Chemistry of Natural Compounds, Vol. 44, No. 4, 2008

MEMBRANE-ACTIVE PROPERTIES AND ANTIRADICAL ACTIVITY OF GOSSYPOL AND ITS DERIVATIVES

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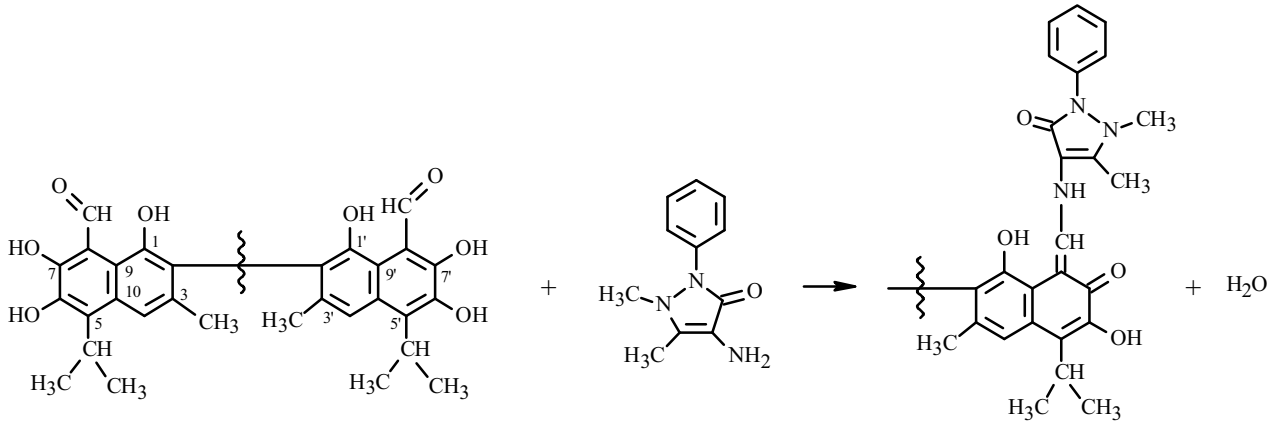
UDC 577.1:577.352.34

A novel asymmetric gossypol derivative was synthesized. The antioxidant activity of gossypol and certain of its derivatives at the aldehyde groups and the interaction of these compounds with model membranes were studied. It was shown that the antiradical and membrane activities of the gossypol derivatives were determined by the structure of the substituent and that gossypol and its derivatives were partially localized in the lipid bilayer and possibly induced formation of a new interdigitating phase.

Key words: gossypol and its derivatives, antiradical activity, bilayer lipid membrane, membrane activity, DSC.

Gossypol [2,2′-bis-(1,6,7-trihydroxy-3-methyl-5-isopropyl-8-formyl)-naphthalene] (**1**) is well known as an antifertility agent [1]. It also exhibits antiviral, anti-inflammatory, antioxidant, and other activities [2]. The toxicity of **1** prevents its broad application [2]. Previously only symmetric Schiff bases have been prepared using its aldehydes [3]. However, semi-apogossypol that was synthesized previously [4] exhibited higher antifertility activity than gossypol itself. This sparked interest in the synthesis of asymmetric derivatives of **1** [5, 6] so that several similar compounds were prepared. Thus, monoamino derivatives of **1** with antipsoriasis activity were produced by partially hydrolyzing the symmetric derivatives [6]. It seemed interesting to synthesize other monoamino derivatives, to study their chemical structure and properties, and to find structure—activity relationships.

Specific receptors in the cellular membrane through which a certain signal within the cell can be induced have not yet been found for gossypol and its derivatives. There is justification for thinking that the target of their action is the membrane lipid matrix. Therefore, an important task is to determine the probable localization of gossypol and its derivatives in model systems and biomembranes and to find the structural disruptions induced by them in the lipid matrix.



