SYNTHESIS AND STRUCTURE OF GOSSYPOL BISDIETHYLAMINOPHENYLIMINES AND THEIR ANTIOXIDANT AND ANTICHOLINESTERASE ACTIVITIES

E. N. Mukhamedzhanova, D. N. Dalimov,
Z. Tilvabaev, V. B. Shneivais,
F. G. Kamaev, L. Biktimirov, and
A. I. Ismailov

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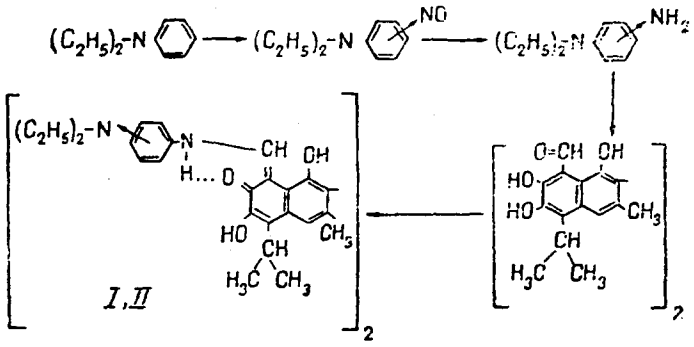
The synthesis has been performed of gossypol gis-p- and -m-diethylaminophenylimines, which possess anticholinesterase activity, antioxidant action on rat brain synaptosome membranes, and an innibiting influence on the oxidase system of zymosan-activated macrophages.

The antitumoral and antiviral activity of gossypol revealed previously [1, 2] has served as a basis for the synthesis of new gossypol derivatives with the aim of imparting to the modified molecules new properties connected with their anticholinesterase activity[3]. It was established at the same time that gossypol derivatives are effective inhibitors of free-radical processes [4].

The gossypol derivatives were synthesized by the following scheme:



The PMR spectrum of gossypol bis-p-diethylaminophenylimine (I) exhibited the signals of gossypol itself: a doublet of methyl protons of the isopropyl fragment was located at 1.46 ppm; a singlet at 2.10 ppm corresponded to an aromatic methyl group; a multiplet at 3.60 ppm corresponded to the methine proton of an isopropyl radical; and the aromatic H_a proton was represented by a singlet at 7.47 ppm. The presence of a doublet at 9.98 ppm and of a broad signal at 14.32 ppm, belonging to exomethylene and H-protons [sic], respectively, indicated the existence of the gossypol bis-p-diethylaminophenylimine molecule in the keto-amine form [5]. When D_2O was added or the H-proton was irradiated with a powerful second high-frequency field (total resonance), the signal of the exomethylene proton was converted into a singlet.

The signals of the other protons were located in the following way: the signals of the protons of the para-substituted benzene ring formed two characteristic doublets at 7.11 and 6.49 ppm, and the ethyl radical caused the appearance of a quartet and a triplet at 3.25 and 1.08 ppm, respectively.

The parameters of the PMR spectrum of gossypol bis-m-diethylaminophenylimine (II) indicated that its molecule was also present in the keto-amine form.

The IR spectra of substances (I) and (II) contained peaks corresponding to the vibrations of the OH group of the gossypol moiety at 3560 cm^{-1} and of a C=N bond at 1685 and 1640 cm^{-1};

A. S. Sadykov Institute of Bioorganic Chemistry, Academy of Sciences of the Uzbek SSR, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 2, pp. 184-188, March-April, 1991. Original article submitted May 14, 1990.

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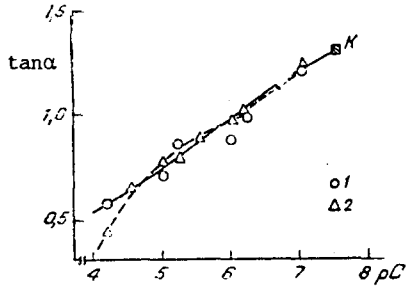

Fig. 1. Interaction of gossypol bis-p- and -m-diethylaminophenylimines ((1) and (2), respectively) with rat brain synaptosomes.

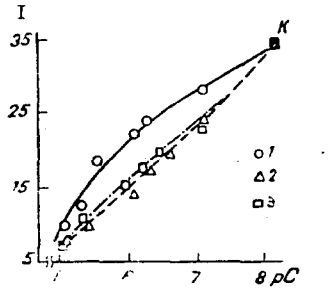

Fig. 2. Interaction of gossypol bis-p- and -m-diethylaminophenylimines ((1) and (2), respectively) and gossypol (3) with peritoneal macrophages.

