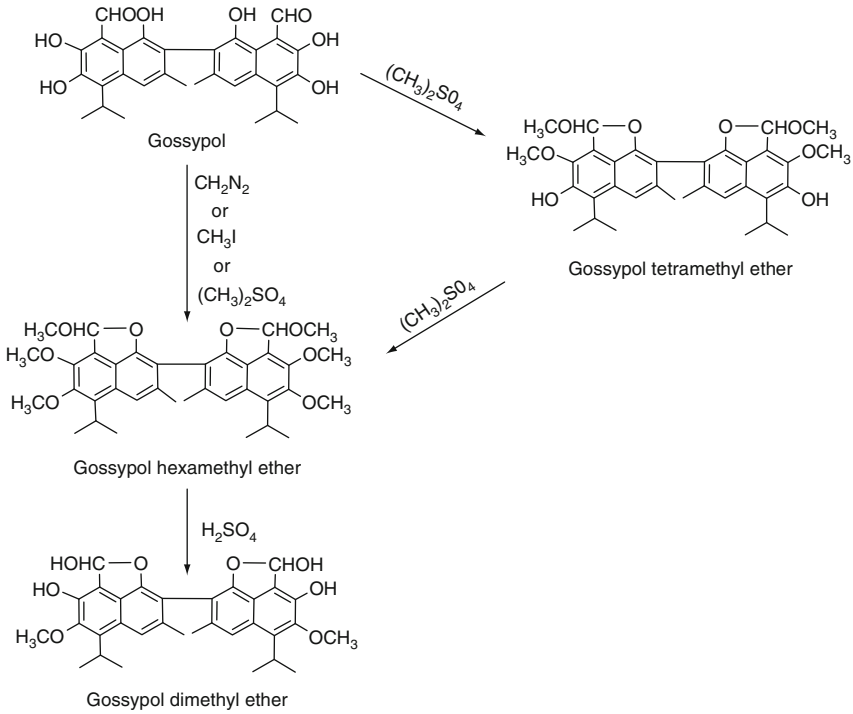


FIGURE 6.7 Methylation of gossypol.

Unlike gossypol, gossypol ethers (Fig. 6.7) are much more stable. Gossypol hexamethyl ethers have been synthesized using three different methylation agents: diazomethane in ether solution, methyl iodide as well as dimethyl sulfate; gossypol offered no resistance to methylation when using less drastic methylation agents such as methyl iodide or diazomethane (Haar and Pominski, 1952). Gossypol hexamethyl ether and gossypol tetramethyl ether were formed at the same time during the reaction between gossypol and dimethyl sulfate in methanolic potassium hydroxide. Gossypol tetramethyl ether could be further methylated into gossypol hexamethyl ether. Moreover, gossypol dimethyl ether can be prepared from demethylation of gossypol hexamethyl ether by treatment with a solution of concentrated sulfuric acid in acetic acid (Fig. 6.7).

With concentrated sulfuric acid the hexamethyl ether of gossypol gives an orange color characteristic in contrast to the tetramethyl ether in a scarlet color (Adams and Geissman, 1938).

Additionally, apogossypol is another important functionalized gossypol derivative (Fig. 6.8). It is a dealdehyde product from the reaction between gossypol and 40% aqueous sodium hydroxide at 85 °C under a nitrogen atmosphere for 1.75 h (Meltzer *et al.*, 1985). Apogossypol is sensitive and subject to change from its original yellow color into brown within a short time of exposure to the air, therefore, it should be freshly prepared whenever needed. By using dimethyl sulfate, apogossypol can be converted to apogossypol hexamethyl ether, which is much more stable than apogossypol. Apogossypol hexamethyl ether can then be treated with concentrated sulfuric acid to obtain didesisopropyl apogossypol hexamethyl ether.

Gossypol can also undergo reduction with $LiAlH_4$, $NaBH_3CN$, or H_2 (Pt as catalyst). During the reduction, the aldehydic moieties are reduced to the methyl group (Shirley and Sheehan, 1955; Shirley *et al.*, 1957) or the methanol group (Dao *et al.*, 2000).

Due to gossypol's multifaceted reactivity and potential wide applications in biological aspects, we also strived to combine gossypol and fullerene in an effort to make a nanoparticle-based therapeutic gossypol–fullerene hybrid through a certain synthetic pathway. Gossypol decomposed and formed several unexpected fulleropyrrolidines including (Fig. 6.9) *N*-methyl, 2-phenylfulleropyrrolidine (**1**), *N*-methylfulleropyrrolidine (**2**), and 1, 2, 2-trimethylfulleropyrrolidine (**3**), which were chemically and physically characterized via the 1D and 2D NMR, IR, MS as well as X-ray methods.

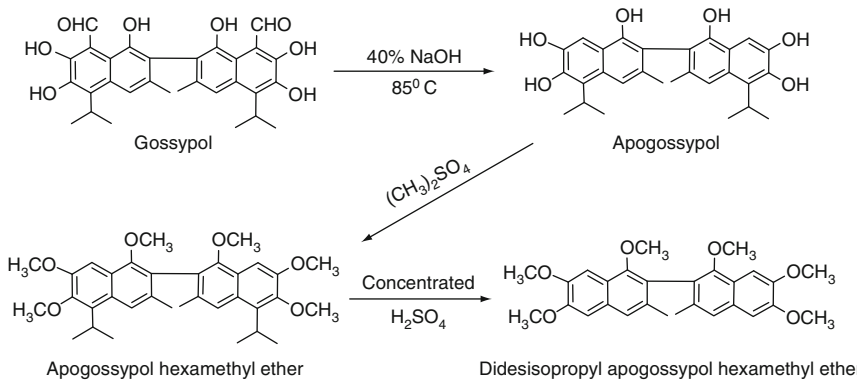


FIGURE 6.8 Formation of apogossypol and its derivatives.

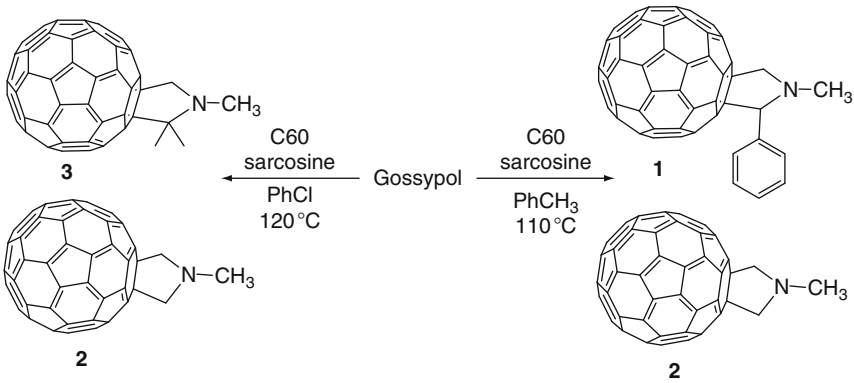


FIGURE 6.9 Syntheses of gossypol–fullerene hybrid.

IV. GOSSYPOL ANALYSES

Methods for the quantitative determination of gossypol in cottonseed, oil, press-cake, and meal have been reviewed by Markman (1968). These methods include gravimetric, volumetric, colorimetric, spectrophotometric, polarographic, and luminescent means.

Gossypol can be derivatized to form a trimethylsilyl (TMS) derivative subject to the gas chromatography (GC) analysis (Raju and Cater, 1967). Near-infrared reflectance, which has been used in many applications for composition analysis (Norries *et al.*, 1976), also offers a possibility for the measurement of gossypol content (Birth and Ramey, 1982). Furthermore, the structural analyses of gossypol, gossypolone, and correlated derivatives are facilitated by using electron impact-mass spectrometer (EI-MS) and infrared spectrometer, which provide ''fingerprint'' mass spectra and IR spectra, respectively (Phillip and Hedin, 1990). Compared with GC, high-performance liquid chromatography (HPLC) is more frequently used for the quantitative and qualitative determination of gossypol and gossypol derivatives due to the existence of chromophoric groups that have strong absorption in the UV range. HPLC has been used to determine gossypol and its derivatives in cottonseed, leaves and flower buds, processed oils, and meals after derivatization (Chamkasem, 1988; Hron *et al.*, 1990; Nomeir and Abou-Donia, 1982; Stipanovic *et al.*, 1988). Cai *et al.* (2004) further demonstrated a simplified HPLC method without gossypol derivatization to analyze gossypol content in various genetic types of cotton varieties on a C18 column with methanol–acetic acid aqueous solution (90:10, v/v) as the mobile phase. Similarly, Aoyama (2008) used a mobile phase of methanol:water (9:1) adjusted to pH 2.6 with phosphoric acid to determine gossypol in feed with UV detector at 254 nm. With the advent of new technology, such as the combination of

HPLC in tandem of mass spectrometer (LC-MS), determination of gossypol has become more sensitive, accurate, and efficient. For example, Orth *et al.* (2007) analyzed BG in insects through forming a Schiff base with aniline by using high-pressure liquid chromatography coupled with a triple quadrupole mass spectrometer.

