NEW ASPECTS OF THE PREPARATION AND
CONTROL OF JUGLONE

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The presence of juglone (5-hydroxy-1,4-naphthoquinone) in a culture of the actinomycete Streptoverticillium hiroshimense, *strain 34, is demonstrated for the first time. A method is proposed for determining the content of derivatives of 1,4-naphthoquinone in the culture medium using EPR spectroscopy.*

Key words: juglone, actinomycete, EPR.

Juglone (5-hydroxy-1,4-naphthoquinone) is used in various applications from medicine to metallurgy [1, 2]. We observed that the seeds of Tashkent-1 cotton variety that are treated with weak solutions of juglone (10^{-3} M) under field conditions differ from the controls in having an increased germination and an added harvest of 3 hundred-weight/hectare. However, the sharply increased cost of juglone in the past decade, which is caused by the expanded application of it as a preservative for bakery products and beverages, prevents wide application of juglone for preplanting treatment of seeds. Industrial syntheses of juglone are known [2]. However, the purification of the final product from side products of the catalytic synthesis increases the cost of pure juglone by two orders of magnitude.

The range of the principal and until recently only source of pure juglone, higher plants of the Juglandaceae family, is limited. Reports [3] of the discovery of juglone sources among lower plants, lower fungi, hold promise that demands for pure juglone can be satisfied. Our task was to demonstrate that actinomycetes represent a new source of juglone without harmful synthetic impurities and that EPR can be used for operative control of the content of 1,4-naphthoquinone derivatives in a growing culture of a microorganism.

Like other polyphenols, free juglone is not synthesized by cells owing to its cytotoxicity [4]. The biosynthesis was found to produce the glycoside form of polyphenols, which is not toxic to cells. The intracellular pH level is usually above 7. This ensures that the nontoxic glycoside forms of polyphenols is retained [5]. Maintenance of the intracellular basic pH level is an energy dependent active metabolic process [5], the disruption of which by external factors leads to the appearance of free aglycones, polyphenols. The isolation of juglone by the methods developed for plant material [2] requires acidification of the starting material. Our attempt to isolate juglone from a culture of the actinomycete *Streptoverticillium hiroshimense*, strain 34, at the native pH of the culture medium was unsuccessful. The juglone yield by the literature method [2] was 1.6 mg/l only after preliminary acidification of the culture with dilute HCl on the 15th day of growth. The growth of a culture of strain 34 was completely suppressed by adding juglone at a concentration of 1 mg/l. This apparent contradiction means that the biosynthesis of juglone in a culture of *S. hiroshimense*, strain 34, occurs with the formation of a nontoxic glycoside form that is unstable in acidic medium.

Significant expenditures of the starting material and time are required to select the optimal conditions and periods for accumulating juglone in a culture of the investigated actinomycete by the method used. Therefore, we attempted to use the high sensitivity (10^{-12} mol/cm^3) of electron paramagnetic resonance (EPR) to estimate the content of semiquinone radicals in basic medium in mini-samples of the culture medium owing to the ability of juglone as a quinone to form them [6]. Treatment of small quantities of the acidified culture medium with extractants and further operations with the extracts produces fractions with R_f 0.78 that contain juglone without destroying the culture growth dynamics. Dissolution of them in a fixed small volume of
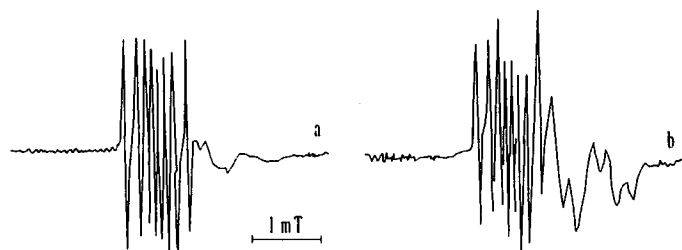
Fig. 1. EPR spectra of a basic solution of the fraction with R_f 0.78 at pH 8.5 (a) and 9.0 (b).

aqueous base produces an EPR signal, the calibration of which by a known amount of juglone enables its content at any growth stage to be quantitatively determined.

It has been reported [6] that the primary semiquinone of juglone converts at increased solution pH to a secondary product that we detected by EPR on a spectrometer operating in the 3-cm range. The parameters of the recorded spectra are given below.

Interpretation of the primary EPR spectra of a basic aqueous solution of juglone at pH 7.2 is difficult [6] owing to the rapid intra- and intermolecular proton exchange that gives rise to additional, besides hyperfine, signal splitting. Increasing the solution pH slows the proton exchange. At pH ~8, the shape of the spectra of the nascent secondary radicals reflects the hyperfine interactions between the unpaired electron and only the ring protons whereas the hydroxyl protons contribute only to line broadening. The signal of the secondary semiquinones as a triplet of triplets indicates that the unpaired electron interacts with two proximal (a_1 = 13 mT) and two distal (a_2 = 4 mT) protons. This may correspond with the structure of the semiquinone product of juglone hydroxylation, namely, 2-hydroxyjuglone.

The triplet of triplets in the EPR changes to a triplet of doublets after replacing ordinary water H_2O by heavy water D_2O at pD ~ 8. This is consistent with the replacement of one of the distal (relative to the unpaired electron in the semiquinone of 2-hydroxyjuglone) protons by deuterium. Much data obtained by us indicates that this proton belongs to the C-3 carbon of the naphthalene framework. At pH ~ 9, the triplet of triplets disappears and eight lines appear, the center of which shifts to weak field compared with the center of the previous triplet of triplets (Fig. 1).

