Inhibitory Effects of Substituted Benzanilides on Photosynthetic Electron Transport in Spinach Chloroplasts

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Received 8 February 1999

The inhibitory activity of 18 benzanilides substituted in the acyl (R^1 = H, 3-Br, 4-Cl, 4-OCH_3, 3-NO_2, 3-F, 4-F) as well as in the anilide part of the molecule (R^2 = 3-Cl, 3-OCH_3, 3-NO_2, 3-F, 4-OCH_3, 4-NO_2, 4-CH_3, 4-$CH(CH_3)_2$, 4-$CH_2CH_2CH_2CH_3$) on oxygen evolution rate in spinach chloroplasts has been investigated. The dependence of photosynthesis-inhibiting activity upon the lipophilicity of the substituents showed a quasi-parabolic course. The IC_{50} values in the investigated set varied in the range from 41 μmol dm^{-3} (R^1 = 3-NO_2, R^2 = 4-$CH(CH_3)_2$) to 497 μmol dm^{-3} (R^1 = 4-F, R^2 = H). Using ESR spectroscopy the site of action of these compounds in the photosynthetic apparatus of spinach chloroplasts has been studied. It was confirmed that the site of action of the studied anilides are the intermediates D^+, *i.e.* tyrosine radicals which are situated in the 161st position in D_2 protein on the donor side of photosystem 2.

Fourty years ago a number of acylanilides have been introduced as herbicides. In this time *Good* investigated a great set of Hill reaction inhibitors containing phenylcarbamates, phenylureas, and acylanilides and thioanilides as well and found that substitution of the hydrogen in the position 3, 4, or 5 of the benzene ring of aniline derivatives by Cl, Br, CH_3O increases the inhibitory effect [1]. More detailed studies concerning the site of herbicide action in the photosynthetic apparatus of plant chloroplasts showed that these compounds are the so-called photosystem (PS) 2 herbicides which interrupt electron transport between the primary and secondary electron acceptors of PS 2 Q_A and Q_B plastoquinones and the mechanism of action is a displacement of Q_B from its binding site at the D_1 protein [2]. Moreover, it was found that acylanilides and *N*-phenylcarbamates beside of inhibition of the photosynthetic electron transport [3] possess also an uncoupling property on the photophosphorylation system [4—6].

Substituted benzanilides and thiobenzanilides represent a group of compounds showing a wide spectrum of biological activities, *e.g.* antimycobacterial [7, 8] and herbicidal [9, 10] as well. It was found that the herbicidal activity of benzanilides can be increased by inserting of chloro substituents in several positions on both aromatic rings of the molecule [10]. Anilides of 2,4,5-trichlorophenoxyacetic acid were found to be Hill reaction inhibitors as well [11]. Derivatives of 3-nitro-2,4,6-trihydroxybenzamide and thiobenzamide which possess a free amino hydrogen atom needed for binding to the receptor site, possibly by forming a hydrogen bond, were found to be efficient inhibitors of photosynthetic electron transport [12]. It was shown that the inhibitory activity of these compounds dominantly depended on the lipophilicity of the *N*-substituent and the effect of electronic properties was not significant. The primary function of the hydrophobic components is to increase the lipid solubility and to improve contacts between the herbicides and the hydrophobic surface of the binding site.

The aim of this paper is to investigate the photosynthesis-inhibiting activity and the site of action of substituted benzanilides, some of which are newly synthesized compounds, in the photosynthetic apparatus of spinach chloroplasts.

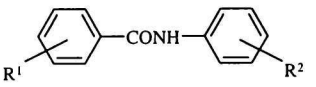
EXPERIMENTAL

The anilides under study were prepared by the reaction of respective benzoyl chloride and aniline in pyridine and their structure has been confirmed by elemental analysis, IR and NMR spectra [8, 13, 14]. The substituents of benzanilides in the acyl moiety are summarized in Table 1.

Chloroplasts were prepared from market spinach by the procedure of *Walker* [15] partly modified by *Šeršeň et al.* [16].

The inhibitory activity of the studied benzanilides concerning oxygen evolution rate (OER) in spinach

Table 1. IC_{50} Values Concerning Inhibition of Oxygen Evolution Rate in Spinach Chloroplasts by Studied Benzanilides (iPr = $CH(CH_3)_2$; Bu = $CH_2CH_2CH_2CH_3$)