Besides the HPLC methods, AOCS official methods (AOCS, Ba 7-58 and Ba 8-78) are also recommended to be used for routine gossypol analyses. However, it is found that the total amount of gossypol determined by HPLC is not always correlated with the results from the AOCS official methods. This phenomenon is ascribed to shortcomings of the AOCS official methods that depend on the aldehydic groups of all the gossypol and gossypol derivatives, which can lead to false readings resulting from the presence of nongossypol aldehydic-containing compounds that can also react with the dye reagent (Stipanovic *et al.*, 1984). Additionally, the AOCS methods are often criticized for their laborious and time-consuming procedures as well as lack of analytical sensitivity and accuracy.

To overcome these obstacles in the quantitative and qualitative analyses of gossypol and gossypol derivatives, polyclonal and monoclonal antibodies to gossypol and relevant corresponding immunochemical methods, or the enzyme-linked immunosorbent assays (ELISA), have been developed to simplify and improve the current gossypol analyses (Wang and Plhak, 2000, 2004; Wang *et al.*, 2004, 2005). Based on the principle of antibody–antigen specific interaction, ELISA tests are generally highly sensitive and specific, and favorably comparable with other methods such as the AOCS methods aforementioned. Besides, this method allows for easy visualization of results and can be completed within a relatively shorter time, and suitable for high throughput screening (analysis) of gossypol and its derivatives. This method has been demonstrated to be able to analyze crude extracts, possibly both soluble and insoluble analytes such as matrix-BG in various complex samples. Moreover, another advantage of the ELISA lies in its lower detection limit that enables analysis of samples in small volume or samples containing low gossypol content.

It was a big challenge to separate gossypol enantiomers, though they are able to be quantified by HPLC after conversion to the Schiff base diastereoisomers (Cass *et al.*, 1991; Stipanovic *et al.*, 2005, 2006b; Zhou and Lin, 1988). Cass *et al.* (1999) developed a new method using a chiral HPLC to separate racemic gossypol without the need of derivatization. Dowd (2003) reported a promising method to obtain large enantiomeric crystals of gossypol–acetone (1:3) from acetone solutions of racemic gossypol–acetic acid (1:1) at low temperature. Later, Dowd and Pelitire (2008) isolated the chiral forms of gossypol, 6-methoxy gossypol and 6,6′-dimethoxy gossypol from the root bark of St. Vincent Island cotton

and quantified the chemicals after the derivatization with *R*-(−)-2-amino-1-propanol. By combining HPLC-PDA-MS-SPE-NMR with CD, Sprogoe *et al.* (2008) were able to detect the (−)-gossypol in crude extracts of *T. danis*.

V. AGRICULTURAL IMPLICATION

A. Insecticidal activity

Gossypol is a secondary metabolite that is present in the pigment glands of cotton plants. It plays an important role in protecting cotton plants from insects (Liu *et al.*, 2008). One study on larvae (El-Sebae *et al.*, 1981) showed that gossypol at a dosage of 1.5% concentration in the feeding diet could significantly reduce the larval weight, and increase the duration of each larval stage. Also, the compound inhibited protease and lipid peroxidase activities as well as ATPase activity in larvae at higher concentrations. Gossypol showed stronger inhibition of mitochondrial ATPase (IC_{50}, 1.7×10^{-4} M) than fenvalerate (insecticide, IC_{50}, 7.0×10^{-4} M). These insecticidal activities could result in slower insect hatchability. The study on *Helicoverpa zea* larvae showed that a diet containing 0.16% racemic gossypol was the most effective on reducing the pest survival compared to (+)- and (−)-gossypol at the same concentration (Stipanovic *et al.*, 2006a). Recent studies demonstrate that the toxicity of gossypol lies in the two aldehydic groups present in the molecule (Przybylski *et al.*, 2008a,b,c), which can lead to changes of the metabolism of sterols, steroids, and fatty acids, and the production of toxic chemicals. Concerning the acute and long-term toxicities of gossypol and some of its derivatives, two methods have been employed to reduce the toxicity of gossypol in an effort to expand its agricultural usage. The first commonly used method is to block its two aldehyde groups by the formation of aza-derivatives and another one is to make a gossypol complex with metal cations. These kinds of chemical treatments can reduce the gossypol toxicity because they are no longer able to covalently bind proteins.

B. Antifeeding activity

Gossypol is chemically reactive due to the reactivity of its carbonyl and phenolic hydroxyl groups as well as its bulky binaphthalene structure. Therefore, it has been the compound of greatest concern in cottonseed.

During the cottonseed oil processing when the oil is removed from the cottonseed, some gossypol will also be extracted and remain in the oil, but it can be removed in the subsequent refining process, although some oil is lost. Meanwhile, some gossypol can react with other compounds in the

cottonseed to form BG. This results in an undesirable color and a low-value protein in CSM (Jones, 1979; Pons *et al.*, 1951).

Some evidence indicates that the majority of gossypol exists in the Schiff bases from the condensation between the aldehydic groups of gossypol and amino groups of proteins during cottonseed processing (Cater, 1968; Lyman *et al.*, 1959). Baliga (1956) found that the ε-amino groups of lysine accounted for most of the free amino groups of the cottonseed protein for the reaction in an experimental model when the purified protein was allowed to react with gossypol. This resulted in a decreased availability of lysine to about one-half of its original value (82.9–48.7%). Additionally, involvement of arginine in the reaction has been reported (Martinez *et al.*, 1961). Although the Schiff base reaction is the most well-known reaction of gossypol, it only represents a part of the known chemical activities of gossypol. In fact, gossypol can form other complexes via chelating with metal ions (Muzaffaruddin and Saxena, 1966), or forming gossypol polymers (Anderson *et al.*, 1984), or being oxidized (Scheiffele and Shirley, 1964). Moreover, not only can gossypol bind with proteins in the CSM to render the adjacent peptides unavailable to the proteolytic action but also directly inhibit the activity of certain enzymes such as pepsinogen, pepsin, and trypsin present in the gastrointestinal tract by binding with their free ε-amino groups of lysine (Sharma *et al.*, 1978), lowering the digestibility. Thus, the presence of gossypol in cottonseed products generally will lower the nutritional values of the CSM.

C. Toxicity

A number of investigators have indicated that gossypol is toxic to monogastric animals as well as young ruminants. Particularly, FG is more toxic to monogastric animals than BG. The most common toxic effect of gossypol was shown in the cardiac irregularity which caused the death of animals due to the prevention of the liberation of oxygen from oxyhemoglobin (Menaul, 1923). Generally, the toxicological doses of gossypol are classified into three levels, which are (1) acute doses causing circulatory failure, (2) subchronic doses causing pulmonary edema, and (3) chronic doses causing symptoms of ill health and malnutrition (Abou-Donia, 1976). However, the toxic doses could not be specifically defined because they depend on different animal models.

Since gossypol possesses dose-dependent toxicity, its content in the CSM is limited if the CSM is used as a livestock feed. An early study on male rats (Eagle and Davies, 1958) showed that the oral LD_{50} values of gossypol ranged from 1061 to 2170 mg/kg, or 2200 to 2600 mg/kg when cottonseed pigment glands (containing 27.0–37.8% of gossypol), were orally administrated, respectively. The cottonseed pigment glands seemed more toxic than pure gossypol for rats in this study. El-Nockerashy

et al. (1963) obtained similar findings in studying the acute oral toxicity by feeding cottonseed pigment glands, gossypol, diaminogossypol, and gossypurpurin for rats with LD_{50} values of 1120, 2570, 3270, and 6680 mg/kg body weight, respectively. Calhoun *et al.* (1990b) compared the toxicity of gossypol acetic acid and FG in CSM and Pima cottonseed by feeding lambs, and found that oral administration of gossypol acetic acid was much more toxic to young lambs than feeding equivalent amounts of FG in CSM or cottonseed. It was hypothesized that gossypol in different chemical states might have different stabilities in the digestive tracts between ruminants and nonruminants, so gossypol administrated in different forms to different animal species exhibited different toxic behaviors.

In the study of gossypol's toxicity on rainbow trout (Herman, 1970), it was found that 95 ppm of FG in the diet could cause histological changes in the fish's liver and kidney while 100 ppm could cause pathological changes. When the gossypol concentration was raised to 290 ppm, growth suppression was observed. At the 531 ppm level, the fish lost weight for a period of time and suffered severe reduction of hematocrit, hemoglobin, and plasma proteins. Another study on channel catfish (8 weeks old) showed that diets containing approximately 0.14% of FG or added gossypol acetate in CSM could depress catfish growth, but a level of 0.09% or less FG in the diet seemed to be safe if all essential amino acids were in balance (Dorsa and Robinnette, 1982). Trischitta and Faggio (2008) investigated the effect of gossypol on transepithelial ion transport of the intestine of *Anguilla anguilla*, an European seawater-adapted eel. They found the addition of gossypol resulted in reduced short-circuit current and increased tissue conductance. However, such effects diminished when the eel was pretreated with a calmodulin inhibitor, suggesting that gossypol operated through the Ca^{2+} calmodulin pathway.