The change in the position of the center (g-value) is consistent with a new structure for the semiquinone. The outer lines of the new spectrum are stronger than the inner lines. Such a combination of amplitudes is encountered only in the EPR spectra of biradicals [7]. This fact forces us to hypothesize that the semiquinone of 2-hydroxyjuglone dimerizes at the C-3 position at pH ~ 9. The remaining three ring protons are split into eight lines owing to their magnetic nonequivalency.

The coupling of the unpaired electrons in the two symmetric halves of the semiquinone 2-hydroxyjuglone dimer is modulated by rotation of these halves by less than 180 owing to steric hindrances from the bulky substituents in the α-positions relative to the central bond. The theory of EPR spectra of biradicals [7] predicts that amplitudes of the outer lines in the spectrum of the biradical will deviate from those of the inner lines at certain ratios of the rates of intramolecular motion to the rate of electron spin exchange [6, 7].

Screening of alkaline solutions of the fractions with R_f 0.78 from the S. hiroshimense culture, strain 34, on an EPR spectrometer showed that the maximum juglone content in the culture medium occurs on the 15th day of growth on peptone under steady-state conditions.

The fraction with R_f 0.78 is not present in all growth stages of S. hiroshimense, strain 51. The dominating fraction with R_f 0.62 from this strain in alkaline solution at pH 7.2 gives a spectrum with two groups of six lines each. The large distance between the centers of these groups suggests that it does not originate from the spin—spin coupling constant in semiquinones [6, 7].

Screening of the EPR spectra of various juglone derivatives revealed an analogous spectrum in alkaline solution (pH 7.2) of flaviolin (2,7-dihydroxyjuglone). Flaviolin is known to dimerize in solutions to form biflaviolin [2, 3]. Further transformations of the spectra of biflaviolin and the fraction with R_f 0.62 completely coincide. This suggests that the fraction with R_f 0.62 represents flaviolin. The PMR spectra of the fraction with R_f 0.62 and flaviolin confirm that they are identical. The signal for the proton on C-3 in both spectra [6.8 ppm in $(CD_3)_2CO$ solution] disappears with dilution by D_2O. This indicates that it undergoes proton exchange in acetone. This indirectly confirms our hypothesis that the proton on C-3 of 2-

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hydroxyjuglone is involved in proton exchange in alkaline solution at pH ~ 8.

The EPR spectrum of flaviolin at pH ~ 8 in alkaline solution also changes. The distance between the furthest groups of lines, with four lines in each group, is less than the distance between the central groups of lines. Obviously the existence of several degrees of freedom for the intramolecular motion in the semiquinone of biflaviolin causes these splittings in the EPR spectra.

The EPR spectrum of biflaviolin semiquinone at pH ~ 9 simplifies into a doublet. However, the alternation of the width and strength of the lines persists. In heavy water the distance between the lines of the doublet decreases by six times. This indicates that the proton is replaced by deuterium.

Obviously the dimerization of 2-hydroxyjuglone at pH ~ 9 prevents the production of flaviolin by a non-enzyme path. This hypothesis is confirmed by the fact that strain 34, which synthesizes juglone, does not produce flaviolin whereas strain 51, which does produce flaviolin, does not contain juglone. The site of blocking in both instances is probably determined genetically by the set of corresponding enzymes.

Thus, the type of 1,4-naphthoquinone produced in the mini-samples from the culture medium can be unambiguously determined from the EPR spectra of their semiquinone forms.

EXPERIMENTAL

A culture of *S. hiroshimense*, strain 34, was grown in rocking flasks at 26-30 C in the following medium (g/l): glucose, 20.0; NaCl, 0.5; $KMnO_4$, 0.5; K_2HPO_4, 0.5; peptone, 10.0; pH, 7.0. The culture medium (5 l) was separated from the mycelium on a double paper filter, acidified with dilute HCl (1:1 by volume) to pH 4, and thrice extracted with ethylacetate using 300 ml of solvent for 1 l of culture medium. The extracts were combined and dried over anhydrous Na_2SO_4. The solvent was removed in a rotary evaporator (RI-1M) at reduced pressure. The solid was fractionated by preparative TLC on glass plates with silica gel Hemapol 5/40 + 13% gypsum using $(C_2H_5)_2O$—C_6H_6—HCO_2H (50:50:1 by vol.). The band with R_f 0.78 was purified from the plates, placed on a 1-cm column, and eluted with ethylether. The ether was removed in a rotary evaporator. The dry solid was recrystallized from petroleum ether (70-100 C). Yield, 3 mg of reddish-orange juglone crystals, mp 154-156 C.

UV spectrum ($CHCl_3$, λ_{max}, nm): 250, 431, 413, 335 (log ε 4.15, 3.57 sh, 3.56, 3.08); mass spectrum (electron impact, 80 eV), m/z (I_{rel}, %): 174 (M+, 100), 173 (M - 1, 19), 146 (M - CO, 10), 92 (M - 2CO - C_2H_2, 28), 63 (M - 2CO - C_2H_2 - HCO, 30), 44 (M - 1 - 2CO - C_2H_2 - HCO - H_2O, 28). ^{13}C NMR [25.15 MHz, $(CH_3)_2CO$]: 190.2 (s, C-4), 184.0 (s, C-1), 161.4 (s, C-5), 139.5 (d, C-2), 138.5 (d, C-3), 136.4 (d, C-7), 131.8 (s, C-9), 124.3 (d, C-6), 119.0 (d, C-8), 114.9 (s, C-10).

The reported chemical shifts are practically the same as those obtained previously for samples of synthetic [1] and natural [3] juglone.

The volume of the mini-samples was 10 ml.

The EPR spectra were recorded on a Bruker ER 200D/SRC EPR spectrometer far from saturating the signal. NMR spectra were recorded on a Varian XL-100/15 spectrometer at 100 MHz with continuous generation (PMR) and pulses (^{13}C NMR). UV spectra were recorded on a Beckman M-25 spectrophotometer.

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