Comp.	R^1	R^2	IC_{50} / μmol dm^{-3}	Comp.	R^1	R^2	IC_{50} / μmol dm^{-3}
I	H	3-NO_2	374	*X*	3-F	H	324
II	3-Br	3-F	86	*XI*	3-F	3-Cl	71
III	4-Cl	3-NO_2	73	*XII*	3-F	4-Me	193
IV	H	4-iPr	50	*XIII*	3-F	3-NO_2	126
V	4-OMe	4-iPr	53	*XIV*	3-F	4-NO_2	109
VI	3-Br	4-iPr	67	*XV*	3-F	4-OMe	484
VII	3-NO_2	4-iPr	41	*XVI*	3-F	3-OMe	263
VIII	H	4-Bu	48	*XVII*	4-F	H	497
IX	3-Br	4-Bu	357	*XVIII*	4-F	3-OMe	365

chloroplasts was investigated spectrophotometrically (Specord UV VIS, Zeiss, Jena) in the presence of the electron acceptor 2,6-dichlorophenol-indophenol (DCPIP) according to [17] and the rate of photosynthetic electron transport was monitored as a photoreduction of DCPIP. The phosphate buffer (0.02 mol dm^{-3}; pH = 7.2) used for dilution of the chloroplast suspension contained sucrose (0.4 mol dm^{-3}), $MgCl_2$ (0.005 mol dm^{-3}), and NaCl (0.015 mol dm^{-3}), the chlorophyll (Chl) content in these experiments was 30 mg dm^{-3} Samples were irradiated from the distance of 1 dm with a halogen lamp (250 W) through a 4 cm water filter to prevent overheating of the samples. This photochemical assay was carried out under saturating irradiance of "white light" ($\approx$ 900 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation)) at 25 °C. The activity of the benzanilides has been expressed by IC_{50} values, *i.e.* by molar concentrations causing a 50 % decrease of OER with respect to the untreated control. For low solubility of the studied compounds in water, these were dissolved in dimethyl sulfoxide. The applied solvent content (up to 4 vol. %) did not affect the photochemical activity in spinach chloroplasts.

The fluorescence emission spectra of chloroplasts were recorded on fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan) using excitation wavelength λ_{ex} = 436 nm for monitoring fluorescence of Chl_a, excitation slit 20 nm and emission slit 10 nm. The samples were kept in the dark 10 min before measuring. The phosphate buffer used for dilution of the chloroplast suspension was the same as described above.

The ERS spectra of the untreated suspension of spinach chloroplasts in the above described phosphate buffer (Chl content in the samples 4 g dm^{-3}) and in the presence of the studied compounds (0.05 mol dm^{-3}) were recorded with an ESR 230 instrument (WG AdW, Berlin) operating in X-band at 5 mW of microwave power and 0.5 mT modulation amplitude. ESR spectra of all samples were recorded in the dark and in the light. The samples were irradiated with $\approx$ 400 μmol m^{-2} s^{-1} PAR directly in the resonator cavity using a 250 W halogen lamp from 0.5 m distance through a 5 cm thick water filter.

RESULTS AND DISCUSSION

The 18 studied benzanilides inhibited oxygen evolution rate in spinach chloroplasts. The IC_{50} values, *i.e.* molar concentrations of inhibitors causing a 50 % decrease of OER in the suspension of spinach chloroplasts vary for the investigated set of benzanilides in the range from 41 μmol dm^{-3} (compound *VII*) to 497 μmol dm^{-3} (compound *XVII*) (Table 1). The correlation of the OER-inhibiting activity with the lipophilic and electronic parameters of individual substituents on the acyl (R^1) and on the anilide moiety of the molecule (R^2) has been studied. The values of π^- parameters expressing lipophilicity of the substituents on the aromatic ring were taken from *Norrington et al.* [18]: 0 (H), 0.47 (3-F), 1.04 (3-Cl), 1.17 (3-Br), 0.54 (3-NO_2), 0.12 (3-OCH_3), 0.31 (4-F), 0.93 (4-Cl), 0.45 (4-NO_2), −0.12 (4-OCH_3), 0.48 (4-CH_3), 1.36 ($CH(CH_3)_2$), 1.98 ($CH_2CH_2CH_2CH_3$). The corresponding Hammett constants σ characterizing electronic properties of the substituent were taken from *Hansch* and *Leo* [19]: 0 (H), 0.391 (3-Br), 0.227 (4-Cl), −0.268 (4-OCH_3), −0.170 (4-CH_3), 0.710 (3-NO_2), 0.337 (3-F), 0.062 (4-F), 0.778 (4-NO_2), 0.115 (3-OCH_3), 0.373 (3-Cl), −0.150 (4-$CH(CH_3)_2$), −0.160 ($CH_2CH_2CH_2CH_3$).

