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FREE-RADICAL GOSSYPOL DERIVATIVES FOR COTTON *Verticillium* WILT

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The ability to bind reversibly molecular oxygen was established for a free-radical product of gossypol oxidation. Conversion of dianhydrogossypol into the stable biradical dioxodianhydrogossypol was viewed as the reason for interruption of the gossypol redox conversion cycle in the extracellular milieu of cotton steles.

Key words: cotton, phytoalexins, oxidation, free radicals, interruption of metabolism cycling.

Modern concepts of the stability of biological species to oxidative stress are based on cyclic reactions of metabolites that involve the formation of free radicals [1-4]. Disruption of the cycling of such metabolites leads to the destruction of biological ultrastructures. In enzymatic metabolism, the cycling is due to an "intrinsic mathematical necessity," the single variable that describes it being time. In this approach, the milieu is ignored [5]. Spatial and temporal nonuniformities in the concentration distribution of extracellular metabolites of fungus and cotton and in phase transitions in cotton steles are common. Such a system can be described by multifactorial analysis.

Our goal was to determine the mechanism of formation and the actual structures of free radicals capable of affecting the stability of host—parasite systems.

One reason for selecting gossypol as the research topic was the fact that it is a sterically hindered polyphenol. Gossypol is the principal antioxidant of cotton and is susceptible to redox conversions. However, it is extremely sensitive to changes in the milieu [6]. Sterically hindered polyphenols are typically oxidized to stable biradicals [7]. The stability of biradicals of sterically hindered polyphenols excludes them from cyclic conversion processes. Therefore, they can be products outside the cycle. Oxidation of gossypol has not been studied from this viewpoint. The formation of radicals via oxidation of phenoxyls was first proposed after observing paramagnetic absorption in polycrystalline gossypol samples [8]. Further development of chemical EPR spectroscopy found that the spectroscopic splitting g-factors in phenoxyl free radicals are concentrated in the narrow range from 2.004 to 2.005 [1]. High levels of UHF power were previously used to record signals because of the low sensitivity of the instrumentation [8]. The EPR spectrum is described as the superposition of a narrow singlet on a broad singlet. The difference in the g-factors of these signals was not determined because they were assumed to be identical.

With the limited UHF power of the modern EPR spectrometer, we observed that the broad singlet in oxidized gossypol samples is resolved as a doublet with a distance of 1.15 mT between components and a g-factor of 2.0030; for the overlapping singlet, 2.0032. Figure 1 shows the EPR spectrum of an oxidized gossypol sample at different UHF power levels.

The intensity ratio of the doublet and singlet vary from sample to sample, which explains the variation in the EPR spectra of oxidized gossypol samples [8-10]. Furthermore, we observed complete reversibility for the temperature effect on the EPR spectrum recorded for the oxidized gossypol samples and noted that the solvent in which the spectra were recorded had a significant effect on its shape. The combination of total reversibility for the temperature changes and their dependence on the properties of the milieu are characteristic for EPR spectra of stable biradicals [11, 12]. The small difference found by us (in the fourth sign) of the g-factors for the singlet and doublet is typical for biradical pairs and the monoradicals corresponding to them [11].

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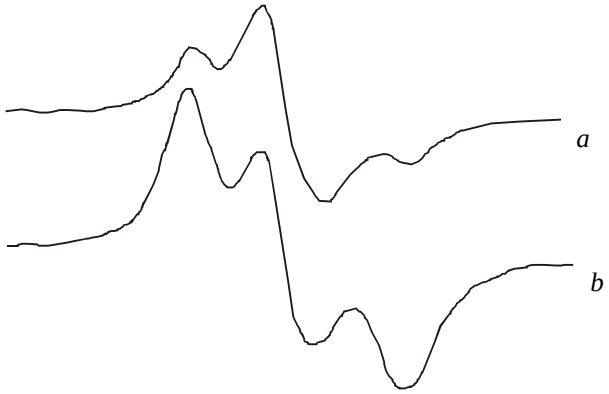

Fig. 1. EPR spectrum of an oxidized gossypol sample at UHF power levels 5 (a) and 20 μW (b).

