Inhibition of Photosynthetic Electron Transport by Some Anilides of 2-Alkylpyridine-4-carboxylic Acids in Spinach Chloroplasts

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The inhibitory activity of some anilides of 2-alkylpyridine-4-carboxylic acids on oxygen evolution rate in spinach chloroplasts as well as the mechanism and site of action of these compounds in the photosynthetic apparatus of chloroplasts have been investigated. Using ESR spectroscopy it was confirmed that the site of action of the studied anilides are the intermediates Z^+/D^+, *i.e.* tyrosine radicals, which are situated in D_1 and D_2 proteins on the donor side of photosystem 2. The biological activity of the tested compounds was affected by lipophilicity of the substituents on the phenyl ring of the anilide part of the molecule (halogen, methyl, hydroxy) and of the 2-alkyl substituent as well.

Many derivatives of heterocyclic compounds, including pyridine, were found to exhibit a wide spectrum of biological activity. Nicotinamide, one of the most known derivatives of pyridine is an essential vitamin occurring also in algae. However, algal mutants deficient in this compound survive only after addition of external vitamin source [1, 2]. The use of isonicotinic acid hydrazide, an efficient antituberculotic agent, as a standard at evaluation of antimycobacterial activity of new compounds is frequently applied [3]. The 4-substituted derivatives of 2-alkyl- or 2-alkylsulfanylpyridine belong to the group of biologically active compounds showing antifungal [4, 5], photosynthesis-inhibiting [6], and antimycobacterial activity [3]. It has been found that the dependence of the antifungal and photosynthesis-inhibiting activity of 2-alkylsulfanylpyridine-4-carbothiamides on the alkyl chain length of the alkylsulfanyl substituent shows a quasi-parabolic behaviour [4—6]. However, the dependence of antimycobacterial activity of these compounds against *Mycobacterium tuberculosis* $H_{37}R_v$ upon the lipophilicity of the alkylsulfanyl substituent shows two maxima of the highest activity [3]. This can be connected with the presence of two pharmacophore groups – the alkylsulfanyl group and thioamide group – in their molecules.

Photosynthesis is the conversion of light energy into chemical energy proceeding in the membrane-bound proteins and cofactors which are located in the photosynthetic centres of photosynthesizing organisms. The photosynthetic electron transport chain is formed by these photosynthetic centres, *i.e.* by the photosystem 1 (PS 1) and the photosystem 2 (PS 2). The interaction of photosynthesis-inhibiting compounds with PS 1 or PS 2 leads to the damage of the photosynthetic electron transport [7].

The aim of this paper is to investigate the photosynthesis-inhibiting activity and the site of inhibitory action of some anilides of 2-alkylpyridine-4-carboxylic acids in the photosynthetic apparatus of spinach chloroplasts.

EXPERIMENTAL

The model anilide compounds have been prepared by reaction of acyl chlorides of 2-alkylpyridine-4-carboxylic acid with corresponding aminophenols. The synthesis and physicochemical characteristics of the compounds are described in [8]. The 2-alkyl substituents in the prepared anilides (Table 1) were propyl (*I*, *X*), isopropyl (*VII*, *IX*, *XIII*), butyl (*II*, *VIII*, *XIV*, *XVI*), isobutyl (*III*), *tert*-butyl (*IV*, *VI*, *XI*, *XV*), and pentyl (*V*, *XII*), the substituents X in the anilide part of the molecules were 3′-Cl, 4′-OH (*I*—*V*); 3′-Br, 4′-OH (*VI*); 4′-CH_3, 2′-OH (*IX*); 5′-Cl, 2′-OH (*VII*, *VIII*); 3′,5′-Cl_2, 4′-OH (*X*—*XII*); 3′,5′-Br_2, 4′-OH (*XIII*—*XV*); and 3′,5′-I_2, 4′-OH (*XVI*).