The photosynthesis-inhibiting activity of the studied benzanilides showed quasi-parabolic dependence on the sum of lipophilicity of R^1 and R^2 substituents expressed as ($\pi_1^- + \pi_2^-$) (Fig. 1). The corresponding correlation can be expressed by the following equation

$$\log\{1/IC_{50}\} = 2.777\ (\pm 0.086) + 1.621\ (\pm 0.128)\cdot (\pi_1^- + \pi_2^-) - 0.439\ (\pm 0.040)(\pi_1^- + \pi_2^-)^2$$

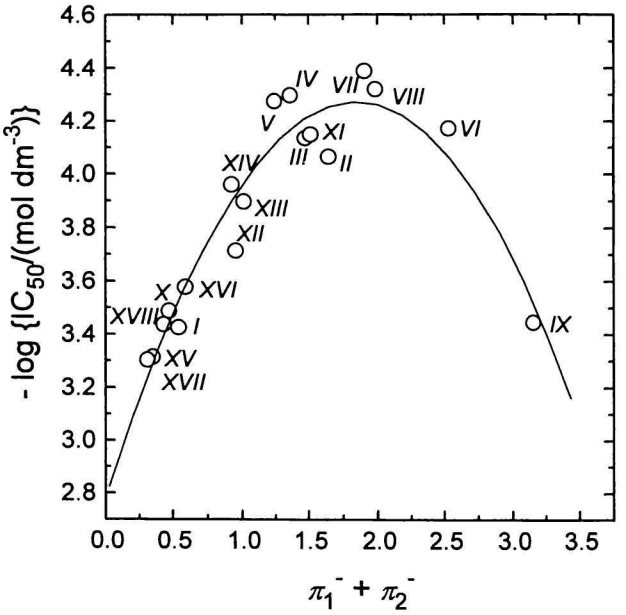


Fig. 1. The dependence of inhibition of OER in spinach chloroplasts on the lipophilicity of the substituents R^1 and R^2 expressed by π^- parameters of substituents on the aromatic ring taken from *Norrington et al.* [18].

$$r = 0.960;\ s = 0.116;\ F = 87.2;\ n = 18$$

After introduction of Hammett constants of substituents (σ_1 and σ_2, respectively) into the above equation the correlation will be modified as follows

$$\log\{1/IC_{50}\} = 2.800\ (\pm 0.079) + 1.674\ (\pm 0.119)\cdot \cdot(\pi_1^- + \pi_2^-) - 0.457\ (\pm 0.037)(\pi_1^- + \pi_2^-)^2 - - 0.129\ (\pm 0.063)(\sigma_1 + \sigma_2)$$

$$r = 0.969;\ s = 0.105;\ F = 72.0;\ n = 18$$

The results of statistical analysis confirm that the Hansch's parabolic model is suitable for description of the correlation between photosynthesis-inhibiting activity and lipophilicity of the studied benzanilides. Similar results have been obtained also for anilides of 2-alkylpyridine-4-carboxylic acids [20]. The results of statistical analysis were not pronouncedly improved by introduction of the Hammett constants of R^1 and R^2 substituents into the former correlation equation indicating that the lipophilicity of the studied benzanilides is decisive for their inhibitory activity. Contrary to these results, the previous study concerning antimycobacterial activity of a set of 3′- and 4′-fluorobenzanilides showed the dominance of the electronic effects, *i.e.* the increase of biological activity with the increase of the electron-accepting power of the substituents [8].

The effects of the studied compounds on the photosynthetic centres of spinach chloroplasts were investigated by studying chlorophyll *a* (Chl_a) fluorescence.

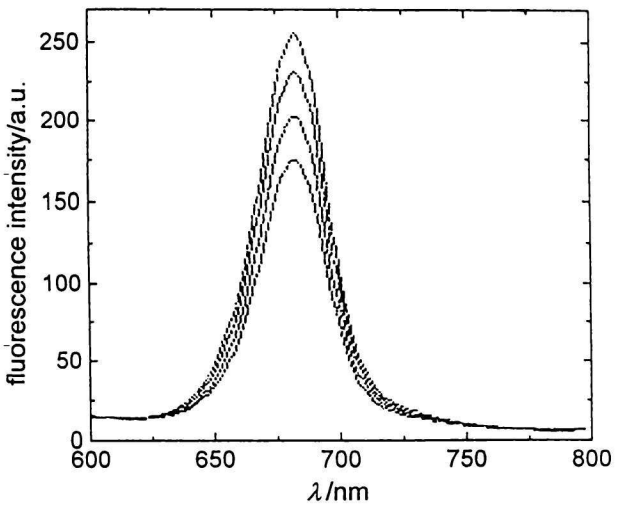


Fig. 2. Fluorescence emission spectra of untreated spinach chloroplasts and in the presence of $c(XIV)/(\mu\text{mol dm}^{-3})$: 10, 20, and 50 (curves from top to bottom; λ_{ex} = 436 nm).