TABLE 1. Anticholinesterase Efficiencies of Gossypol Bis-p- and -m-diethylaminophenylimines ((I) and (II), respectively)

Compound	ACE			BuCE		
	$p\bar{K}_i$	pK_i	pK'_i	$p\bar{K}_i$	pK_i	pK'_i
I	2,91	2,78	2,00	3,42	3,35	3,00
II	3,69	3,48	3,15	4,07	3,82	3,55

*$\bar{K}_i$, K_i, and K'_i are the generalized, competitive, and uncompetitive inhibitory constants.

consequently, these substances were subject to keto-imine tautomerism, passing from the keto into the imino form, as was also confirmed by their PMR spectra.

The optical absorption spectra of subsatnces (I) and (II) each had an absorption peak at $\lambda_{max}^{CH_3COCH_3}$ 470 nm.

The action of these substances on the level of peroxide oxidation of the lipids (POL) of rat brain synaptosomes by the method of Fe-induced chemiluminescence (CL) [6] has been studied. The rate of oxidation of the lipids in the presence of these compounds was evaluated from the change in the angle of slope of the slow flash [7].

It can be seen from Fig. 1 that, in the range of concentrations of from $1 \cdot 10^{-7}$ to $1 \cdot 10^{-4}$ M, both derivatives exhibited an antioxidant action. Up to $1 \cdot 10^{-5}$ M the p- and m- derivatives lowered the level of chemiluminescence of the synaptosomes equally, but in concentrations of from $1 \cdot 10^{-5}$ to $1 \cdot 10^{-4}$ M the gossypol bis-m-diethylaminophenylimine possessed a stronger antioxidant action. Substance (II) caused 50% inhibition of POL in the synaptosomes at a concentration of $2 \cdot 10^{-5}$ M, while the p- derivative required a concentration of $3 \cdot 10^{-5}$ M.

The influence of the substances on the activity of the oxidase systems of peritoneal macrophages was studied by the method of luminol-dependent chemiluminescence activated by opsonized zymosan [8].

A kinetic curve of the dependence of the intensity of luminesence on the amount of preparation added is given in Fig. 2. The kinetics of luminescence were recorded for 15 minutes, the peak being reached in the seventh minute and the intensity at the maximum being 35 rel. units in the absence of inhibitors. In all cases, native gossypol and its derivatives lowered the level of CL. It can be seen from Fig. 2 that gossypol and the m- derivative affected the level of luminescence similarly, while the p- derivative quenched the chemiluminescence more feebly. For gossypol and the m- derivative, 50% inhibition took place at a concentration of $6.4 \cdot 10^{-7}$ M, and for the p- derivative at $3 \cdot 10^{-6}$ M.

It can be seen from the results obtained that the antioxidant action of the p- and m-isomers of the gossypol derivatives depends on the position of substituents in the aniline ring: the m- derivative possessed a greater antioxidant activity than the p-isomer. For the m-isomer a 50% lowering of the level of luminol-dependent CL of macrophages took place at a concentration ($6.4 \cdot 10^{-7}$ M), an order of magnitude smaller than for the p-isomer ($3 \cdot 10^{-6}$ M). A comparison of the antioxidant effect sof gossypol bis-p- and -m-diethylaminophenylimines with native gossypol in macrophagic oxidation showed that antioxidant action may be enhanced or weakened with the aid of introduced functional groups.

A study of the interaction of the compounds obtained with human blood erythrocyte acetylcholinesterase (ACE) and horse-blood serum butyrylcholinesterase (BuCE) showed that both these substances were typical reversible inhibitors, since the effect of inhibition by these compounds did not depend on the time of incubation with tbe enzymes and set in immediately after the addition of the inhibitor to the reaction mixture. Both substances inhibited the activities of ACE and BuCE by the mixed type of action. Table 1 gives results obtained in a study of the influence of the gossypol derivatives on the rate of hydrolysis of acetylcholine under the action of ACE adn BuCE. As the characteristics of the reactivities of the reversible inhibitors are given the values of the inhibition constants expressed in pK_i values, which reflect the binding of the inhibitor with the enzyme. It can be seen from Table 1 that the position of the diethylamino group in the benzene ring exerts an influence on anticholinesterase activity. When this group was present in the meta-position the inhibition of ACE and BuCE was more powerful than when it as in the para-position.