Another interesting phenomenon was observed for yolk discoloration when hens were fed with the gossypol-containing feed, due to chemical combination of gossypol with ferric Fe released from yolk (Kemmerer *et al.*, 1966; Schaible *et al.*, 1934). Lordelo *et al.* (2007) found that the ingestion of (+)-gossypol in the diet for 18 days reduced egg production in laying and broiler hens, and had a greater effect on egg yolk discoloration than (−)-gossypol. Both (−)- and (+)-gossypol can bind with Fe, so the greater potency of (+)-gossypol to trigger egg yolk discoloration indicates that the mechanisms by which gossypol causes egg yolk discoloration are more complicated than previous hypothesis (Kemmerer *et al.*, 1966). In addition, gossypol caused lower diet consumption in laying hens compared with others fed by alternative dietary treatments.

A study on calves (Zelski *et al.*, 1995) showed that a diet containing 33% CSM, in which the concentration of FG was 100–220 ppm, resulted in the death of 24 out of 57 calves, each between 7 and 15 weeks of age. It was believed the gossypol in CSM caused large volumes of serous fluid in the

body cavities, hard livers of ''nutmeg'' appearance, and pulmonary congestion. Several calves showed hemosiderosis and fibrosis in some pulmonary vessels of the lungs, in addition to the livers which exhibited periacinar necrosis in acute cases and periacinar fibrosis in chronic cases.

Studies on the effects of different processing methods on the availability of gossypol through feeding lambs (Calhoun *et al.*, 1990a,b) and cattle (Calhoun, 1996) demonstrated that gossypol toxicity was influenced by the methods of cottonseed processing, dietary concentrations of iron and calcium, the presence of other toxic terpenoids and chemicals found in cottonseed, as well as the amount and duration of gossypol consumption. Lindsey *et al.* (1980) studied the physiological responses of lactating cows to gossypol after the animals were fed with CSM containing high FG, and suggested that the rumen microorganisms were responsible for the detoxification that occurred in mature ruminants. It was also proposed that gossypol formed a gossypol–microbial protein complex with the soluble protein in the rumen liquor. This complex was very stable against the enzymatic hydrolysis, and not absorbed in the digestive tract (Reiser and Fu, 1962). It is believed the higher microbial yield characteristic of the rumen is important in supplying enough protein to capture the gossypol, although the microbial population in the rumen is regulated by the ecological balance of conditions that tend to prevail (Van Soest, 1982). This may explain why cattle were found to be more tolerant to gossypol than young calves due to the action of the rumen (Morgan, 1989).

However, if the gossypol content is too high in the diet, it will still surpass the rumen's ability to detoxify. For example, it was found that 348–414 mg of FG per day would cause congestive heart failure in adult goats (East *et al.*, 1994). Similarly, 8 mg of FG and 222 mg of TG (in extracted CSM)/kg body weight per day could cause physical and hematological changes at 2 weeks for cows (Lindsey *et al.*, 1980). Also, the intake of 36.8 mg of FG per kg of body weight per day could have detrimental effects on embryo development in pregnant heifers, and contribute to fertility problems in dairy cows (Villasenor *et al.*, 2008). Besides, gossypol was toxic to swine when the animal was fed by 200–400 ppm of FG. Thus, it was suggested that FG should be less than 100 ppm for growing and fattening swine (Haschek *et al.*, 1989).

It is arguable whether FG content is a good predictor of the toxicity of the cottonseed products (Calhoun, 1996; Calhoun *et al.*, 1990a; Eagle and Davies, 1958; Eagle *et al.*, 1956) because measurements of BG, FG, and TG are insufficient. For example, the BG fraction is not always entirely inactive depending on the mechanism(s) of gossypol binding that occurs during the cottonseed processing, the type of animals consuming the gossypol, and the physical form of the feed. Moreover, different gossypol derivatives also yield different physiological activities, and the (+/−)-two gossypol enantiomers, possess different biological activities. Study of

the gossypol enantiomers revealed that only (−)-gossypol was toxic to humans, but different ratios of (+)- and (−)-gossypol enantiomers in cotton plants did not affect the toxicity toward insects and pathogens (Liu *et al.*, 2008). Calhoun (1996) suggested that different forms of BG might have different stabilities *in vitro* and *in vivo*, possibly because different cottonseed processing conditions favor various forms of BG. The animals selected for bioactivity experiments would also affect the results because of differing digestive environments. Therefore, current results related to the relationship between the available gossypol and its toxicity must be evaluated and interpreted carefully. Also, it should take into account of other factors such as dose and route of administration, the possibility of other toxic materials, and the degree of inactivation of administered gossypol before and/or after administration by reaction with components of the diet (Yu, 1987).

D. Detoxification

Due to the toxicity of gossypol to animals, a glandless variety of cotton was developed in the early 1960s. It was believed that the value of cottonseed oil and meal would be improved if gossypol were not present. However, this glandless strain was easily susceptible to insect infestation, and attracted field mice and other rodents (Lusas and Jividen, 1987; Stipanovic *et al.*, 1986). Dilday (1986) developed a cotton plant through an interspecific cross of tetraploid ($2N = 52$) *G. hirsutum* L. × diploid ($2N = 26$) *G. sturtianum* Willis. This plant showed gossypol glands in vegetative foliar and fruiting tissues but not in the seed. However, this approach needs more investigation to prove the stability and yield of this plant.

Another approach for gossypol removal is by a flotation process based on the specific gravity difference or by centrifugal force, such as the liquid cyclone process which is a physical separation of intact pigment glands (Ridlehuber and Gardner, 1974). Nevertheless, solvent extraction is more popular to remove gossypol because it is widely soluble in various organic solvents like ethyl alcohol, hexane, aqueous acetone, acidic butanol, methylene chloride, aniline, and aliphatic amines, which allows an extraction process with more choices in regard to the safety of solvent, volatility, cost, etc. Among the various extraction methods, extraction with 95% ethanol is more promising for a reduction in TG content up to about 70% in CSM (Hron *et al.*, 1994). Aqueous acetone is also a desirable choice to reduce the FG in CSM (Damaty and Hudson, 1975; Pons and Eaves, 1971). A mixture of ethanol and hexane also reduce both FG and BG in flakes and oil tremendously (Liu *et al.*, 1981). A two-step extraction technique utilizing aqueous and anhydrous acetone, successfully reduced the FG content in gossypol protein concentrate to below FDA standards for human consumption without compromising organoleptic characteristics

(Gerasimidis *et al.*, 2007). Although solvents have generally been successful in reducing the gossypol content of cottonseed products to quite low levels, how to completely remove the potentially harmful solvent residual is another challenging subject.

Chemical methods have been involved in gossypol detoxification. Bressani *et al.* (1964) reported that an alkaline pH of cooking, associated with calcium ions, was effective in reducing FG and TG in cottonseed flour used for human foods. The addition of calcium increased the effectiveness of the gossypol–iron complex formation, resulting in full protection from gossypol toxicity. Nagalakshmi *et al.* (2002, 2003) also confirmed the effectiveness of calcium hydroxide for the gossypol detoxification.

Iron sulfate is an inexpensive source of Fe that could also reduce gossypol intoxication in nonruminant animals (Ullrey, 1966). Kemmerer *et al.* (1966) reported that the addition of iron sulfate to feed could prevent yolk discoloration caused by gossypol. Muzaffaruddin and Saxena (1966) showed that a 1:1 molar ratio of ferric iron to gossypol formed an iron–gossypol complex, in which the two perihydroxyl groups of gossypol were the most plausible sites for the ferric irons to be chelated. Barraza *et al.* (1991) investigated the efficacy of iron sulfate and feed pelleting to detoxify FG in cottonseed diets for dairy calves. Ferrous sulfate added to diets for swine contained 244 and 400 ppm FG, a molar ratio of 0.5:1 for iron to gossypol resulted in partial detoxification and, furthermore, a 1:1 ratio gave complete detoxification. The addition of phospholipids to CSM or CSM coupled with cooking could eliminate some FG and improve the protein quality (Yannai and Bensal, 1983).

Microbial fermentation of CSM, which was proposed to detoxify FG in the CSM (Zhang *et al.*, 2006a,b), seems promising because some exoenzymes such as cellulolytic enzymes, amylase, protease, and lipolytic enzymes that are secreted by certain microorganisms, and some vitamins, as well as some unknown active substances are produced in the fermented CSM (Brock *et al.*, 1994), which adds nutritional value of the fermented CSM. Recently, Qian *et al.* (2008) reported that *in situ* alkaline-catalyzed transesterification could produce a CSM with FG and TG contents below the FAO standard. However, the requirement for a high amount of methanol usage in the *in situ* transesterification and the potential energy consumption to remove the methanol in the meal may be an obstacle for its practical application.

VI. BIOLOGICAL PROPERTIES

Gossypol is a reactive compound due to the presence of six phenolic hydroxyl groups and two aldehyde groups. This reactivity also contributed to its biological activities, which will be discussed in the following sections.

