

## FREE RADICAL OF THE SEMIQUINONE TYPE GENERATED IN THE REDOX REACTION OF HYDROXYBENZOTROPOLONE

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Participation of hydroxybenzotropolones in the free radical processes may be relevant to the transformation of environmentally important organic residues to humus substances. To elucidate this possibility we autooxidized PPG = 2,3,4,6-tetrahydroxy-5H-benzocyclohepten-5-on with O<sub>2</sub> in Na<sub>2</sub>CO<sub>3</sub> solution. An intermediate stable radical of the semiquinone type SQ<sup>•</sup> revealed the hyperfine structure and a strong absorption band in the spectral region. The influence of reducing agent (ascorbate), stabilizer of the EPR signal (Zn<sup>2+</sup>) and spin trap (DMPO) on SQ<sup>•</sup> EPR spectra was measured. The obtained results suggest that the PPG SQ<sup>•</sup> may undergo several processes such as dismutation, polymerization and addition. These processes are leading to humus-like dark macromolecular paramagnetic products probably containing tropolone moieties. Their physicochemical and physiological properties have not been yet carefully studied.

### INTRODUCTION

The molecule of purpurogallin (PPG = 2,3,4,6-tetrahydroxy-5H-benzocyclohepten-5-on) contains the extraordinary tropolone 7-C-element aromatic ring condensed with the 1,2,3-trihydroxybenzene ring (pyrogallol) with the hydrogen bonds between C=O and –OH groups. The structure of the PPG, hydroxybenzotropolone-type molecule is shown in Fig. 1. In the 7-C ring the amount of delocalized electrons = 6, therefore the Hückel rule 4n+2 for this structure is fulfilled.

PPG reveals relatively strong absorption, diamagnetism and microbiological resistance (Sławiński, 1971; Sławińska, Lichszeld & Michalska, 1978; Sławiński, Szczodrowska & Włodarczyk-Graetzer, 1973). The latest research has shown that PPG may be a particularly promising antioxidant scavenging reactive oxygen species (ROS)

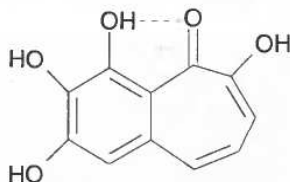


Fig. 1. The structure of purpurogallin (PPG=2,3,4,6-tetrahydroxy-5H-benzocyclohepten-5-on)

generated by polymorphonuclear leukocytes (Prasad, Kapoor & Lee, 1994) and by three types of human cardiovascular cells (Wu, Zeng, Wu, Fung, Weisel, Hempel & Camerman, 1996). PPG occurs as a purple pigment in galls (*Cecidia, Galla*) on the leaves of *Quercus pendunculata*, *Salix fragilis*, *Ciprinus betulus* and other plants (e.g. in certain barks in the form of glucosides). It plays also the role of an active cytoprotector (Rootman, Bindish, Zeng, Hasany & Wu, 1994), effectively chelates ferrous (II) ions and suppresses the formation of the OH<sup>•</sup> radical in the Fenton reaction (Wu *et al.*, 1996). PPG oxidized enzymatically or in an alkaline solution exhibits relatively strong chemiluminescence and a deep-blue stable free radical of the semiquinone type (Sławiński, 1971; Sławińska *et al.*, 1978; Sławiński *et al.*, 1973; Nilsson, 1964). Recently it has been shown that the 3,4,6-trihydroxy-5H-benzocyclohepten-5-one radical plays a dominant role in the transformation of theaflavins and their physiological activity (Jovanovic, Yuki-hiko, Steen & Simic, 1997). It has been reported that PPG and its analogues formed from polyphenols can be precursors of the most complex and ubiquitous compounds in the biosphere, humic acids (HA) and melanin-like polymers in nature (Sławiński, 1971; Sławińska *et al.*, 1978; Sławiński *et al.*, 1973; Nilsson, 1964; Sławińska, Sławiński & Sarna, 1975; Schulten, 1995; Senesi & Miano, 1994; Lipski, Manikowski, Skwarek &