It must be mentioned that in all cases, regardless of the position of the ammonium grouping, the decrease in the rate of hydrolysis under the action of BuCE was considerably greater than in the case of ACE. This may be the result of additional hydrophobic interactions taking place both through the gossypol moiety itself and through the aromatic ring. The decrease in anticholinesterase activity for gossypol bis-m-diethylaminophenylimine is apparently connected with its noncomplementarity to the active surface of ACE.

EXPERIMENTAL

The IR spectra of the substances in carbon tetrachloride solution were taken on a Specord-71-IR spectrophotometer in NaCl cells in the wavelength interval of 4600-700 cm^{-1}. PMR spectra were recorded on a XL-200 spectrometer (Varian) with a working frequency of 200 MHz. The specimens used were 5% solutions of the compounds under investigation in CCl_4 or $CdCl_3$. Chemical shifts were measured in the δ-scale relative to the internal standard HMDS. UV spectra were taken on a SF-26 spectrophotometer using 0.002% solutions in acetone.

m- and p-Diethylaminoanilines were synthesized according to [9]. The boiling point of the p-diethylaminoaniline was 115°C (3 mm), yield 1.98 g (65% of theor.). The boiling point of the m-diethylaminoaniline was 120°C (1 mm), yield 1.25 g (41% of theor.).

Synthesis of Gossypol Bis-m- and -p-diethylaminophenylimines. With heating on the water bath for 2 h, 1 g (0.0019 mole) of gossypol was dissolved in 16 ml of ethanol. To this solution was added 0.63 g (0.0038 mole) of m-diethylaminoaniline dissolved in 10 ml of ethanol. The mixture was heated at the boiling point of the solvent for 3 h and was left overnight at room temperature. The red precipitate that had deposited was filtered off and was washed first with ethanoland then with diethyl ether to eliminate unchanged gossypol. Then it was dried in a vacuum drying chest at 50-60°C. mp 182°C (decomp.), yield 0.81 g (52% of theor.) Gossypol bis-p-diethylaminophenylimine was obtained analogously. mp 186-187°C (decomp.), yield 0.87 g (56% of theor.). N_{found} 6.91%, $N_{calc.}$ 6.93%.

The apparatus for measuring chemiluminescence consisted of a low-noise photoelectron multiplier, FEU-39A, and an electomeric amplification system, with recording on the chart of a KSP-4 recording potentiometer.

The synaptosomes were obtained by the method of Gray and Whittaker [10]. Their activity was determined by Ellman's method [11]. The concentrations of protein in the biosubstrates were determined by Bradford's method [12].

Fe-induced chemiluminescence was brought about as in [7]. At room temperature, 0.5 ml of a solution of synaptosomes (3 mg of lipids, 1-2 mg of protein) was added to 8 ml of 105 mM KCl, 20 mM phosphate, buffer, pH 7.4, followed by 0.5 ml of 10 mM $KMnO_4$. The mixture was introduced into the thermostated cell of a chemiluminometer fitted with a stirrer, the shutter was opened, the recorder was switched on and the level of spontaneous CL was recorded; then 1 ml of a freshly prepared solution of $FeSO_4$ was added to the cell and the parameters of the CL were recorded for 10 min. On the recorder chart a tangent was drawn to the curve describing the slow flash and the angle formed by this line with the X-axis was determined and its tangent was obtained. The CL of the substances studied was determined in triplicate.

System of Peritoneal Macrophages. To obtain the peritoneal macrophages, we used random-bred female rats weighing 120-150 g. The rats were decapitated. Into the abdominal cavity was introduced 5 ml of medium 199 (based on Hanks' solution at pH 7.4). The abdomen was kneaded for 3 min and then it was opened up and the contents were withdrawn with the aid of a Pasteur pipette, filtered through a double layer of gauze, and centrifuged at 1200g for 10 min. The deposit was washed twice and was resuspended in medium 199 and, after the number of cells had been counted under the microscope, the concentration was brought to $5 \cdot 10^6$ cells per ml. All the manipulations in the isolation if the cells were performed at 4°C. The viability of the cells in the suspension was determined with the aid of the test with Trypan Blue and usually amounted to 90-95%. The luminol-induced chemiluminescence of the macrophages activated by opsonized zymosan was brought about as described in [8].