A. Antioxidant property

Gossypol is a polyphenolic compound from the viewpoint of its chemical structure. Like many other phenolic chemicals, such as butylated hydroxytoluene (BHT), coumaric acid, gallic acid, quercetin, myricetin, catechin, gallocatechin, etc., gossypol is an effective and potent natural antioxidant. For example, gossypol was found to be able to protect carotene *in vitro* against preformed fat peroxides many decades ago (Hove and Hove, 1944). Hove (1944) confirmed that cottonseed products containing gossypol could inhibit carotene destruction and rancidity development *in vitro*, and gossypol could act as a carotene-protecting antioxidant *in vivo*. Gossypol has shown potential in inhibiting rat liver microsomal peroxidation, which is caused by an incubation with ferric/ascorbate ($IC_{50} < 0.1$ μM) (Laughton *et al.*, 1989). Gossypol also exhibited a significant positive effect on oil and biodiesel stability. With a concentration of 0.1% gossypol, the oxidative stability indices (OSI) of cottonseed oil biodiesel could increase to 17.2 h from 4.15 h at 110 °C (Fan *et al.*, 2008).

In some cases, the modification of the functional groups on gossypol may not affect its original chemical and biological activities. For instance, the modification of aldehydic groups on gossypol to form dianilinogossypol, of which the free carbonyl groups were tied up by the anilido complex, did not decrease the antioxidant activity of the free compound (Bickford *et al.*, 1954; Hove, 1944). Bickford *et al.* (1954) also found the other Schiff base-formed gossypol derivatives, gossypol-urea, gossypol-aminobenzene-thiol, and gossypol-glycineindicates, have roughly equivalent antioxidative ability to gossypol on a molar basis. Gossypol bis(piperinoethylimine) and bis(morpholinoethylimine) also demonstrate potent antioxidant action in human blood serum and rat brain synaptosomes. At equal concentrations, these substances suppressed the peroxidation of lipids in enzymatic and nonenzymatic systems regarding the oxidation of rat liver microsomes (Dalimov *et al.*, 1989).

On the contrary, in many other cases, the modification of phenolic hydroxyl groups on gossypol could significantly decrease the ''chemical'' antioxidative abilities regarding free radical scavenging activity, reducing power, and DNA damage prevention activity (Wang *et al.*, 2008), demonstrating that the hydroxyl groups are critical for the antioxidative activities. For example, 6-methoxy gossypol exhibited a similar free radical scavenging activity as 6,6′-dimethoxy gossypol, while gossypol possessed a stronger radical scavenging activity than either of the methylated derivatives. The concentrations of 6-methoxy gossypol and 6,6′-dimethoxy gossypol needed to scavenge 50% of the free radicals in the test system were twofold higher than that of gossypol (about 16 ppm vs. 8 ppm). Although gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol all reduced ferric ions to ferrous ions in a dose-dependent manner, gossypol

again showed greater reducing power and higher efficiency than 6-methoxy gossypol or 6,6′-dimethoxy gossypol. However, all three test compounds showed much stronger reducing power than BHT; 6,6′-dimethoxy gossypol at a concentration of 10 ppm exhibited the same reducing power as BHT at a 100-ppm concentration. The relative capability of gossypol and its methylated derivatives to prevent DNA damage caused by ultraviolet light and hydrogen peroxide was consistent with the compounds' antioxidant effects. This suggests that gossypol's protection of DNA may occur partially by quenching free radicals, therefore alleviating oxidative stress. A previous study (Li *et al.*, 2000) also found that gossypol demonstrated the ability, in a dose-dependent manner, to protect supercoiled plasmid DNA from damage caused by exposure to Fe^{3+}/ascorbate.

B. Antifertility activity

Gossypol has been studied extensively and intensively as a potential contraceptive agent through *in vivo* models including rats (Hadley *et al.*, 1981; Lin *et al.*, 1980, 1985), mice (Coulson *et al.*, 1980), monkeys (Shandilya *et al.*, 1982), hamsters (Matlin *et al.*, 1987; Waller *et al.*, 1984), rabbits (Chang *et al.*, 1980), bulls (Arshami and Ruttle, 1988), and male humans (National Coordinating Group on Male Antifertility Agents, 1978). The following section is going to cover some research findings on antifertility activity of gossypol.

Oral administration of gossypol acetic acid at a dose of 5 or 10 mg/kg body weight/day for 12 weeks induced sterility in male hamsters and rats. Treatment in male rabbits at doses ranging from 1.25 to 10 mg/kg body weight/day for 5–14 weeks decreased the sperm mortality but did not affect the average number of sperm per ejaculate (Chang *et al.*, 1980). In another study reported by Lin *et al.* (1980), gossypol treatment at a dose of 30 mg/kg body weight/day was found to markedly diminish sperm production in rats. Hoffer (1982) observed no differences in the morphology of the testis or epididymal sperm in male rats at a low dosage of 7.5 mg/kg body weight/day for 7 weeks. At the higher dosages (20 and 30 mg/kg body weight/day), deleterious changes in epididymal sperm and a limited extent of testicular damage were observed.

In a study through oral administration of gossypol at a level of gossypol acetic acid of 5 or 10 mg/kg body weight/day for 6 months, it was found that adult male cynomoglus monkeys showed a significant decrease in sperm concentration and motility, even though there was neither a significant decrease in circulating levels of testosterone nor a significant difference in plasma testosterone levels (Shandilya *et al.*, 1982). Similarly, daily feeding of gossypol acetic acid (40 mg/kg body weight/day) to chickens for 18 days resulted in a decrease in semen volume and

sperm concentration. The treatment also decreased the activities of acrosin, hyaluronidase, and angiotensin converting enzyme, and fertility dropped to zero at the end of the treatment period. Meanwhile, other obvious side effects including a loss of appetite, loss of body weight, and morphological abnormalities in spermatozoa were observed in the treated cocks (Mohan *et al.*, 1989).

It was also found that (−)-gossypol is more active in its antifertility function than (+)-gossypol (Matlin *et al.*, 1987). (+)-Gossypol at the dosage of 30 mg/kg orally for 14 days has neither antifertility effects nor toxicity in male rats, but slight damage was found in the germinal epithelium of the testis in animals dosed for 4 weeks. (−)-Gossypol at 30 mg/kg orally for 7 days showed an antifertility effect in male rats (Wang *et al.*, 1987). Gossypolone, an oxidized metabolite of gossypol, displayed less spermicidal activity than gossypol isomers on spermatozoa from human, monkey, rabbit, mouse, rat, and hamster subjects (Kim *et al.*, 1984).

The concurrent treatment of gossypol and steroid hormones showed potential in contraceptive activity. For example, the combination of gossypol (12 mg/kg body weight/day) with steroid hormones methyltestosterone at 20 mg/kg body weight/day and ethinyl estradiol at 100 μg/kg body weight/day for 6 weeks, along with a low-dose gossypol alone for 12 weeks as a maintenance dose could damage the epididymal sperms in male rats (Xue, 2000). A similar study on adult Wistar rats under the treatment with a combination of a low dose of gossypol and steroid hormone (methyltestosterone and ethinyl estradiol) via gastric intubation also caused a similar decrease of epididymal sperm motility and epididymal sperm deterioration (Wang *et al.*, 2000).

The mechanism illustrating how gossypol exerts contraceptive activity has been exploited by some researchers. Spermatogenesis is a process involving a specific ratio of cells with unique DNA ploidy (1C, 2C, and 4C). Treatment of 20 mg/kg body weight/day of gossypol in rats revealed a significant shift from the 1C stage to the 2C and 4C stages, indicating less sperm available for female fertilization (Ojha *et al.*, 2008). In another study, Bai and Shi (2002) found that the concentration $\geq$5 μM gossypol could completely block the T-type Ca^{2+} currents in mouse spermatogenic cells, and the gossypol-induced inhibition of T-type Ca^{2+} currents could be responsible for the antifertility activity of the compound. A decreased seminal plasmid lipid concentration contributed to the reduction in sperm counts in rabbits (Shaaban *et al.*, 2008) and bulls (Kelso *et al.*, 1997).

Studies on the activity of rabbit sperm acrosomal enzymes have indicated that gossypol at 12–76 μM could significantly inactivate azocoll proteinase, acrosin, neuraminidase, and arylsulfatase. Hyaluronidase, β-glucuronidase, and acid phosphatase were also inhibited at a higher concentration of gossypol (380 μM) (Yuan *et al.*, 1995). Since acrosomal enzymes play important roles in the fertilization process, the inhibition of

these enzymes would alter the acrosome reaction, and interrupt the fertilization process.

ATPases are a type of enzyme that can catalyze the decomposition of adenosine triphosphate (ATP) and provide energy for cell growth. Gossypol was found to inhibit the human spermatozoa ATPase activity, lowering the energy available for cell motility (Wu *et al.*, 1998). Taitzoglou *et al.* (1999) found that plasminogen activator activity from man and ram extracts can be completely inhibited by 350 and 300 μM of gossypol, respectively. Also, a low concentration of gossypol (2.5–40 μM) was found to be able to significantly inhibit plasmin activity in a dose-dependent manner. Since the plasminogen activator/plasmin system plays a role in the entire process of ovum fertilization, the inhibition of both acrosomal plasminogen activator and plasmin activity is a possible mechanism by which gossypol exerts its antifertility effect. In addition, the inhibition of LDH also contributes to the antifertility. Selective inhibitors of human LDH (LDH-C4, -B4, and -C4) targeted to the dinucleotide fold hold promise as male antifertility drugs. Gossypol is a nonselective competitive inhibitor of NADH binding to LDH, with K_i values of 1.9, 1.4, and 4.2 μM for LDH-A4, -B4, and -C4, respectively (Yu *et al.*, 2001).