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 0009-3130/08/4404-0440

TABLE 1. Effective Constants of DPPH Reduction by Studied Polyphenols Calculated by Nonlinear Regression

Parameter	1	2	3	4	5
A_{un}	0.21±0.01	0.57±0.01	0.94±0.01	0.64±0.01	0.55±0.02
A_{re}	0.78±0.02	0.41±0.03	0.06±0.01	0.34±0.02	0.41±0.03
k1, min^{-1}	2.62±0.31	2.05±0.34	2.54±0.31	2.29±0.31	1.16±0.21

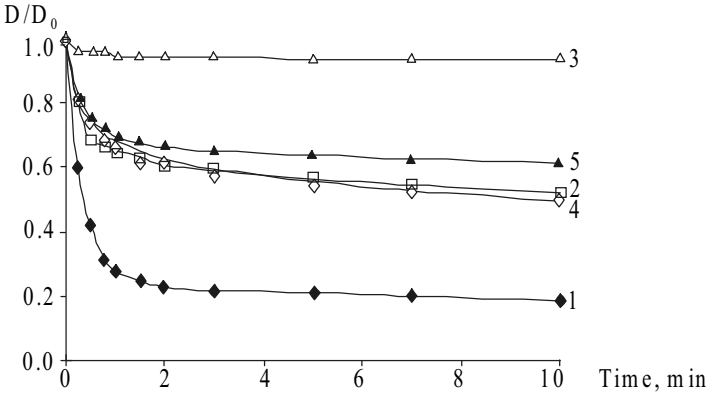

Fig. 1. Change of relative optical density of DPPH solution in ethanol upon adding gossypol and its derivatives. DPPH concentration, 0.1 mM; concentration of polyphenolic compounds, 0.01 mM.

Herein asymmetric amino derivatives of gossypol are synthesized. These include semiragosin 1,1′,6,6′,7-pentahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7′-oxo-8-formyl-8′-methin-[4″-imino-(1″-phenyl-2″,3″-dimethyl-5″-pyrazolone)]-2,2′-dinaphthalene (**2**) by the reaction of gossypol and 4-aminoantipyrine (see below) in order to compare it with the symmetric analog ragosin 1,1′,6,6′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7,7′-dioxo-8,8′-dimethin-[4″,4‴-diimino-1″,1‴-diphenyl-2″,2‴,3″,3‴-tetramethyl-5″,5‴-dipyrazolone)]-2,2′-dinaphthalene (**3**), which is known to be an effective hepatic protector.

4-Aminoantipyrine added to only one aldehyde of gossypol upon mixing the reagents in a 1:1 molar ratio at low temperature. This prevented rapid reaction of both aldehydes of gossypol with the amino compound. The relatively low solubility of the desired product in $CHCl_3$ at such a low temperature enabled precipitation to be used successfully to separate them with subsequent filtration and recrystallization of the precipitate.

We studied the antiradical activity and the reaction mechanism of **1**, **2**, and **3** and two symmetric gossypol derivatives, namely diaminogossypol [2,2′-bis-(1,6-hydroxy-3-methyl-7-oxo-5-isopropyl-8-methinamino)-naphthalene] (**4**) and dianhydrogossypol [6,6′-dihydroxy-5,5′-diisopropyl-3,3′-dimethyl-7,7′-dioxo-bis(3*H*-naphtho[1,8-*bc*]furan-3-one] (**5**), with artificial membranes by differential scanning calorimetry (DSC) and established the possible localization of these polyphenols in the lipid bilayer.

Uncontrolled peroxide oxidation of lipids occurring in the lipid matrix of biomembranes can cause irreversible destructive changes that thereby initiate cell death [7]. The antiradical activity (ARA) of compounds is frequently related to their antioxidant properties [8]. In order to estimate the ARA, we used a method based on the ability of antioxidants to reduce the stable radical 2,2-diphenyl-1-picrylhydrazide (DPPH) during measurement of the recombination kinetics of the preparations with it [9]. The color of an alcohol solution of DPPH changed upon adding the studied polyphenols. This corresponded to conversion of DPPH to the nonradical form. Figure 1 shows the kinetics of the change of optical density of the DPPH solution upon adding the studied preparations. The path of the experimental points was modeled with high accuracy by the sum of two exponential curves. This suggested that rapid and slow DPPH reduction phases were present. For simplification, the experimental curve was approximated by a single exponent, the path of which agreed rather accurately with the experimental points of the rapid DPPH reduction phase. The first-order constant of the chemical reaction can act as a criterion of the antiradical effectiveness of the studied preparations. Table 1 gives the constants of the antiradical reaction for the studied compounds.