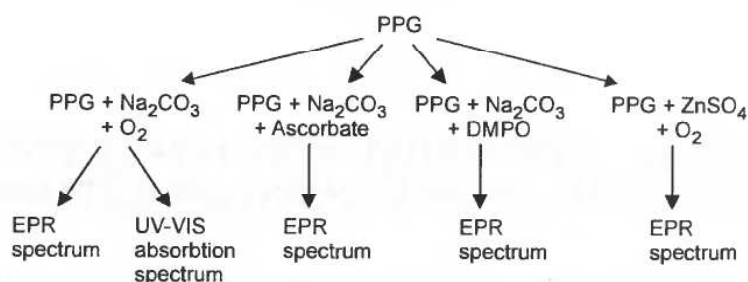
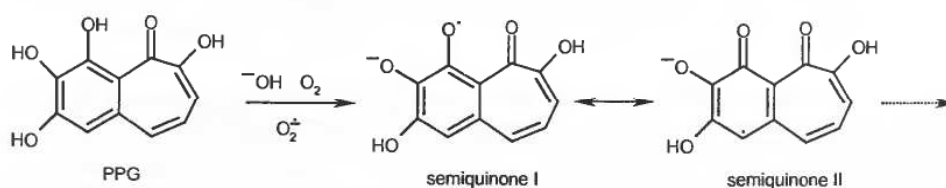


Fig. 2. Scheme of experiment

Fig. 3. Reaction of  $\text{SQ}^\cdot$ 

Sławiński, 2000; Gwoździński, Lipski & Sławiński, 2000; Sławińska & Sławiński, 1967)).

## MATERIALS AND METHODS

The oxidation process of PPG alkaline solution (PPG +  $\text{Na}_2\text{CO}_3$  1:1, [5 mM]) in the presence of atmospheric oxygen ( $[\text{O}_2] = 0.7$  mM) to macromolecular humus substances was performed. The influence of reducing agent (ascorbate), stabilizer of the EPR signal ( $\text{Zn}^{2+}$ ), and a spin trap (DMPO) was measured.

A technical grade commercial PPG (Aldrich P-5590-2, 15 g) was crystallized from diethyl ether solution. The reducing agent ascorbate (puriss.) and a spin trap DMPO (puriss.) were from Sigma, and stabilizer  $\text{ZnSO}_4$  (puriss.) was from POCh, Gliwice.

The experiment was performed according to the scheme (Fig. 2).

Aliquots of the PPG solution were analyzed by EPR spectroscopy (Bruker 300E with microwave resonator 9105TM274) and UV-VIS spectrophotometry (Jasco V-530).

## RESULTS AND DISCUSSION

### Electron paramagnetic resonance

To investigate the PPG oxidation reaction four experiments using EPR technique have been performed.

(i) In the first experiment the formation of PPG-semiquinone free radical  $\text{SQ}^\cdot$  during the oxidation process has been investigated. The reaction equation and structures of  $\text{SQ}^\cdot$  are shown on Fig. 3.

The EPR spectrum of PPG-semiquinone formed during the reaction of PPG with  $\text{Na}_2\text{CO}_3$  and  $\text{O}_2$  (5 mM), 4 min after mixing, was measured. The intermediate stable radical (Fig. 4) of the semiquinone type  $\text{SQ}^\cdot$  revealed the hyperfine structure ( $B = 336.5\text{--}334$  mT,  $g = 2.0033\text{--}2.0044$ ). The  $g$  giromagnetic splitting factor was calculated according to the basic equation – resonance condition:

$$h\nu = g\mu_B B$$

where:  $h$  – Planck's constant,  $\nu$  – microwave fixed frequency,  $g$  – giromagnetic splitting factor,  $\mu_B$  – Bohr's magneton,  $B$  – magnetic field intensity =  $334.0\text{--}336.5$  mT

For comparison, value of free electron's giromagnetic split factor is 2.0023. The difference with the obtained value for  $\text{SQ}^\cdot$  is the result of influence of electron magnetic orbital angular momentum on  $\text{SQ}^\cdot$ .