However, gossypol's antifertility activity may be influenced by many endogenous effectors. Javed and Khan (1999) found that histidine, cysteine, and glycine could block the effect of gossypol acetic acid on the inhibition of purified LDH-X, while arginine, glutamic acid, phenylalanine, and valine were ineffective against the inhibitory action of gossypol acetic acid. In humans, 5-alpha-reductase activity is critical for certain aspects of male sexual differentiation and may be involved in the development of benign prostatic hyperplasia, alopecia, hirsutism, and prostate cancer. Gossypol is a selective noncompetitive inhibitor of the type 1 steroid 5-alpha-reductase isoenzyme and, therefore, may have potency for the prevention or treatment of androgen-dependent disorders. This inhibition seems to require the presence of catechol moieties on the gossypol molecule (Hiipakka *et al.*, 2002).

C. Anticancer activity

Gossypol is capable of inhibiting the growth of a variety of cell lines including breast, colon, prostate, and leukemia cells (Balci *et al.*, 1999; Benz *et al.*, 1990; Huang *et al.*, 2006; Zhang *et al.*, 2003). Table 6.2 summarizes the antitumor activities of gossypol against several cancer human cell lines *in vitro*. These disruptions include inhibition of cytoplasmic and mitochondrial enzymes involved in energy production (Ueno *et al.*, 1988) and uncoupling of oxidative phosphorylation (Abou-Donia and Dieckert, 1974; Flack *et al.*, 1993). In addition, depletion of cellular ATP has been demonstrated in cultured tumor cells (Keniry *et al.*, 1989). Gossypol also

TABLE 6.2 Antitumor activity of gossypol against several human cancer cell lines *in vitro*

Human cancer cell lines	Gossypol activity IC_{50} (μM)			References
Ovarian	(+)/(−)-	(−)-	(+)-	Band *et al.* (1989)
OVCA-420	3.8	1.6	11.7	Band *et al.* (1989)
OVCA-429	2.4	0.9	11.3	Band *et al.* (1989)
OVCA-433	2.5	1.2	8.5	Band *et al.* (1989)
OVCA-432	2.4	1.1	10.2	Band *et al.* (1989)
OVCAR-3	1.5	0.6	5.8	Band *et al.* (1989)
Prostate				
PC-3	10			Zhang *et al.* (2007b)
Breast				
MCF-7	5			Coyle *et al.* (1994)
MCF-7	24			Zhang *et al.* (2007a)
T47-D	5	3	20	Benz *et al.* (1990)
MCF-7 WT	3.4			Jaroszewski *et al.* (1990)
MCF-7 ADR	4.3			Jaroszewski *et al.* (1990)
SKOV-3	5.7	[a]		Le Blanc *et al.* (2002)
Endomentrial				
RL95-2	3.4			Le Blanc *et al.* (2002)
Carvix				
KB-3	3.8			Jaroszewski *et al.* (1990)
KB-A1	2.9			Jaroszewski *et al.* (1990)
KB-V1	4			Jaroszewski *et al.* (1990)
Hela	4			Coyle *et al.* (1994)
SiHa	47	30	>50	Shelley *et al.* (2000)
SiHa	14			Zhang *et al.* (2007a)
Adrenocortical				
H295R	2.9			Le Blanc *et al.* (2002)
SW-13	1.3			Le Blanc *et al.* (2002)
Medullary thyroid				
TT	18.9			Le Blanc *et al.* (2002)

(*continued*)

TABLE 6.2 (*continued*)

Human cancer cell lines	Gossypol activity IC_{50} (μM)			References
Lung				
A549	0.5			Chang *et al.* (2004)
H69	30	20	50	Shelley *et al.* (2000)
Colon				
Caco 2	17			Zhang *et al.* (2007a)
Colo201	4			Coyle *et al.* (1994)
SW407	8.2			Tuszynski and Cossu (1984)
SW1084	6.5			Tuszynski and Cossu (1984)
SW1116	7			Tuszynski and Cossu (1984)
HCT-8		5	50	Benz *et al.* (1990)
HT-29	5			Zhang *et al.* (2003)
Leukemia				
JURKAT T		20		Oliver *et al.* (2005)
HL-60	50			Jarvis *et al.* (1994)
HL-60	8.3			Hou *et al.* (2004)
HL-60		20	>50	Shelley *et al.* (2000)
HL-60	≈15			Moon *et al.* (2008b)
K562	≈15			Moon *et al.* (2008b)
U937	≈15			Moon *et al.* (2008b)
THP-1	≈15			Moon *et al.* (2008b)
Pancreatic				
MiaPaCa		3	20	Benz *et al.* (1990)
Head and neck				
UM-SCC		2.5–10		Oliver *et al.* (2004)
Melanoma				
SK-MEL-19	25	20	>30	Blackstaffe *et al.* (1997)
SK-MEL-28	23	16.5	>30	Blackstaffe *et al.* (1997)
WM9	6.2	3.1	14.3	Band *et al.* (1989)
WM56	6			Tuszynski and Cossu (1984)
WM164	5.5			Tuszynski and Cossu (1984)

(*continued*)

TABLE 6.2 (*continued*)

Human cancer cell lines	Gossypol activity IC_{50} (μM)	References
FEMX	2.4	Jaroszewski *et al.* (1990)
FEMX4AP	5	Jaroszewski *et al.* (1990)
SK-EML-3	4	Coyle *et al.* (1994)

[a] No data provided; IC_{50}: median inhibitory concentration. Adapted from Dodou *et al.* (2005).

inhibits key nuclear enzymes responsible for DNA replication and repair, including DNA polymerase α (Rosenberg *et al.*, 1986) and topoisomerase II, and blocks DNA synthesis in HeLa cells (Wang and Rao, 1984). Hou *et al.* (2004) found that gossypol at 50 μM for 6 h could induce apoptosis in human promyelocytic leukemia cells (HL-60) (DNA fragmentation, poly (ADP) ribose polymerase cleavage), and also induce the truncation of Bid protein, the loss of mitochondrial membrane potential, cytochrome *c* release from mitochondria into cytosol, and activation of caspases-3, -8, and -9. At a low dose of 5 μM, gossypol also could cause a significant elevation of caspases-3, -8, and -9, which resulted in cell apoptosis of human colon cancer cell line HCT 116 (Zhang *et al.*, 2007b). Recent studies on human leukemia U937 cells showed gossypol at >10 μM resulted in significant cell cytotoxicity and DNA fragmentation, induced caspase-3 activation and poly(ADP) ribose polymerase cleavage. These properties make gossypol a potential antineoplastic agent.

It is reported that inhibition of DNA synthesis can be achieved with 10 μM gossypol by blocking the G1/S checkpoint in MCF-7 cells at 24 h of incubation (Ligueros *et al.*, 1997). Gossypol might regulate cell cycles by modulating the expression of cell-cycle regulatory proteins Rb and cyclin D1 and the phosphorylation of Rb protein. A similar conclusion was obtained from Jiang *et al.* (2004) that inhibitory effects of gossypol on the proliferation of human prostate cancer PC3 cells were associated with induction of TGF-β1, which in turn influenced the expression of the cell-cycle regulatory protein, cyclin D1. In human alveolar lung cancer cells, gossypol induces Fas/Fas ligand-mediated apoptosis (Moon *et al.*, 2008a). Also, gossypol induces transcriptional downregulation and posttranslational modification of hTERT in human leukemia cells, causing inactivation of c-Myc and Akt, respectively. Both c-Myc and Akt are able to regulate various Bcl-2 proteins, the proapoptosis protein family members.

Treatment with gossypol also downregulated the expression of NF-kappa B-regulated gene products, including inhibitor of apoptosis protein (IAP)-1, IAP-2, and X-linked IAP. These results suggest that

gossypol-induced apoptosis partially involves suppression of NF-kappa B activity (Moon *et al.*, 2008b). Treatment of Ramos cells with gossypol not only induced cell arrest on the G_0/G_1 phase but also increased apoptosis and growth inhibition induced by etoposide (VP-16), doxorubicin hydrochloride (ADM), vincristine (VCR), and paclitaxel (taxol) (Li *et al.*, 2008). Liu *et al.* (2002) found that (−)-gossypol was more active in inhibiting breast cancerous epithelial cells (cEC) and cancerous stromal cells (cSC). Meanwhile, the inhibitory activity of (−)-gossypol was related to the reduction of the cell-cycle regulator, cyclin D1, and the induction of the cell proliferation inhibitor, TGF-β. In the study of human prostate cancer cells, it was found that (−)-gossypol-induced apoptosis was mediated by the regulation of Bcl-2 and caspase families (Huang *et al.*, 2006). Another *in vitro* study (Mohammad *et al.*, 2005) demonstrated (−)-gossypol had significant inhibitive effects against the growth of lymphoma cell line WSU-DLCL2 and fresh cells obtained from a lymphoma patient with no effect on normal peripheral blood lymphocytes. (−)-Gossypol also induced complete cytochrome *c* release from mitochondria, increased caspases-3 and -9 activity, and caused apoptotic death without affecting protein levels of Bcl-2, Bcl-X(L), Bax, and Bak. Recent research has revealed that (−)-gossypol acts as a BH3 mimetic, binding to the BH3-binding domain in various proapoptotic proteins of the Bcl-2 family, displacing prodeath partners to induce apoptosis (Balakrishnan *et al.*, 2008; Meng *et al.*, 2008).