The decreased intensity of the emission band at 686 nm, belonging to the pigment-protein complexes in photosystem 2 [21] (Fig. 2) suggested PS 2 as the site of action of the studied inhibitors.

Chloroplasts of higher plants exhibit ESR signals belonging to both photosystems (the so-called signal I and signal II) in the region of free radicals ($g \approx 2.00$) [22]. Signal I belongs to the chlorophyll dimer in the core of PS 1 (P700) [22]. Signal II consists of two constituents (signal II_{slow} and signal $II_{very\ fast}$) belonging to the intermediates Z^+/D^+, which are situated on the donor side of PS 2 and secure the electron transfer from the oxygen-evolving complex to the core of PS 2 (P680). The ESR signals I and II can be affected by compounds causing inhibition of photosynthetic electron transport. From the change of the intensity and the shape of ESR signals in the presence of an inhibitor its site of action in the photosynthetic apparatus can be determined. Fig. 3 presents ESR spectra of the untreated chloroplast suspension as well as that in the presence of compound *IX* in the dark and in the light. Signal II_{slow} corresponds practically to the whole ESR signal registered in the dark at $g = 2.0046$ and line width $\Delta B = 2$ mT (Fig. 3 (A), full line). It is stable in the dark during several hours and it belongs to the intermediate D^+, *i.e.* to the tyrosine 161 (Tyr_D) which is situated in D_2 protein on the donor side of PS 2 [23, 24]. ESR signal induced by light practically corresponds to signal $II_{very\ fast}$ (the difference of the signal intensity in the light and in the dark is shown in Fig. 3 (A)) and it belongs to the intermediate Z^+, *i.e.* to the tyrosine 161 which is located in D_1 protein [23, 24]. From Fig. 3 (B) it is evident that the intensity of ESR signal II, mainly the intensity of its constituent signal II_{slow} (Fig. 3 (B), full line), has been decreased

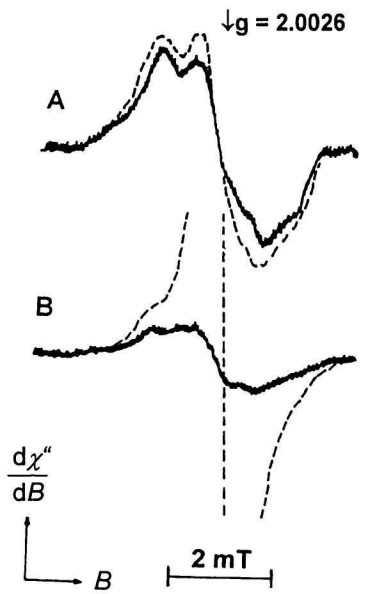


Fig. 3. ESR spectra of untreated spinach chloroplasts (A) and of chloroplasts treated with $c(IX) = 0.05$ mol dm^{-3} (B). The full lines correspond to chloroplasts kept in the dark, the dotted lines to the illuminated chloroplasts.

by the studied compounds. That means that the studied compounds interact with D^+ intermediate. Due to the interaction of benzanilides with this part of PS 2, the photosynthetic electron transport between PS 2 and PS 1 is impaired and consequently a pronounced increase of signal I intensity in the light (Fig. 3 (B), dashed line; $g = 2.0026$, $\Delta B \approx 0.7$ mT) belonging to the cation radical of the chlorophyll dimer in the core of PS 1 can be observed. The site of action of previously investigated anilides of 2-alkyl-substituted 4-pyridinecarboxylic acid was not restricted only to the intermediate D^+ and these compounds interfered also with the intermediate Z^+, *i.e.* tyrosine 161 (Tyr_Z) which is situated in D_1 protein on the donor side of PS 2 [20]. This is probably connected with the presence of polar hydroxy substituent in the anilide part of these compounds enabling interaction with the intermediate Z^+ which is located in more polar environment of thylakoid membrane than the intermediate D^+ [24].

The diphenylcarbazide (DPC) is an artificial electron donor of PS 2 acting in the intermediate Z^+/D^+ on the donor side of PS 2. Its addition to the chloroplasts inhibited by compounds which do not impair the own core of PS 2 (P680) can restore the photosynthetic electron transport. Upon addition of DPC (0.5 mmol dm^{-3}) to spinach chloroplasts inhibited by the studied benzanilides the oxygen evolution rate was practically completely restored and consequently we assume that in the presence of the studied compounds the own core of PS 2 (P680) remains intact. However, similar experiment with DPC performed at the same conditions confirmed that P680 is partially damaged by anilides of 2-alkyl-substituted 4-pyridinecarboxylic acid with hydroxy substituent in the anilide part of the compound [20].

Acknowledgements. This work is supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences under the No. 1/4013/97.

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