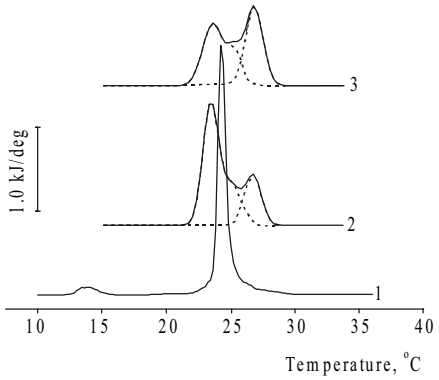

Fig. 2. Melting thermograms of multilamellar dispersions formed from dimyristoylphosphatidylcholine (DMPC) (0.3 mM): control (1), with added gossypol ($C_{\mathbf{1}}/C_{lip}$ = 1:100) (2), ($C_{\mathbf{1}}/C_{lip}$ = 1:50) (3). Results of the third scanning are given. Dashed lines correspond to deconvolution of the experimental curve into Gaussian curves.

Parameter A_{un} in the first-order reaction defines the mole fraction of DPPH that was not reduced during the reaction with the polyphenols during the experiment. Parameter A_{re} is a measure of that fraction of DPPH molecules that reacted with the studied compounds with rate constant k1. Based on Table 1, the ARA of these compounds can be placed in the following order: **1** > **2** ≈ **5** > **4** >> **3**. It is known that the two hydroxyls in the 7- and 7′-positions are most mobile in **1** [10]. The ARA of **1** is determined mainly by just these hydroxyls. There is only one of these groups in **2** and no hydroxyls in these positions for **3**. Apparently this explains the lack of ARA for **3**. Compound **5** is unstable. It is partially converted into **1** on dissolution in alcohol because of hydration [11]. This explained its ARA. Protons on N atoms are apparently responsible for ARA in **4**. Thus, the ARA of gossypol derivatives can vary over wide ranges and is determined by the structure of the substituent.

The kinetic curves showed that most DPPH was reduced in the first 2-3 min. Then the reduction proceeded slower. Apparently both direct reactions of the studied preparations with DPPH to form inactive products (first-order kinetics) and reactions connected with the ability of DPPH to form intermediate donor—acceptor complexes that react with new DPPH molecules (second-order kinetics) occurred in this instance [9].

Peroxide oxidation of lipids developed as chain reactions in the lipid phase of the biomembranes and lipoproteins. The initial (and possibly intermediate) stages of this complicated system of reactions occurrred in the aqueous phase [12]. It seemed logical to study the ARA not only in an isotropic micro-environment (alcohol, water) but also in anisotropic media, of which the lipid matrix of biomembranes is one. Similar investigations were performed for **1** and several of its derivatives [13]. However, the important question of the possible localization of **1** and its derivatives in bilayer lipid membranes, where their antioxidant activity can be manifested, remained unanswered. Furthermore, being rather bulky molecules, they should induce structural disruptions in the initial packing of lipids in the bilayer systems. Characterizing the disruptions induced by the polyphenols is also a critical task for explaining the molecular reaction mechanisms of polyphenols and biomembranes. We established by studying the ARA of the polyphenols that gossypol is the best reductant of the free valence of DPPH. This is probably due to the presence of the OH groups in it. Therefore, any chemical modification at these groups changes the ARA of the derivatives and can affect the nature of their interaction with lipid bilayers.

We used differential scanning microcalorimetry (DSC) and multilamellar dispersions of dimyristoylphosphatidylcholine (DMPC), which is a synthetic analog of lecithin, the basic structural component of the lipid matrix of most biomembranes, in order to characterize the reaction of the studied compounds with lipid systems.