Sikora *et al.* (2008) found that the combination of gossypol with the antioxidant *N*-acetyl-cysteine (NAC) to block reactive oxygen species (ROS) would increase the (−)-gossypol-induced cytotoxicity in tumor cells, but not normal cells, indicating that concurrent treatment with antioxidants to block ROS prevents oxidative inactivation of (−)-gossypol and limits off-target toxicity allowing more potent (−)-gossypol-induced antitumor activity. An *in vivo* study also showed that (−)-gossypol significantly enhanced the antitumor activity of X-ray irradiation, leading to tumor regression in the combination therapy by inhibiting both antiapoptotic proteins Bcl-2 and/or Bcl-xL (Xu *et al.*, 2005). A combination of docetaxel and (−)-gossypol synergistically enhanced the antitumor activity of docetaxel both *in vitro* and *in vivo* in the human prostate cancer PC-3 xenograft model in nude mice. (−)-Gossypol exerts its antitumor activity through inhibition of the antiapoptotic protein Bcl-xL accompanied by an increase of proapoptotic Noxa and Puma (Meng *et al.*, 2008). One study on gossypol derivatives (Arnold *et al.*, 2008) showed that apogossypolone could inhibit the growth of the lymphoma cell line WSU-FSCCL with an IC_{50} of 109 nM, and the activation of caspases-9, -3, and -8 was observed. Hu *et al.* (2008) found that apogossypol selectively inhibited proliferation of three NPC cell lines (C666-1, CNE-1, and CNE-2) that highly expressed the antiapoptotic Bcl-2 proteins with release of cytochrome *c*, activation of

caspases-9 and -3, and apoptosis of sensitive NPC cells (Hu *et al.*, 2008). The toxicity and efficacy study on mice (Kitada *et al.*, 2008) showed that mice tolerate doses of apogossypol two- to four-times higher than gossypol. Apogossypol displayed superior activity to gossypol in terms of reducing splenomegaly and reducing B-cell counts in the spleens of Bcl-2-transgenic mice, indicating the potential of gossypol derivatives for cancer therapy. Gossypolone was less potent than gossypol in inhibiting human breast cancer cells (Gilbert *et al.*, 1995). The reduced effectiveness of gossypolone compared to gossypol in breast cancer cells agrees with the antifertility effects (Kim *et al.*, 1984), but is in contrast to the antisteroidogenic and antireproductive effects of gossypolone, which have shown similar potency as gossypol (Gu and Lin, 1991). Methylated gossypol, 6-methoxy gossypol, and 6,6′-dimethoxy gossypol, compared with the parent compound, showed superior anticancer activity against cervical (SiHa), breast (MCF-7), and colon (Caco-2) cancer cells (Wang *et al.*, 2008).

In summary, gossypol is believed to arrest cell growth at the G_0/G_1 phase and induce cell apoptosis, in cancer cells, by regulating the cell cycle, enzymes, antiapoptosis, and proapoptosis proteins.

D. Antivirus activity

Lin *et al.* (1989, 1993) reported that gossypol inhibited the replication of human immunodeficiency virus type 1 (HIV-1) and found (−)-gossypol to be more inhibitory ($IC_{50} = 5.2$ μM) compared to the (+)-gossypol ($IC_{50} = 50.7$ μM). Besides HIV-1, gossypol also showed antiviral activity in multiple enveloped viruses including herpes simplex virus type II (HSV-II), influenza virus, and parainfluenza virus (Vander Jagt *et al.*, 2000).

Gossypol and a series of periacylated gossylic nitriles (Fig. 6.10) were compared for their antiviral activities against HSV-II and for their toxicities to the host Vero cells. All of the periacylated gossylic nitriles exhibited lower cytotoxicities to the host cell than did the parent compound gossypol. Both gossypol and the series of derivatives exhibited antiviral activities against HSV-II when the virus was treated with concentrations as low as 5×10^{-7} M. Two of the derivatives, gossylic nitrile-1,1′-diacetate and gossylic nitrile-1,1′-divalerate, were capable of inhibiting viral multiplication in Vero cells that were infected with virus before administration of the drug. Radloff *et al.* (1986) concluded that modification of gossypol's aldehydic groups lowered its toxicity to the host Vero cells but did not abolish the compound's antiviral (HSV-II) activity. Derivatives of gossypol may be useful as antiviral agents.

Later, Royer *et al.* (1991) found that gossypol and its derivatives, gossylic nitrile-1,1′-diacetate, gossylic iminolactone, and gossylic lactone (Fig. 6.10), inhibited the replication of HIV-1 *in vitro*. Gossylic iminolactone displayed the greatest inhibition, followed by gossypol, gossylic

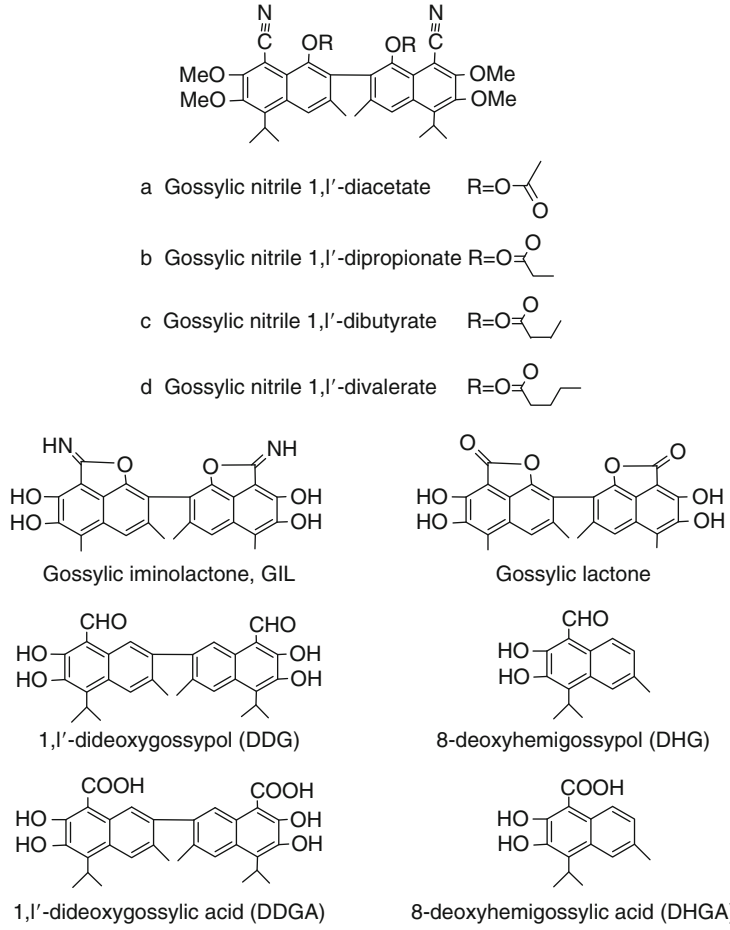
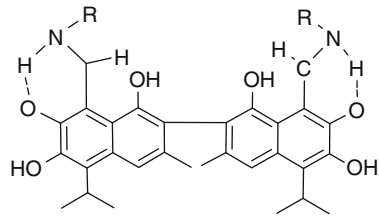


FIGURE 6.10 Gossypol derivatives.

nitrile-1,1′-diacetate, and gossylic lactone, indicating that derivatives of gossypol can retain antiviral activities. Then, Royer *et al.* (1995a,b)) tested several other gossypol derivatives for inhibition of HIV 1,1′-dideoxygossypol (DDG), 1,1′-dideoxygossylic acid (DDGA), 8-deoxyhemigossypol (DHG), and 8-deoxyhemigossylic acid (DHGA) (Fig. 6.10). The result showed that DDGA was the most effective in inhibiting the replication of HIV *in vitro* with $EC_{50} < 1$ μM. Meanwhile, DDG was less effective than DDGA. DHG showed some anti-HIV activity, and DHGA was ineffective against HIV. Since all four gossypol derivatives were found to have much lower affinities for albumin than the parent compound gossypol, this would possibly enhance the antivirus activity of the gossypol derivatives *in vivo* with less interference from *in vivo* proteins.