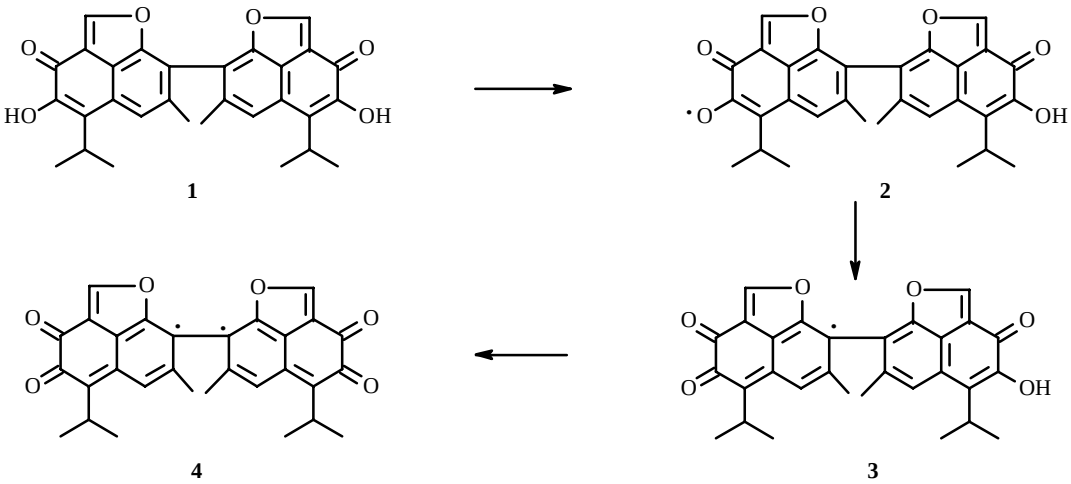

Fig. 2. Oxidation scheme of dianhydrogossypol.

The g-factors measured by us for the observed doublet and singlet do not fall within the range of values given above for phenoxyl radicals. Phenoxyl radicals of polyphenols are unstable in solutions [12] although in our instance the EPR spectra of solutions of oxidized gossypol samples in various media remain unchanged for a prolonged storage period. Apparently primary phenoxyl radicals of starting gossypol are oxidized further at the hydroxyls remaining in them and disappear through intramolecular oxidative coupling. The product of these processes is diamagnetic dianhydrogossypol **1** (DAG, Fig. 2), the appearance of which can be observed in the PMR spectra [6] by dissolving gossypol in ethylacetate at 20°C in the presence of air. These facts indicate that only oxidation of DAG gives a stable EPR spectrum.

The first stage of DAG oxidation should give phenoxyl monoradical **2** which, in contrast with primary phenoxyl radicals of gossypol, can be stabilized by resonance through rapid transfer into the quinoid form **3**. Oxidation of **3** at the next hydroxyl in an analogous manner leads to a biradical with the dioxodianhydrogossypol structure **4**. The persistence of steric hindrance from bulky substituents in the positions α to the central bond, which create atropoisomerism in gossypol and its derivatives, prohibit the rings in **4** from lying in one plane. Formation of a central double bond by the unpaired electrons in biradical **4** requires overlap of orbitals, the axes of which are orthogonal to the planes of the binaphthyl rings and, because these rings are not coplanar, are located in different planes and do not overlap. Therefore, hindrances to inner rotation in **4** ensures that the lifetime of the resulting biradical will be indeterminately long both in a solid matrix of starting gossypol and in solutions.

The structures of **4** and **3**, a monooxodianhydrogossypol, are consistent with the presence in the mass spectrum of oxidized gossypol of impurity peaks with *m*/*z* 480 and 481, which do not appear in the mass spectrum of starting pure gossypol [13]. Coupling between the unpaired electron spins in **4** appear as a splitting of their signal into the aforementioned doublet. The singlet corresponds to monodioxodianhydrogossypol. The magnitude of the splitting in the doublet depends on the ratio

of the integrals of direct and exchange coupling between the unpaired electrons. This makes it sensitive to the dielectric characteristics of the milieu. Assembly of biradicals into chains is predicted for concentrated solutions of organic stable biradicals in view of their triplet ground state [12]. This is reflected in the dominance of the monoradical singlet owing to the spatial separation of the unpaired electrons at the chain ends. The literature describes just such a strong singlet [8]. However, it was mistakenly assigned to a charge-transfer complex between gossypol and oxygen. The mentioned assembly provides experimental confirmation of the triplet ground state for the unpaired electrons in dioxodianhydrogossypol. A theoretical calculation of the state of the unpaired electrons in **4** performed using the integrated program set HyperChem (Hypercube) confirms that their ground state in **4** is a triplet.