It should be noted that all protons in PPG are not-equivalent. There are protons H-C at various positions connected directly to the ring, and protons H-O-C connected indirectly, through oxygen atoms. In addition, the hydrogen bonds between H atoms connected indirectly (through oxygen) to 7- and 6-C-rings and carbonyl O=C group was observed (Sławiński *et al.*, 1973). Thus, the protons in PPG molecule could not be ascribed to any

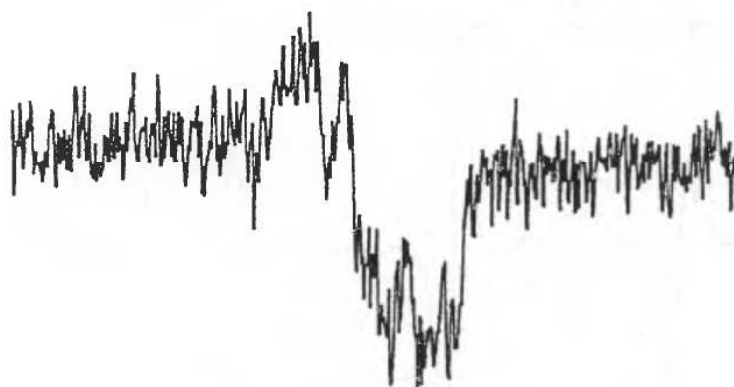


Fig. 4. EPR spectrum of PPG-semiquinone formed during the reaction of PPG with  $O_2$  in 0.01 M  $Na_2CO_3$  solution, 4 min after mixing (5 mM). Range: 336.5–334 mT. Gain:  $2 \times 10^4$ . Time const.: 20.48 ms, modulation amplitude: 1.013 G



Fig. 5. EPR spectrum of a PPG +  $Na_2CO_3$  + Ascorbate 1:1:1 mixture (3.3 mM) in the presence of the  $O_2$ . A typical doublet of ascorbyl radical. Range: 336.5–334 mT. Gain:  $2 \times 10^4$ . Time constant: 20.48 ms, modulation amplitude: 1.013 G

particular line intensity in EPR spectrum. This makes interpretation of the PPG EPR spectra exceptionally difficult.

The spectrum shape and its parameters reminds that of HA ( $B = 336.5\text{--}334$  mT,  $g = 2.0039 \pm 0.0002$ ) (Sławińska, Sławiński & Sarna, 1975; Lipski, Manikowski & Sławiński, 1997; Senesi & Miano, 1994; Cheshire, Bedrock, McPhail & Fraser, 1997).

The obtained results suggest that the PPG  $SQ^\bullet$  may participate in several processes such as dismutation, polymerization and addition. These reactions lead probably to humus-like dark macromolecular paramagnetic products (Sławińska *et al.*, 1978; Sławińska *et al.*, 1975; Sławińska *et al.*, 1975; Lipski *et al.*, 1997; Senesi & Miano, 1994; Cheshire *et al.*, 1997).

(ii) In the second experiment the influence of reducing agent, ascorbate ion (Asc) has been investigated.

Thus, PPG +  $Na_2CO_3$  + Ascorbate ( $pK_1 = 4.17$ ,  $pK_2 = 11.57$  for ascorbic acid (*The Merck Index*, 1998)) were mixed at a 1:1:1 ration (3.3 mM, in the presence of  $O_2$ ). We propose the following scheme of this redox reaction, that proceeds *via* free-radical mechanism:



Therefore, according to the above mentioned scheme, the typical doublet of ascorbyl radical has been obtained (Fig. 5). It means that ascorbate have stronger reductive properties and more negative redox potential (0.166 V at pH 4 (*The Merck Index*, 1998)) than PPG. This is also confirmed by

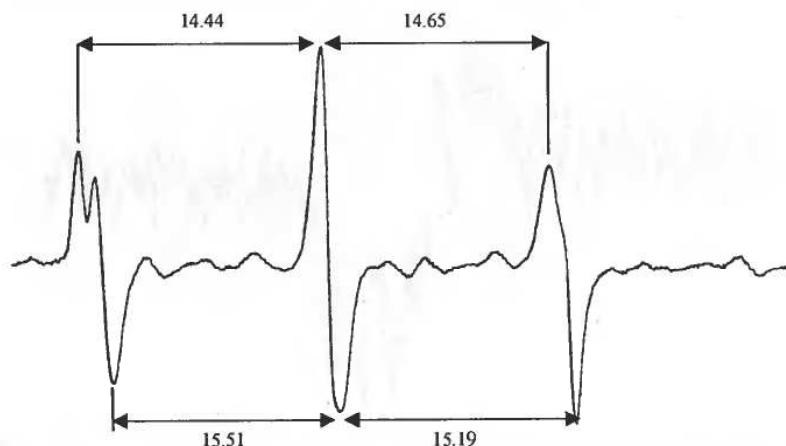


Fig. 6. EPR spectrum of PPG +  $\text{Na}_2\text{CO}_3$  + DMPO 1:1:1 mixture (3.3 mM) in the presence of the  $\text{O}_2$ . Hyperfine splitting constants are given. Range: 336.5–334 mT. Gain:  $2 \times 10^4$ . Time constant: 20.48 ms, modulation amplitude: 1.013 G