The inhibitory activity of the studied anilides concerning oxygen evolution rate in spinach chloroplasts was investigated spectrophotometrically (Specord UV VIS, Zeiss, Jena) in the presence of the electron acceptor 2,6-dichlorophenol—indophenol (DPIP) according to the method described in [9]. 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}) and the samples were irradiated (30 s) from the distance of 1 dm with a halogen lamp (250 W) using a water filter to prevent overheating of samples. The activity of the compounds has been expressed by IC_{50} values, *i.e.* by molar concentrations causing a 50 % decrease of oxygen evolution rate with respect to the untreated control. For the low solubility of the studied compounds in water, they were dissolved in dimethyl sulfoxide. The applied solvent content (up to 4 %) did not affect the photochemical activity of spinach chloroplasts.

The fluorescence emission spectra of chloroplasts were recorded on a fluorescence spectrophotometer F-2000 (Hitachi, Tokyo) using excitation wavelength λ_{ex} = 436 nm, excitation slit of 20 nm, and emission slit of 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. Chlorophyll (Chl) content in the samples was 10 mg dm^{-3}

The ESR 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 ERS 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 directly in the resonator using a 250 W halogen lamp and a water filter to exclude warming of the samples.

RESULTS AND DISCUSSION

Eleven compounds from sixteen studied anilides of 2-alkylpyridine-4-carboxylic acids (Table 1) inhibited oxygen evolution rate in spinach chloroplasts (Fig. 1). For compounds with X = 3′-Cl, 4′-OH (*I*, *IV*, *V*); 3′-Br, 4′-OH (*VI*), or 2′-OH, 5′-Cl (*VII*, *VIII*)

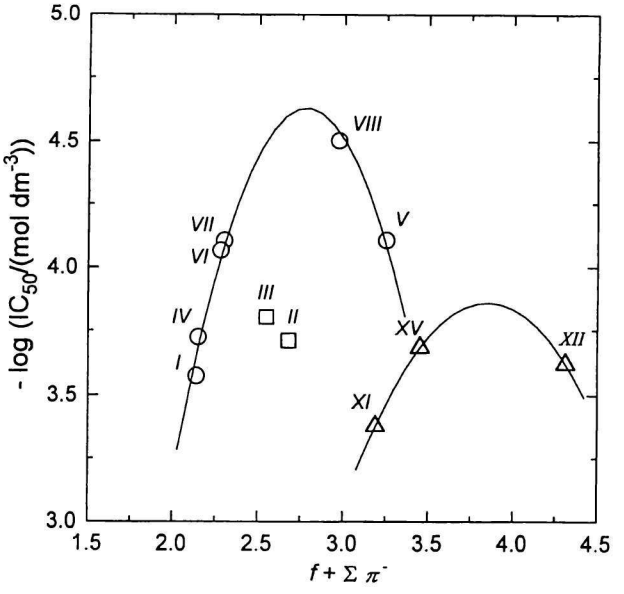
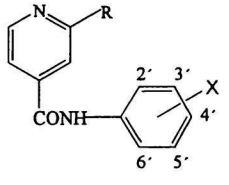


Fig. 1. Dependence of $-\log\{IC_{50}\}$ on hydrophobicity of R and X substituents ($f + \Sigma\pi^-$) of the studied anilides of 2-alkylpyridine-4-carboxylic acids.

the biological activity of the studied anilides (expressed by $-\log(IC_{50}/(\text{mol dm}^{-3}))$ values) showed quasi-parabolic dependence on the sum of hydrophobic fragment constants of R substituent (f) and corresponding π^- parameters of X substituents (halogen, hydroxy) of the phenyl ring of the anilide part of the molecule ($\Sigma\pi^-$) (Fig. 1). The resulting hydrophobicity of substituents was calculated using the following f and π^- values of individual substituents (taken from *Hansch* and *Leo* [10] and *Norrington et al.* [11]): 1.97 (propyl), 1.84 (isopropyl), 2.51 (butyl), 2.38 (isobutyl), 1.98 (*tert*-butyl), 3.10 (pentyl), 1.04 (3-Cl), 1.17 (3-Br), −0.58 (2-OH), −0.66 (4-OH), 0.48 (4-CH_3). The presence of a second halogen substituent in the phenyl ring of the studied compounds caused