The melting thermogram of multilamellar dispersions of DMPC showed a narrow peak with a maximum at 24.2°C, called the temperature of the principal gel—liquid crystal phase transition, and a smaller peak of a pretransition at 13.8°C (Fig. 2). The half-width of the peak characterizes the cooperativity of the melting process. The area under the curve is proportional to the enthalpy change or, in other words, to the thermal effect of this process [14].

The melting thermogram changed considerably upon adding **1** to the multilamellar dispersions of DMPC. The nature and degree of the changes depended on the concentration ratio of **1** and the lipid ($C_{\mathbf{1}}/C_{lip}$) (Fig. 2). The peak of the pretransition disappeared.

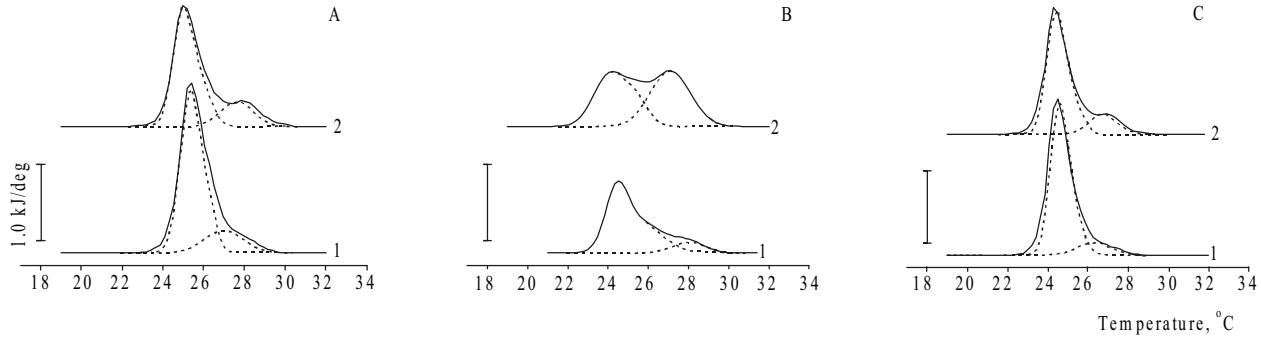

Fig. 3. Melting thermograms of multilamellar dispersions formed from DMPC (0.3 mM) upon adding gossypol derivatives at various lipid:derivative concentration ratios: semiragosin (C_2/C_{lip} = 1:100, 1; C_2/C_{lip} = 1:50, 2) (A), anhydrogossypol (C_5/C_{lip} = 1:100, C_5/C_{lip} = 1:50) (B), ragosin (C_3/C_{lip} = 1:50) and diaminogossypol (C_4/C_{lip} = 1:50) (C). Results of the third scanning are given. Dashed lines correspond to deconvolution of the experimental curve into Gaussian curves.

A high-temperature shoulder appeared on the phase-transition curve. The high-temperature shoulder appeared as a separate melting peak upon repeated scanning. Considering that the phase transition obeys a Gaussian distribution law, the experimental curve should deconvolute into several curves, the sum of which should give the initial melting thermogram. The solid lines in Figs. 2 and 3 correspond to the experimental curves; the dashed lines, deconvolution into Gaussian curves. Such deconvolution can reveal separate lipid phases and determine their thermodynamic parameters.

The greatest changes in the thermograms of multilamellar bilayers were observed upon adding **1** and **5**, which caused a substantial broadening of the melting peak and separation of the high-temperature phase. Adding **3** had practically no effect on the melting thermogram of the lipid. Adding **2** gave a more significant change than for **3**, changing the shape of the melting curve and giving a more distinct high-temperature shoulder. Increasing the concentrations of the studied compounds produced more substantial changes in the melting thermograms (Fig. 3). Thus, adding **1** and its derivatives **2**, **4**, and **5** to multilamellar dispersions of DMPC caused the structure of the lipid bilayer to become heterogeneous. At least two types of lipid clusters (low- and high-temperature) were present in it.