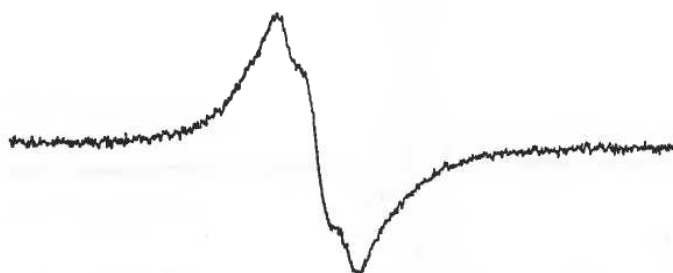


Fig. 7. EPR spectrum of PPG +  $\text{ZnSO}_4$  [+  $\text{O}_2$ ] 1:1 mixture (3.3 mM) after 20 min. The sample turns black and shows a very strong EPR signal typical of humic acids and melanins. Range: 336.5–334 mT. Gain:  $2 \times 10^4$ . Time constant: 20.48 ms, modulation amplitude: 1.013 G

of the reaction mixture, color changes from blue to yellow.

(iii) One of the most popular spin traps, DMPO, has also been used in the reaction: PPG +  $\text{Na}_2\text{CO}_3$  + DMPO +  $\text{O}_2$  1:1:1 (3.3 mM, in the presence of the  $\text{O}_2$ ).

It has been found that C-centered free radical has been formed (Fig. 6).

(iv) The specific EPR signal amplifier (signal stabilizer) for melanins,  $\text{ZnSO}_4$ , has been used. The obtained EPR spectrum of PPG +  $\text{ZnSO}_4$  1:1 (3.3 mM, in the presence of the  $\text{O}_2$ ) after 20 min of reaction initiation is shown on Fig. 7. It is evident that a humus-like substance is formed.

#### UV-VIS absorption

Absorption spectrum of PPG in neutral solution ( $\text{EtOH} : \text{H}_2\text{O} = 1:10$  v/v,  $[\text{PPG}] = 0.1$  mM) shows maxima at 245 nm ( $A = 1.2$ ) and at 319 nm (strongest maximum,  $A = 2.35$ ) (Fig. 8 —). The

origin of the strongest maximum at the wavelength of 319 nm can be explained as follows. Due to the conjugation of the electrons from the  $\text{C}=\text{O}$  chromophore with the  $\pi$ -delocalized electrons from the tropolone and benzene aromatic rings the bathochromic shift takes place, and absorption at this wavelength indicates a  $\pi \rightarrow \pi^*$  transition in  $\text{C}=\text{O}$  chromophore. Without conjugation with the  $\pi$ -delocalized electrons,  $\pi \rightarrow \pi^*$  transition in  $\text{C}=\text{O}$  occurs at 250 nm.

PPG autooxidized in the alkaline solutions (0.1 mM PPG in 0.1 mM  $\text{Na}_2\text{CO}_3$ ,  $\text{EtOH} : \text{H}_2\text{O} = 1:10$  v/v) formed the deep-blue anion of PPG $^-$  semiquinone-type stable free radical  $\text{SQ}^-$  (Fig. 8 ----), strongly absorbing at 612 nm (Sławiński, 1971; Sławińska *et al.*, 1978; Sławiński *et al.*, 1973; Sławiński *et al.*, 1975; Lipski *et al.*, 2000; Gwoździński *et al.*, 2000). In this step, the OH-groups of the phenolic ring of PPG undergo oxidation, leading to the  $\text{SQ}^-$  intermediate.