Table 1. The Studied Anilides of 2-Alkylpyridine-4-carboxylic Acids



Compound	R	X	Compound	R	X
I	C_3H_7	3′-Cl, 4′-OH	*IX*	$CH(CH_3)_2$	4′-CH_3, 2′-OH
II	C_4H_9	3′-Cl, 4′-OH	*X*	C_3H_7	3′,5′-Cl_2, 4′-OH
III	$CH_2CH(CH_3)_2$	3′-Cl, 4′-OH	*XI*	$C(CH_3)_3$	3′,5′-Cl_2, 4′-OH
IV	$C(CH_3)_3$	3′-Cl, 4′-OH	*XII*	C_5H_{11}	3′,5′-Cl_2, 4′-OH
V	C_5H_{11}	3′-Cl, 4′-OH	*XIII*	$CH(CH_3)_2$	3′,5′-Br_2, 4′-OH
VI	$C(CH_3)_3$	3′-Br, 4′-OH	*XIV*	C_4H_9	3′,5′-Br_2, 4′-OH
VII	$CH(CH_3)_2$	5′-Cl, 2′-OH	*XV*	$C(CH_3)_3$	3′,5′-Br_2, 4′-OH
VIII	C_4H_9	5′-Cl, 2′-OH	*XVI*	C_4H_9	3′,5′-I_2, 4′-OH

a pronounced decrease of biological activity (Fig. 1, compounds *XI*, *XII*, *XV*). The activity of compounds *II* and *III* (R = butyl or isobutyl; X = 3′-Cl, 4′-OH) was lower than expected with respect to their hydrophobicity, probably due to their lowered aqueous solubility (Fig. 1). Because of very low aqueous solubility of compounds *IX*, *X*, *XIII*, *XIV*, and *XVI* it was not possible to determine the corresponding IC_{50} values of these anilides.

The effect of the studied compounds on photosynthetic centres of chloroplasts was investigated by studying the room temperature fluorescence of chlorophyll *a* (Chl_a) using suspension of spinach chloroplasts in phosphate buffer. In the presence of the studied compounds the intensity of the Chl emission band at 684 nm (belonging to the pigment-protein complex of PS 2 [12]) obtained with untreated control sample showed a decrease reflecting interaction of these compounds with the photosynthetic apparatus of spinach chloroplasts (Fig. 2).

Due to the fact that the chloroplasts of higher plants exhibit in the region of free radicals ($g \approx 2.00$) ESR signals (the so-called signal I and signal II), the ESR spectroscopy is a suitable experimental method for studying the photosynthetic apparatus. For more precise determination of the site of action of the studied anilides the ESR spectra of the untreated suspension of spinach chloroplasts and in the presence of the studied compounds (0.05 mol dm^{-3}) have been recorded in the dark and in the light. The effect of the studied compounds on ESR signals I and II belonging to the photosynthetic centres PS 1 and PS 2 [13] is illustrated in Fig. 3. It is evident from Fig. 3 that in the presence of compound *VI* the intensity of ESR signals II of chloroplasts (signals II_{slow} and $II_{very\ fast}$, respectively) decreases (Fig. 3B, full line). This means that the site of action of the studied compounds are

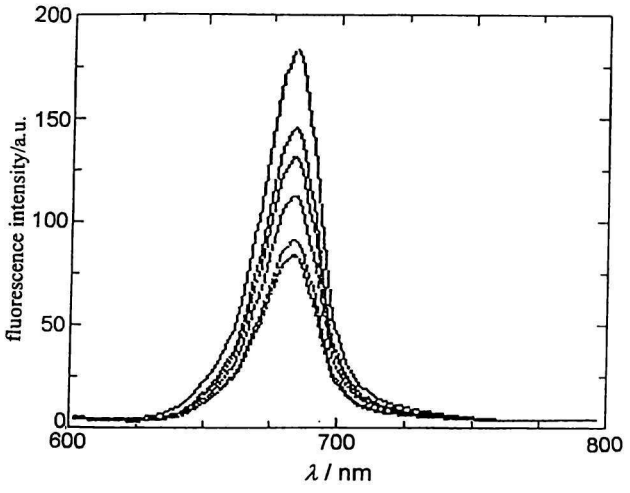


Fig. 2. Fluorescence emission spectra of untreated spinach chloroplasts and in the presence of $c(IV)/(\mu mol\ dm^{-3})$: 20, 50, 100, 200, and 300 (curves from top to bottom; λ_{ex} = 436 nm).