Heating diluted solutions of oxidized gossypol in vegetable oils up to 40°C produces reversibly in the EPR spectrum side components on the doublet, as expected from the theory of EPR spectra of biradicals [12] for a change in the modulation rate of interelectron coupling by intramolecular rotation. At 80°C, all spectral components exhibit fine structure as a result of line narrowing caused by decreased solubility of O_2 at 80°C and simultaneous loss of paramagnetism in O_2 [14]. At 10°C, reversible disappearance of the EPR spectrum is observed owing to spin exchange in collisions of O_2 with dioxodianhydrogossypol. The decomposition of these complexes at elevated temperature is accompanied by restoration of the EPR spectrum and the appearance of active forms of O_2 (AFO).

Pure piperidine was added to a cooled (10°C) solution of oxidized gossypol in castor oil and the solution was heated to 30°C for 30 min to confirm the presence of AFO. An elongated EPR spectrum of N-oxypiperidine was superimposed on the signal of the biradicals (**4**) in the resulting EPR spectrum, which proved the appearance of AFO upon decomposition of the complexes. The covalent bond in the peroxide cleaves because of Brownian motion of increased energy. Therefore it should be accompanied by interconversion of spins in the leaving O_2. Obviously, O_2 will occur in the singlet state upon decomposition of this complex. This increases its reactivity and leads to the development of an oxidative chain reaction.

The appearance of AFO during decomposition of dioxodinahydrogossypol complexes is important for plants. The first symptoms of *Verticillium* infection, the appearance of necrotic zones in cotton leaves, are usually ascribed to large variations in night and day temperatures. A source of AFO in the necrotic zones could be peroxidic and hydroperoxidic forms of dioxodianhydrogossypol that decompose during temperature inversions.

DAG in aqueous acetone is reduced to starting gossypol whereas DAG is stable in anhydrous acetone [6]. Therefore, lipid deposits in plant vessels with inclusions of gossypol glands should be considered to act as a "depot" for formation of biradicals **4** in view of their hydrophobicity. However, gossypol samples in which an impurity of plant lipids was observed in the PMR spectra (signals at 0.8 and 1.2 ppm) are oxidized in light by atmospheric O_2 and then give an EPR signal like the aforementioned superposition of a singlet on a doublet. Obviously, it is important that the AFO formed by light have a lifetime long enough to oxidize gossypol. In lipids, the lifetime is one of the longest [14]. Changing O_2 to an oxidant such as diphenylpicrylhydrazyl (DPPH) does not require illumination. Adding DPPH to gossypol solutions in benzene and in oils results in formation of dioxodianhydrogossypol and reduction of DPPH. Both processes occur at 20°C.

Thus, the differences in the milieu conditions can be considered to be a result of: a) oxidation of gossypol to DAG; b) reduction of DAG to starting gossypol; and c) oxidation of DAG to mono- and biradicals.

Considering that gossypol, like other polyphenols, is synthesized by the plant only as water-soluble glycosides, the distribution of gossypol throughout the plant results in the decomposition of extracellular glycosides if the milieu pH shifts into the acidic region (pH < 7). Oxidative stress caused by a pathogen introduced into the plant creates these conditions as a result of decreased energy exchange in the plant. This is confirmed by quantitative data for the determination of the free gossypol content in various plant parts before and after infection with the pathogenic strain *V. dahliae* [16]. At pH ≤ 6.5, lipophilic gossypol is poorly soluble in water. Therefore, the bulk of liberated gossypol settles in lipid deposits in the plant vessels. The water—lipid phase boundary can be viewed as a region where redox transformations of gossypol can occur. Lipophilic oxidants such as AFO and juglone, which is produced by *V. dahliae*, can oxidize gossypol whereas contact of the resulting DAG with water leads to its reduction to starting gossypol.