The low-temperature asymmetric peak could be viewed as the phase transition of lipids of DMPC that were reacting with the polyphenols and were perturbed by them. The high-temperature peak had parameters consistent with an interdigitated phase (IDP). Hydrocarbon chains of DMPC molecules located in different monolayers of the bilayer typically interpenetrate each other (in the middle of the bilayer) in this phase [15, 16]. The packing density in this portion is higher than in the portion where the hydrocarbon chains are located along the phospholipid profile above or below the bilayer outside the IDP level. Consequently, a higher transition energy is needed for the IDP from the gel state into the liquid-crystalline one. This may explain the appearance of the high-temperature shoulder on the melting thermogram. According to the literature, the IDP can be formed in the bilayer upon adding membrane-active compounds that satisfy certain requirements. First, the reacting molecules in the lipid bilayer should be localized at the level of the membrane—water interphase boundary [16]. Second, its nonpolar part should penetrate into the region of the phospholipid acyl chains to a level not more than the fifth C atom [16]. For this, the ability to induce an IDP does not depend on the presence or absence of charge on the membrane-active molecule [17].

It is known that **1** is an amphiphile with the nonpolar part associated with the naphthalene and isopropyl groups and the polar parts, the carbonyl and hydroxyls. Most probably **1** is incorporated into the lipid region by the hydrophobic naphthalene rings. The partial penetration of **1** and certain of its derivatives is interpreted most correctely in light of data obtained using EPR spin markers [13]. The fraction of lipid in the IDP increased in our experiments with each repeated scan of the same sample. If the molecules of the studied compounds had penetrated fully into the hydrophobic region, then their complicated three-dimensional structure should have produced a more substantial perturbation of the initial packing of the lipid molecules. In turn, this would exclude part of the lipids from the phase transition. In this instance the number of polyphenols incorporated into the depth of the hydrophobic region should increase with each new scan. Thus, the total enthalpy and the temperature of the principal phase transition would decrease considerably. However, the temperature of the principal phase transition in our experiments shifted weakly either to lower temperature or to higher temperature. The increase in the fraction of IDP after each scan produced an increase in the overall enthalpy of the phase transition because more energy was required for IDP melting [18]. The change of half-width for samples containing polyphenols compared with the control was due to

incorporation of membrane-active molecules into the hydrophobic region. This decreased the size of cooperative clusters of lipids that were unaffected by the polyphenols [14].

The effect of **5** on the thermodynamic properties of the multilamellar phospholipid dispersions was comparable with that of **1** (Fig. 3). This was explained by its instability in aqueous solution, which was mentioned above. Compound **5** in all probability converted to **1** during the recording of the melting curves. Thus, a high-temperature phase similar to that induced by **1** appeared on the experimental thermograms. Molecule **3** was the most hydrophobic of all studied compounds and had identical substituents on both aldehydes, 4-aminoantipyrine. These substituents have steric bulk comparable with that of the naphthalene rings in **1**. Considerable energy is required to incorporate such a bulky molecule into the hydrophobic region of the lipid bilayer because large voids that require significant bending of the phospholipid acyl chains to fill them are formed in the interchain space of the lipids upon its incorporation.

According to the experimental results, **3** caused the high-temperature phase to appear slightly (Fig. 3). Therefore, **3** interacted weakly with the lipid bilayer and was practically not incorporated into the hydrocarbon interchain space of the lipid bilayer. Compared with **3**, **2** has as a substituent only one 4-aminoantipyrine group on one of the naphthalene rings whereas the other part of the molecule has the same structure as **1**. The experimental results suggest that **2** was incorporated to a certain depth of the hydrophobic region of the lipid bilayer, most likely that part which was free of the bulky substituent and induced formation of the high-temperature IDP (Fig. 3). Compound **4** was the most polar of all the studied derivatives. Therefore, **4** apparently reacted more effectively with the polar part of the membrane bilayer without significant penetration into the hydrophobic region of the bilayer.