the intermediates Z^+/D^+ which are situated on the donor side of PS 2. These intermediates correspond to tyrosine radicals Tyr_Z 161 and Tyr_D 160 located in D_1 and D_2 proteins [14, 15]. The intensity increase of ESR signal I (belonging to the chlorophyll dimer in PS 1 [15]) in ESR spectra of illuminated chloroplasts treated with the studied compounds (Fig. 3B, dashed line) shows that these compounds do not interact with PS 1. This leads to the conclusion that the site of action of the studied compounds differs from those of structurally similar 2-alkylsulfanylpyridine-4-carbothiamides which interact only with the intermediate D^+ (corresponding to Tyr_D) [5].

The use of the artificial electron donor 1,5-diphenylcarbazide (DPC) acting in Z^+/D^+ [14] can

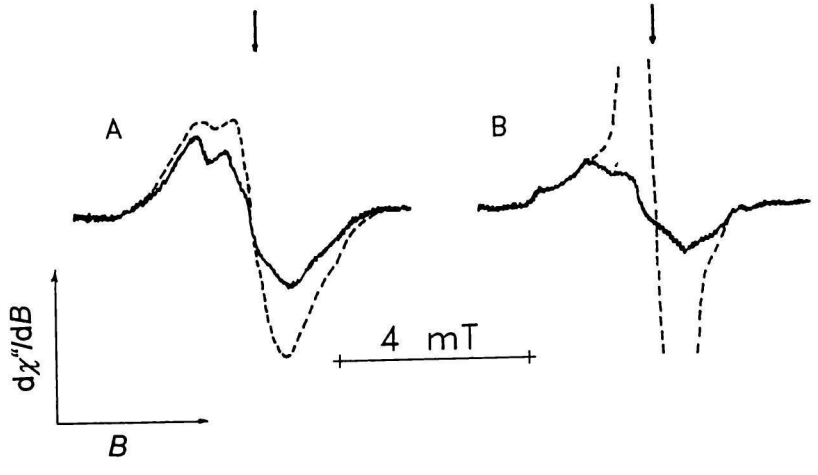


Fig. 3. ESR spectra of untreated spinach chloroplasts (A) and of chloroplasts treated with $c(VI)$ = 0.05 mol dm^{-3} (B). The full lines correspond to chloroplasts kept in the dark, the dotted lines to the illuminated chloroplasts. The arrows denote g = 2.0026.

be helpful for determination of incidental action of the studied anilides on the core of PS 2 (P 680). It is evident that the core of PS 2 is partially impaired by the investigated compounds while the reduction of DPIP in chloroplasts inhibited by the studied anilides was not completely restored after addition of 5×10^{-4} mol dm^{-3} DPC. However, it was shown that in the presence of structurally similar 2-alkylsulfanylpyridine-4-carbothiamides the core of PS 2 (P 680) and a part of the electron transport chain – at least up to plastoquinone – remain intact [6].

The quasi-parabolic course of the dependence of $-\log(IC_{50}/(\text{mol dm}^{-3}))$ values upon the hydrophobicity of R and X substituents ($f + \Sigma\pi^-$) of compounds *I, IV, V, VI, VII,* and *VIII* (Fig. 1) can be connected with the fact that the highest inhibitory activity will be exhibited by compounds having sufficiently high lipophilicity for securing their passage through the lipidic parts of biological membranes and simultaneously enabling sufficiently high anilide concentration in the aqueous phase which is indispensable for the interactions with Z^+/D^+ intermediates situated on the lumenal side of photosynthetic membranes in D_1 and D_2 proteins. The presence of the second halogen substituent on the phenyl ring of the anilide part of the tested compounds causes a strong decrease of biological activity due to their intensively lowered aqueous solubility.

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