E. Antiparasitic protozoan activities

Malaria is a vector-borne infectious disease caused by protozoan parasites. Human malaria is usually caused by the infection of *Plasmodium falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* (Mendis *et al.*, 2001). It is widespread in tropical and subtropical regions, including Asia, Africa, and parts of the Americas. Each year there are about 350–500 million cases of malaria, and more than 1 million people die (CDC, 2009). A series of gossypol derivatives with modified aldehydic groups and hydroxyl groups (Figs. 6.10 and 6.11**)** have been shown to inhibit the growth of *P. falciparum* (Razakantoanina *et al.*, 2000; Royer *et al.*, 1986). Table 6.3



Derivatives	R
Methyl gossypol	$-CH_3$
Ethyl gossypol	$-CH_2CH_3$
Propyl gossypol	$-CH_2CH_2CH_3$
Isopropyl gossypol	$-CH(CH_3)_2$
Butyl gossypol	$-CH_2CH_2CH_2CH_3$
s-butyl gossypol	$-CH_2\,CH(CH_3)-CH_3$
t-butyl gossypol	$-C(CH_3)_3$
Pentyl gossypol	$-CH_2CH_2CH_2CH_2CH_3$
Hexyl gossypol	$-CH_2CH_2CH_2CH_2CH_2CH_3$
Heptyl gossypol	$-CH_2CH_2CH_2\,CH_2\,CH_2CH_2CH_3$
Dodecyl gossypol	$-CH_2\,(CH_2)_{11}CH_3$
Mephenet[a] gossypol	$-CH_2(CH_3)CH-C_6H_5$
Phemet[b] gossypol	$-CH(COOCH_3)-CH_2-C_6H_5$

a: mephenet: ß-methyl phenylalanine ethyl
b: phemet: phenylalanine methyl ester

FIGURE 6.11 Chemical formula for gossypol Schiff bases.

TABLE 6.3 Antimalarial activity of gossypol and its derivatives against *P. falciparum in vitro*

	IC_{50} (μM)[a]			IC_{50} (μM)[a]	
	Strain			Strain	
Drug	PFB[b]	FCB1[b]	Drug	FCB/NC-1[c]	CDC/I/HB-3[c]
Gossypol	15.3	28.8	Gossypol	13	7
Methyl gossypol	ND	66.2	Gossylic nitrile 1,1′-diacetate	76	36
Ethyl gossypol	22	22.5	Gossylic nitrile 1,1′-dipropionate	69	46
Propyl gossypol	16	20.8	Gossylic nitrile 1,1′-dibutyrate	26	21
Isopropyl gossypol	16.6	17.6	Gossylic nitrile 1,1′-divalerate	16	12
Butyl gossypol	42.4	ND			
s-Butyl gossypol	37.8	54			
t-Butyl gossypol	39.5	40			
Pentyl gossypol	ND	ND			
Hexyl ossypol	67.2	ND			
Heptyl gossypol	ND	33.2			
Dodecyl gossypol	43.2	37			
Mephenet gossypol	47	56			
Phemet gossypol	83	70.3			

[a] IC_{50} represents the drug concentration producing 50% inhibition of the growth of *P. falciparum* in drug free control wells.
[b] Chloroquine-resistant strains of *P. falciparum.*
[c] Strains FCB/NC-1 and CDC/I/HB-3 are the chloroquine-resistant and chloroquine-sensitive strains of parasite, respectively. Adapted from Royer *et al.* (1986) and Razakantoanina *et al.* (2000).

summarizes the antimalarial activity of gossypol and its derivatives *in vitro*. The IC_{50} values are between 13 and 83 μM for gossypol and gossypol derivatives against *P. falciparum*. The derivatives with ethyl, propyl, or isopropyl side chains as well as gossylic nitrile 1,1′-divalerate with IC_{50} values close to gossypol ($IC_{50} = 16$ μM) showed stronger inhibition than other gossypol derivatives against the growth of *P. falciparum*.

Royer *et al.* (1986) proposed that the antimalarial activity of gossypol and gossypol derivatives was through the inhibition of LDH. LDH is the most active and essential enzyme for anaerobic life cycle of *P. falciparum*. Any compound showing inhibition of this enzyme also kills the parasites (Razakantoanina *et al.*, 2000; Royer *et al.*, 1986). A study on *Toxoplasma gondii*, a protozoan parasite causing toxoplasmosis, also demonstrated that the inhibition of *T. gondii* LDH activity is correlated with the inhibition of *T. gondii* growth in cultures (Dando *et al.*, 2001). In the study on *Entamoeba histolytica* (Gonzalez-Garza *et al.*, 1993a,b), gossypol also showed the inhibition to alcohol dehydrogenase and malic enzymes, and (−)-gossypol was found more active than racemic gossypol and (+)-gossypol. The (−)-gossypol was 3.6 and 13 times more potent than (+/−)- and (+)-gossypol, respectively, in inhibiting the malic enzyme, and 1.9 and 2.9 times more potent than (+/−)- and (+)-gossypol, respectively, against the alcohol dehydrogenase.

Trypanosomes, protozoan parasites belonging to the subphylum Mastigophora, can cause a chronic infection called sleeping sickness. It has seriously affected the health of people in western and central African countries, and exerted significant mortality in man and livestock. Over 60 million people living in 36 sub-Saharan countries are threatened by sleeping sickness (WHO, 2001) and 48,000 deaths were reported in 2002 (WHO, 2004). In addition, 46 million cattle are exposed to the risk of the sleeping disease. The disease costs an estimated 1340 million USD per year (Kristjanson *et al.*, 1999). However, few drugs are available for the treatment of trypanosomal infections that cause significant mortality in man and livestock in Africa. Gossypol was reported to be able to inhibit trypanosomes (Blanco *et al.*, 1983; Kaminsky and Zweygarth, 1989; Montamat *et al.*, 1982). Montamat *et al.* (1982) reported that a 5-min exposure to 100 μM gossypol (∼50 ppm) immobilizes cultures of *Trypanosoma cruzi*. Blanco *et al.* (1983) reported that a 30-min exposure to 25 μM gossypol (∼12 ppm) immobilizes and alters the cell morphology of *T. cruzi*. Later, Kaminsky and Zweygarth (1989) reported that, for three separate *T. brucei* strains (including one drug-resistant strain), the IC_{50} value for a 24-h gossypol exposure was >10 ppm. Our study showed a similar level of gossypol's antitrypanosomal activity with IC_{50} value of 7.8 ppm after 24-h exposure. Moreover, methylated gossypol, both 6-methoxy gossypol (IC_{50} value, 3.98 ppm) and 6,6′-dimethoxy gossypol (IC_{50} value, 3.21 ppm) showed more effective inhibition of growth than

gossypol. In *T. cruzi*, gossypol has been reported (Gerez de Burgos *et al.*, 1984; Montamat *et al.*, 1982) to inhibit some oxidoreductases, such as, alpha-hydroxyacid and malate dehydrogenases, NAD-linked enzymes, and glutamate dehydrogenase, malic enzyme and glucose-6-phosphate dehydrogenase, NADP-dependent enzymes. Gossypol also inhibits the MDH enzyme of *T. cruzi* (Gerez de Burgos *et al.*, 1984).

Accordingly, the possible mechanism of the antiparasitic effect of gossypol and gossypol derivatives could be the selective inhibition of vital enzymes in the parasites.

F. Antimicrobial activity

The antimicrobial properties of gossypol have been reported by several research groups. Gossypol has general antifungal activities with LD_{50} values from 20 to 100 ppm of pure gossypol (Bell, 1967), and has an inhibitory effect on microorganisms including aerobic sporeformers and lactobacilli and some yeasts (Table 6.4) (Margalith, 1967). Gossypol showed strong antibiotic activity against aerobic sporeformers and lactobacilli, and displayed antagonistic property to some of the more oxidative yeasts.

Later, Vadehra *et al.* (1985) investigated the effects of gossypol on the growth of a variety of bacteria and on spore formation and germination in *Bacillus cereus*. It has been found that gossypol has more potent

TABLE 6.4 Inhibitory effect of gossypol on microorganisms effect of gossypol on microorganisms

Organism: minimal inhibitory concentration			
Bacteria	μg/ml	Yeasts	μg/ml
Staphylococcus aureus	10	*Saccharomyces cerevisiae*	>200
Sarcina lutea	25	*S. carlsbergensis*	>200
Bacillus polymyxa	50	*Zygosaccharomyces mellis*	>200
B. megaterium	50	*Hansenula anomala*	200[a]
B. licheniformis	25	*Hanseniaspora* sp.	200[a]
B. cereus	50	*Candida utilis*	>200
B. thermoacidurans	50	*Debaryomyces nicotianae*	100
Leuconostoc mesenteroides	10	*Pichia membranefaciens*	25[b]
Lactobacillus delbruckii	20	*Cryptococcus neoformans*	25
Escherichia coli	>200	*Rhodotorula mucilaginosa*	>200
Proteus mirabilis			>200
Pseudomonas aeruginosa			>200

[a] Caused slight inhibition.
[b] Caused complete suppression of film growth. Adapted from Margalith (1967).

antibacterial properties against Gram-positive organisms (i.e., *Streptococcus* spp., *Bacillus* spp., *Staphylococcus aureus*) than Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella* spp., *Klebsiella pneumoniae*, *Shigella* spp., *Proteus* spp., and *Escherichia coli*. All of the Gram-positive organisms tested were completely inhibited at a concentration of 100 ppm. None of the tested Gram-negative strains was inhibited at 100 ppm of gossypol, and only one-third of the tested strains were inhibited at 200 ppm of gossypol. The authors proposed that the antibacterial activity of gossypol was related to the Gram character of the organisms. Besides, the chemical and quantitative differences of the cell wall and cell membrane of the Gram-positive and -negative groups may influence the transport of gossypol to its target site (i.e., Gram-positive organisms have high amount of peptidoglycan in the cell wall, and lack the outer membrane found in Gram-negative bacteria). The same research group also found that yeasts, such as *Saccharomyces cereviseae*, *S. uvarum*, *S. diasticu*, were sensitive to gossypol, and the growth were completely inhibited at 50 ppm of gossypol. Subsequent research (Poprawski and Jones, 2001) found that fungi *Paecilomyces fumosoroseus* (associated with cutaneous and disseminated infections in dogs and cats) were highly tolerant to gossypol even at 500 ppm, but could be strongly inhibited at 1000 ppm of gossypol.