The reaction of **1** and its derivatives with the lipid bilayer occurs due to Van-der-Waals forces. The bilayer in the gel phase was not destabilized through formation of an IDP. Polar parts of the membrane-active molecules were responsible for the localization of the polyphenols at the interphase membrane boundary in the gel phase whereas nonpolar parts were incorporated into the interchain space of the phospholipid acyl chains. Such incorporation should form voids between lipid hydrocarbon chains. The formation energy of such voids is rather high so that they should be eliminated [18]. The voids can be removed through cooperative formation of bends in lipid acyl chains or through formation of an IDP [18]. The IDP formation process is more preferred and requires less energy than the other cooperative structural changes.

Thus, the proposed localization of gossypol and its derivatives at the polar interphase boundary of the lipid bilayer and the partial incorporation of their nonpolar parts into the hydrophobic region of the membrane enabled them to recapture effectively free radicals and to break the chain of peroxide lipid oxidation.

EXPERIMENTAL

Synthesis of Asymmetric Amino Derivative of Gossypol 1,1′,6,6′,7-Pentahydroxy-5,5′-diisopropyl-3,3′-dimethyl-7′-oxo-8-formyl-8′-methine-[4″-imino-(1″-phenyl-2″,3″-dimethyl-5″-pyrazolone)]-2,2′-dinaphthalene (semiragosin). A mixture of gossypol (104 mg, 0.0002 mol) and 4-aminoantipyrine (39 mg, 0.0002 mol) was dissolved in $CHCl_3$ (5 mL) and stored at -15°C for 3 d. The course of the reaction was monitored by TLC (on Silufol UV 254 plates with elution by $CHCl_3$:EtOAc, 9:1). The resulting precipitate (R_f 0.5) was filtered off and recrystallized from acetone (mp 200-202°C). Yield 43%. The contents of gossypol and the diamino derivative in the reaction mixture were 30 and 27%, respectively.

PMR spectrum (100 MHz, $CDCl_3$): 16.24 (1H, br.s, –NH), 15.25 (1H, s, OH-7), 11.12 (1H, s, –CHO), 10.98 (1H, br.s, ketoimine proton =CH–N–), 7.73-7.75 (5H, m, $–C_6H_5$ substituent), 7.71* (1H, s, H-4), 7.62* (1H, s, H-4′), 3.84 (1H, m, isopropyl $–^5C–CH$), 3.11 (3H, s, $N–CH_3$ substituent), 2.45 (3H, s, $C–CH_3$ substituent), 2.16* (3H, s, $^3C–CH_3$), 2.11* (3H, s, $^3C–CH_3′$), 1.59** (6H, s, isopropyl CH_3), 1.52** (6H, s, isopropyl $CH_3′$). Portons OH-1, OH-1′ and OH-6, OH-6′ could not be identified individually because of proton exchange.

Compounds **3-5** were synthesized as before [19-20].

Optical Method of Studying Antioxidant Activity. The ARA of the preparations was determined as before [9].

Microcalorimetric Measurements. Samples for DSC studies were prepared by drying a solution of DMPC (Sigma, USA) and the polyphenols in a rotary evaporator and then forming multilamellar bilayers in Tris-HCl buffer (10 mM, pH 7.5) by the literature method [21]. The resulting suspension was placed in the differential adiabatic scaning microcalorimeter chamber (DASM-4, Russia) and cooled to 5°C. Then the thermogram was recorded at a rate of 1°C/min. The enthalpies of fusion of the lipids were determined from the area under the peak by comparison with the corresponding thermal standards of

the instrument. The accuracy of the transition enthalpy determination was at most 8%. Transition temperatures were determined from the position of the maxima of the corresponding peaks to an accuracy up to 0.05°C. Deconvolution of asymmetric melting peaks into Gaussian components was performed using an algorithm developed by us for adjusting experimental curves to the baseline using the programs EXEL and ORIGIN.

ACKNOWLEDGMENT

The research was performed with financial support of Project P181 of the foundation STSU/ARS USDA.

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