The enzymes used in the experiments were purified preparations of human erythrocyte ACE (EC 3.11.7) and horse-blood serum BuCE (EC 3.11.8) produced by the Perm Scientific Research Institute of Vaccines and Sera. The catalytic properties of the enzymes were determined by Ellman's colorimetric method [11] on a KFK-2 TsKhL-42 colorimeter at 25°C in 0.2 M phosphate buffer, pH 7.5. A commercial preparation of acetylcholine was used as substrate. The anti-acetylcholinesterase activities of the preparations synthesized were evaluated from the magnitude of the generalized inhibitory constant K_i, which, in the mixed type of inhibition, is connected with the competitive and uncompetitive constants K_i and K_i', respectively. To determine the values of K_i and K_i' we measured the rates of hydrolysis of acetylcholine at various concentrations of it both in the presence of the inhibitors and in their absence. The inhibition constants were calculated graphically [15] [sic] and were expressed in the form $pK_i = -\log K_i$, etc. The type of inhibition was determined from the ratio of the magnitudes pK_i and pK_i' competitive when $K_i' \to 0$; mixed when $pK_i > pK_i'$ and uncompetitive when $pK_i = pK_i'$.

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CHEMICAL COMPOSITION OF THE RHIZOMES OF THE Rhodiola rosea BY THE HPLC METHOD

A. G. Dubichev, V. A. Kurkin, G. G. Zapesochnaya, and E. D. Vorontsov

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The component composition of extracts of the rhizomes of Rhodiola rosea has been studied by the HPLC method; the amount of rosavin has been determined and its lability on the autofermention of the raw material has been established.

The chemical composition of the rhizomes of roseroot stonecrop Rhodiola rosea L. (family Crassulaceae) is represented by substances of various natures: flavolignans (rhodiolin), flavonoids (rhodionin, rhodiosin, acetylrhodalgin, 8-methylherbacetin, kaempferol, kaempferol 7-O-rhamnoside, tricin, and tricin 5-O- and 7-O-glucosides), phenolic compounds (salidroside, tyrosol, gallic acid, methyl gallate), phenylpropenoids (cinnaymyl alcohol and its glycoside rosin, rosavin, rosarin), sterols (β-sitosterol, daucosterol), and monoterpenes (rosiridin, rosidirol) [1-8].

On the basis of results of chemical investigations a number of new biologically active substances have been discovered (cinnamyl alcohol glycosides, rosiridin) [6-10], which, together with salidroside, are among the main components of the plant. On the basis of these facts, new approaches have been proposed to the problem of the standardization of the raw material and of roseroot stonecrop preparations [11, 12]. In partiuclar, procedures have been developed for the quantitative determination of salidroside [11] and of rosavin (rosavidin) [12] which have been used for the study of the dynamics of their accumulation in the rhizome during the ontogenesis of the plant [13] and also for choosing the temperature conditions for drying the raw material [14].

The aim of the present investigations was to determine the component composition of an extract of the rhizomes of roseroot stonecrop by high-performance liquid chromatography (HPLC) and to develop a method for the quantitative determination of rosavin in raw material from this plant.

In the development of a method of qualitative and quantitative analysis with the aid of reversed-phase HPLC on silica gel-C18, we made a choice of the chromatographic conditions (composition of the organic phase of the eluent, the buffer, the rate of elution, and the method of detection). The detection of the separated substances was made from the absorption of UV light at two wavelengths simultaneously: λ_1 = 254 nm (the absorption maximum of rosavin) and λ_2 = 280 nm (the absorption maximum of salidroside). The assignment of the peaks of the substances on chromatograms of an extract of roseroot stonecrop was made on the basis of the retention times of the individual components isolated previously from roseroot stonecrop rhizomes (Table 1).

Chromatograms of the extract (1) contained peaks corresponding in their retention times to rosavin, rosarin, rosin, cinnamyl alcohol, salidroside, tyrosol, gallic acid, and methyl gallate (Table 1). The noncoincidence of the elution profiles of an extract at λ_1 = 254 nm and λ_2 = 280 nm shows that the components separated had different chromophores and, consequently, differed in chemical structure.

A separation of the main active substances (rosavin, rosarin, rosin, and salidroside) optimum with respect to time of chromatography and resolution was achieved by the use as elu-

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