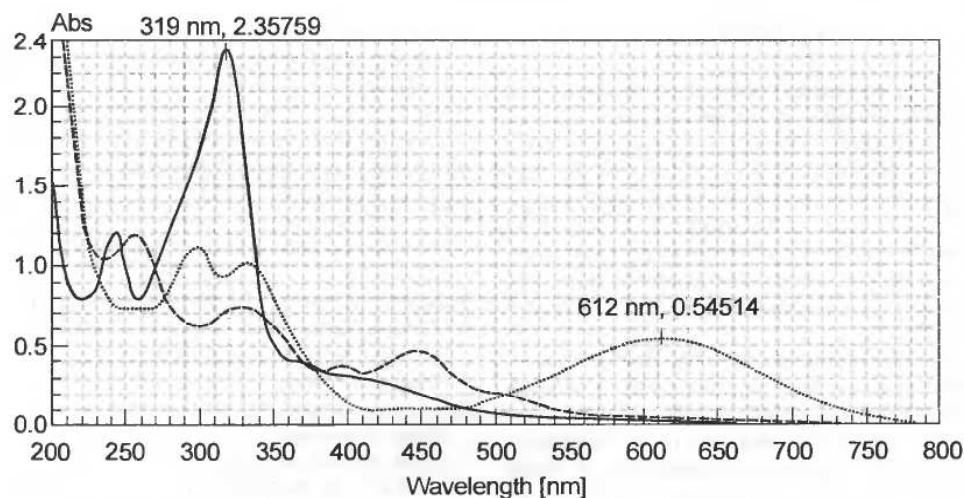


Fig. 8. Absorption spectrum of purpurogallin (PPG) in EtOH : H<sub>2</sub>O = 1:10 v/v solution [PPG] = 0.1 mM (—). PPG + 0.7 mM O<sub>2</sub> + 0.1 mM Na<sub>2</sub>CO<sub>3</sub> solution 3 min after the redox reaction initiation. The absorption maximum at 612 nm corresponds to the deep-blue PPG-semiquinones (.....). Absorption spectrum 60 min after the reaction initiation, resembling that of humic acids or melanins (---)

Absorption spectrum of the reaction solution, 60 min after the reaction initiation, has an almost monotonic shape (with few shoulders), resembling that of humic acids or melanins (Lipski, Sławiński & Zych, 1999; Auger & Richard, 1996; Senesi & Miano, 1994; Lipski, Sławiński, Manikowski & Górski, 1997; Górski, Lipski, Sławińska & Sławiński, 1997; Sławińska & Sławiński, 1967), is shown in Fig. 6. It is known that absorption in the range 250–385 nm is characteristic for carbonyl derivatives of tropolone (Sławiński, 1971; Sławińska *et al.*, 1978; Sławiński *et al.*, 1973).

It should be noted that a distinct isosbestic point at 378 nm ( $A = 0.35$ ) was obtained. The total absorbance value at this wavelength consists of at least absorbances of the substrate PPG, deep-blue SQ<sup>•</sup> and products HA.

The oxidation process of PPG, leading to humus-like polymers, proceeds via several steps. This seems to be confirmed by the fact that several types of absorbing species are formed in the course of PPG oxidation, as described above.

## CONCLUSIONS

Results of above experiments lead to the following conclusions: multi-step scheme of PPG redox conversions to HA proceeds *via* a free-radical mechanism. The formation of PPG-semiquinone free radical is accompanied by color changes, from

yellow to blue. When the reaction commences, the HA is formed and the color changes to brown. These color changes are visible in the UV-VIS absorption spectrum (Fig. 8). Thus, the proposed scheme is given as follows:

Stage I. PPG → SQ<sup>•</sup>

Stage II.

1<sup>st</sup> possibility SQ<sup>•</sup> + SQ<sup>•</sup> (recombination) → dimer (SQ-SQ), without HA formation

2<sup>nd</sup> possibility SQ<sup>•</sup> + SQ<sup>•</sup> (dismutation) → PPG + quinone PPG

Stage III. After stage II p. 2. further polymerization is possible:

Quinone PPG → → → humic acids (probably PPG polymers)

The ecological role of these conversions is not clear. Due to role SQ<sup>•</sup> radicals in the transformation and physiological activity of theaflavins these reactions of PPG may have an important influence on the human health. However, the exact mechanisms and possible ecological role of this process remains to be elucidated.