G. Lowering plasma cholesterol levels

Cholesterol is a fat-soluble compound found in the body. Having high ''bad'' cholesterol means you have too much low-density lipoprotein (LDL) in your blood, which is linked to serious problems, such as atherosclerosis and coronary heart attack or stroke. A study on adult male cynomolgus monkeys (Shandilya and Clarkson, 1982) found that gossypol administered orally at 10 mg/kg/day for 6 months could cause a significant decrease in total plasma cholesterol (TPC) and LDL without any significant decrease in plasma high-density lipoprotein (HDL) cholesterol levels. It was proposed that this cholesterol lowering activity may be attributed to (a) gossypol might possibly reduce the intestinal absorption of dietary cholesterol and (b) gossypol may reduce the hepatic synthesis of LDL. Studies with rabbits also showed that dietary cottonseed protein effectively lowers the concentration of plasma cholesterol when compared to the animal protein casein (Beynen and Liepa, 1987), which may be attributed to the present of gossypol in the cottonseed protein. Thrice weekly subcutaneous injection doses of 20 mg/kg body weight to rats for 4 weeks also resulted in lower serum cholesterol (Akingbemi *et al.*, 1995). Another study on rats (Achedume *et al.*, 1994) demonstrated that gossypol consumption had a significant effect on alcohol dehydrogenase and had a profound influence on the regulation of cholesterol level in the liver. A subsequent study on rats (Nwoha and Aire, 1995) showed that

the administration of gossypol at 20 mg/kg body weight/rat/day for 8 weeks could significantly decrease the serum level of cholesterol in both low- and normal-protein-fed male Wistar rats. The combined administration of gossypol and chloroquine (chloroquine, a 4-aminoquinoline, used for treatment of malaria) to the protein-malnourished rats had more profound effects in decreasing the levels of serum cholesterol and triglycerides compared to normal-protein-fed rats, indicating the implication of the treatment and dietary effect on the level of serum cholesterol. However, the mechanism by which gossypol lowers the serum cholesterol still needs further investigation.

VII. CLINICAL IMPLICATION

Gossypol initially spurred a lot of interest due to its contraceptive activity. A large-scale clinical trial that involved 10,000 healthy volunteers was conducted in China in 1978. A dose of 20 mg/day by mouth for 75 days (loading dose), and then 50 mg/week (maintenance dose) were administered to the volunteers. A small portion of the volunteers (0.75%) developed severe hypokalemia and 10% of the men taking gossypol for >1 year acquired irreversible aspermatogenesis (National Coordinating Group on Male Antifertility Agents, 1978). Another international study on 151 men from Brazil, Nigeria, Kenya, and China found that 15 mg/day of gossypol for 12 or 16 weeks, followed by either 7.5 or 10 mg/day for 40 weeks did not cause hypokalemia, and spermatogenesis was recovered after gossypol discontinuation (Coutinho, 2002). A study from Xue (2000) on male volunteers found that taking low doses of gossypol (15 mg/day) could induce antifertility within 12 weeks. Furthermore, all of the volunteers remained infertile without developing hypokalemia and irreversible azoospermia after a low-maintenance dose of gossypol (10 mg/day) for 44 weeks. In contrast, the fertility, induction of abnormal histone-to-protamine replacement reaction, as well as alteration of nuclear basic proteins could be recovered 10 weeks after the withdrawal of drug treatment (Xue, 2000).

Two key side effects of high-dose gossypol treatment include irreversible infertility and hypokalemia. The inhibition of gossypol on 11-β-hydroxysteroid dehydrogenase (11-β-OHSD) results in hypokalemia. The enzyme, 11-β-OHSD, is present near mineralocorticoid receptors. It oxidizes hydrocortisone to inactive cortisone in the kidney and is an important regulator of renal K^+ clearance. Inhibition of 11-β-OHSD leads to the production of mineralocorticoid in excess, hypokalemia, and hypertension (Reidenberg, 2000). Gossypol inhibits purified 11-β-OHSD from rat liver and human kidney microsomes in a competitive manner. The degree of physiological symptoms due to potassium excretion is correlated to the initial serum potassium level of the individual and can be

changed by dietary 11-β-OHSD inhibitors, such as polyphenols from tea, naringerin from grapefruit juice, and glycyrrhizic acid from licorice in a synergistic manner (Reidenberg, 2000; Song *et al.*, 1992). The hypokalemia of concern may have been caused by an improper diet of the test subjects (Coutinho, 2002). Hypokalemia is a common occurrence in Chinese men and Chinese people frequently consume tea, providing a possible explanation for the 10% of patients who developed hypokalemia during gossypol trials. Also, permanent infertility could potentially be a manageable side effect by limiting the use of gossypol to patients who are ready to accept the consequences or have already established families as an alternative to a vasectomy.

The anticancer activity of gossypol has also gained a big interest in the past several decades. A preliminary study investigated the effects of an increasing dosage of gossypol on 34 patients with advanced cancer (cancer that has spread to other places in the body and usually cannot be cured or controlled with treatment). The resulting emesis was the dose-limiting adverse effect in most patients (Stein *et al.*, 1992). A clinical trial conducted on 21 patients with metastatic adrenal cancer revealed that oral gossypol given in doses of 30–70 mg/day was able to induce a tumor response (induce tumor size). All of these patients had little or no response to previous treatments. Three of the eighteen patients who finished the study over the course of 6 weeks showed a $\geq$50% decrease in tumor volume (Flack *et al.*, 1993). Patients' side effects included xerostomia, transient transaminitis, dry skin, fatigue, intermittent nausea, vomiting, transient ileus, and minor hair thinning, yet none of the 18 patients had to withdraw due to these side effects. There was no mortality observed due to administration of gossypol. Also, the highest dosage of gossypol that patients could tolerate was found to be 0.8 mg/kg body weight/day. Another group of 27 patients with progressive or concurrent glial tumors that had already undergone radiation therapy were administered 10 mg gossypol orally twice a day. Two patients exhibited a partial response, one of which lasted 78 weeks. Development of mild thrombocytopenia, hypokalemia, grade 2 hepatic toxicity, and peripheral edema occurred (Bushunow *et al.*, 1999). Twenty women with refractory metastatic breast cancer were involved in a phase I/II study in which each was given a dose of 30–50 mg/day oral gossypol. A minor response was observed in one patient and two patients exhibited >50% reduction in serum tumor markers. Adverse effects included nausea, fatigue, emesis, altered taste sensation, and diarrhea. Dermatologic toxicity limited the dosage, and the maximum tolerable dosage was established as 40 mg/day (Van Poznak *et al.*, 2001). Table 6.5 lists the percentage of patients with the most noted side effects in gossypol clinical trials.

A study conducted on one patient with chronic lymphocytic leukemia, in which malignant immunologically incompetent lymphocytes

TABLE 6.5 Percentage of patients with most noted side effects in gossypol clinical trials

Side effect	Adrenal cancer patients (%) (Flack *et al.*, 1993)	Glioma patients (%) (Bushunow *et al.*, 1999)	Breast cancer patients (%) (Van Poznak *et al.*, 2001)
Xerostemia	93	3.7	—
Transient transaminitis	93	—	—
Fatigue	64	—	15
Nausea	36	—	35
Emesis	21	—	20
Transient ileus	21	—	—
Hypokalemia	—	33.3	—
Thrombocytopenia	—	7.4	5
Hepatic toxicity	—	33.3	—

accumulate, proposed that the detoxified gossypol found in fresh bovine milk decreased the lymphocyte count over a period of 5 years (Politzer, 2008). Derivates, such as apogossypol, have been shown to have similar antitumor activity with less toxicity (Hu *et al.*, 2008). Perhaps future clinical trials may utilize gossypol derivates that have comparable antitumor activity with less toxicity.

VIII. CONCLUSIONS

Gossypol is a polyphenolic aldehydic compound, and it has been studied for its versatile biological activities. Gossypol's biological activities are based on direct chemical reactions, the inhibition of enzymes, and the regulation of signal transduction pathways. However, due to its toxicity, the application of gossypol is sometimes limited.

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