Spatial and temporal inhomogeneities in the milieu within the plant satisfies the first requirement for participation of metabolites in cyclic transformations within the plant, namely, both formation and disappearance of a metabolite should be independent of each other and spontaneous [4]. The stability of biradical **4** in various media leads to irreversible exclusion of gossypol from the redox cycle, where the expended energy is thermal in all transformations. The exclusion of gossypol from the cycle owing to its oxidation to stable dioxodianhydrogossypol leads to a loss of information that is necessary for plant functioning. These properties appear over a wide gossypol concentration range and are fundamentally attributed to it.

Therefore, gossypol satisfies the requirements [4] for low-molecular-weight metabolites for participation in cyclic transformations. Persistence of the cycle of reversible gossypol transformations can cause plants to ripen with all signs of fungus colonization. This is observed in practical cotton production. This can explain the absence of absolutely pathogenic races of *V. dahliae* and the lack of cotton varieties that are absolutely resistant to wilt.

EXPERIMENTAL

Pharmacopoeiac gossypol was recrystallized from acetone. The resulting yellow polycrystalline powders gave no EPR spectrum at the maximum instrument (ER-200D-SRC) power at working frequency 9.8 GHz.

Oxidation of Gossypol. A suspension of gossypol polycrystals in CCl_4 was left in Petri dishes in contact with air at 15°C for 2 d until CCl_4 was completely evaporated. This solvent was selected as a milieu in which the AFO lifetime is much longer than in other organic solvents [14]. Samples of oxidized gossypol were powders with a brownish tint. Their solutions in pharmacopoeiac castor oil (known to have no paramagnetic impurities) were used to record EPR spectra at various temperatures. EPR spectra were recorded at slow scan rates under conditions far from signal saturation.

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REFERENCES

1. Ya. I. Azhipa, *Medical-Biological Aspects of Electron Paramagnetic Resonance* [in Russian], Nauka, Moscow (1983).
2. R. D. Stipanovic, M. E. Mace, M. H. Elissalde, and A. A. Bell, *ACS Symp. Ser.*, No. 449, "Naturally Occuring Pest Bioregulators," Am. Chem. Soc., Washington, DC (1991), p. 336.
3. V. P. Reutov and E. G. Sorokina, "Magnetic Resonance in Chemistry and Biology," April 20-27, 2001, Zvenigorod, Russia (2001), p. 53.
4. M. Eigen and P. Schuster, *The Hypercycle. A Principle of Natural Self-Organisation*, Springer-Verlag, Berlin (1979).
5. B. C. Goodvin, *Analytical Physiology of Cells and Developing Organisms*, Academic Press, London (1976).
6. N. D. Abdullaev, A. A. Tyshchenko, I. P. Nazarova, N. T. Ul′chenko, M. R. Yagudaev, and A. I. Glushenkova, *Khim. Prir. Soedin.*, 166 (1990).
7. D. Barton and D. Ollis, eds., *Comprehensive Organic Chemistry, The Synthesis and Reactions of Organic Compounds*, Pergamon Press, New York (1979), Vol. 1.
8. A. S. Sadykov, A. P. Ibragimov, and A. Ismailov, *Dokl. Akad. Nauk Uzb. SSR*, No. 11, 43 (1966).
9. A. Sultanov, A. Ibragimov, Sh. Aripdzhanov, A. I. Ismailov, and A. S. Sadykov, *Uzb. Biol. Zh.*, No. 2, 3 (1967).
10. A. de Peyster and Y. Y. Wang, *Mutat. Res.*, 297, No. 3, 293 (1993).
11. A. L. Buchachenko, in: *Free-Radical States in Chemistry* [in Russian], L. A. Blumenfel′d and Yu. N. Molin, eds., Nauka, Novosibirsk (1972), p. 87.
12. V. N. Parmon, A. I. Kokorin, and G. M. Zhidomirov, *Stable Biradicals* [in Russian], Nauka, Moscow (1980).
13. R. R. Razakov, M. M. Dolmatov, and A. K. Kosimov, *Research in Organic and Bioorganic Chemistry* [in Russian], Universitet, Tashkent (1992), p. 71.
14. S. D. Razumovskii, *Oxygen, Elemental Forms and Properties* [in Russian], Khimiya, Moscow (1979).
15. W. Adam, K. Hanneman, and R. M. Wilson, *J. Am. Chem. Soc.*, **108**, 929 (1986).
16. G. Ya. Gubanov, *Cotton Wilt* [in Russian], Kolos, Moscow (1974).