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## REFERENCES

- Auger J. & Richard C. (1996). Reactive species produced on irradiation at 365 nm of aqueous solutions of humic acids. *J. Photochem. Photobiol. A: Chem.*, **93**, 193–198.
- Cheshire M., Bedrock C., McPhail D. & Fraser A. (1997). The origin of free radicals in humic acid: increase in the free radical content of straw during humification. *The Role of Humic Substances in the Ecosystems and in Environmental Protection*, Wrocław, pp. 309–314.
- Głębska J. & Gwoździński K. (1998). Rodniki semichinonowe jako produkty pośrednie reakcji w układach biologicznych. *Curr. Topics Biophys.*, **22**, 27–55.
- Górski Z., Lipski M., Sławińska D. & Sławiński J. (1997). UV-C induced photodegradation of humic acids, [In:] *XVIIIth International Conference on Photochemistry*. Warszawa, pp. 3P37.
- Gwoździński K., Lipski M. & Sławiński J. (2000). Free Radical of semiquinone type generated in the redox reaction of hydroxybenzotropolone. *5th Symposium Free Radicals in Biology and Medicine*, Łódź, pp. 136–137.
- Jovanovic S., Yukihiko H., Steen S. & Simic M. (1997). Antioxidant potential of theaflavins. *J. Am. Chem. Soc.*, **119**, 5337–5343.
- Lipski M., Manikowski H. & Sławiński J. (1997). Changes in the EPR signal of UV-C-irradiated humic acids. *Application of Magnetic Resonance in Chemistry and Related Areas*, Warszawa, pp. C-10.
- Lipski M., Manikowski H., Skwarek R. & Sławiński J. (2000). Fluorescence, UV-Vis absorption and EPR spectra of purpurogallin during oxidation. *International Conference on Photobiophysics in Technology and Medicine*, Poznań, pp. P16.
- Lipski M., Sławiński J. & Zych D. (1999). Changes in the luminescent properties of humic acids induced by UV-radiation. *J. Fluoresc.*, **9**, 133–138.
- Lipski M., Sławiński J., Manikowski H. & Górski Z. (1997). Chemiluminescence and spectroscopic properties of oxidated humic acids. *Eur. J. Clin. Chem. Clin. Biochem.*, **9**, A 87.
- Nilsson R. (1964). On the mechanism of HRP-catalyzed reaction studied by means of CL. *Acta Chem. Scand.*, **19**, 389–401.
- Prasad K., Kapoor R. & Lee P. (1994). Purpurogallin, a scavenger of polymorphonuclear leukocyte-derived oxyradicals. *Mol. Cell. Biochem.*, **139**, 27–32.
- Rootman D., Bindish R., Zeng L., Hasany S. & Wu T. (1994). Purpurogallin as a cytoprotector of cultured rabbit corneal endothelium. *J. Ophthalmol.*, **29**, 220–223.
- Schulten H. R. (1995). The three-dimensional structure of humic substances and soil organic matter studied by computational analytical chemistry. *Fres. J. Anal. Chem.*, **351**, 62–73.
- Senesi N. & Miano T. (1994) *Humic Substances in the Global Environment and Implications on Human Health*, Elsevier, Amsterdam.
- Sławińska D. & Sławiński J. (1967). Chemiluminescence of humic acids. *Nature*, **213**, 902–903.
- Sławińska D., Lichtszeld K. & Michalska T. (1978). Singlet oxygen and chemiluminescence in autooxidation of pyrogallol. *Pol. J. Chem.*, **52**, 1729–1740.
- Sławińska D., Sławiński J. & Sarna T. (1975). Photoinduced luminescence and EPR – signals of polyphenol and quinone polymers. *Photochem. Photobiol.*, **21**, 393–396.
- Sławińska D., Sławiński J. & Sarna T. (1975). The effect of light on the ESR spectra of humic acids. *Soil Sci.*, **26**, 127–131.
- Sławiński J. (1971). Chemiluminescence in the process of oxidative ring-opening of purpurogallinoquinones. *Photochem. Photobiol.*, **13**, 489–497.
- Sławiński J., Szczodrowska B. & Włodarczyk-Graetzer M. (1973). Studies on the chemiluminescence of the system: purpurogalline-peroxidase-hydrogen peroxide. *Acta Biochim. Pol.*, **20**(2), 119–132.
- The Merck Index* (1998), Chapman & Hall.
- Wu T., Zeng L., Wu J., Fung K., Weisel R., Hempel A. & Camerman N. (1996). Molecular structure and antioxidant specificity of purpurogallin in three types of human cardiovascular cells. *Biochem. Pharmacol.*, **52**, 1073–1080.