Inhibition of Photosynthetic Electron Transport in Spinach Chloroplasts by 2,6-Disubstituted Pyridine-4-thiocarboxamides

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Inhibition of oxygen evolution rate in spinach chloroplasts by 2,6-disubstituted pyridine-4-thiocarboxamides (R^1 = SCH_3, SC_2H_5, SC_3H_7, SC_4H_9, SC_5H_{11},SC_6H_{13}, SC_7H_{15}, SC_8H_{17}; R^2 = SCH_3, SC_2H_5, SC_3H_7, SC_4H_9, SC_5H_{11}, Cl, Br) was investigated. The determined IC_{50} values varied in the range from 9.3 μmol dm^{-3} (R^1 = SC_3H_7; R^2 = Cl) to 342.0 μmol dm^{-3} (R^1 = R^2 = SCH_3). The dependence of the biological activity on the lipophilicity of the compounds showed a bilinear course for compounds with R^2 = halogen and a quasi-parabolic course for compounds with R^1 = R^2 = alkylsulfanyl. At comparable log $\{P\}$ values the compounds containing halogen atom in their molecules exhibited significantly higher biological activity than the 2,6-dialkylsulfanyl derivatives. Using EPR spectroscopy it was found that the studied compounds interacted with the intermediate $D^{\bullet}$, *i.e.* tyrosine radical ($Tyr_D^{\bullet}$) situated in the 161st position in D_2 protein on the donor side of photosystem (PS) 2. Due to this interaction the photosynthetic electron transport between PS 2 and PS 1 was interrupted. The primary donor of PS 2 (P680) remained intact.

A great variety of pyridine derivatives was found to exhibit significant biological activity. *Soskic* and *Sabljic* [1] investigated substituted 4-hydroxypyridines acting as phenol-type inhibitors of photosystem (PS) 2 and found that the size and lipophilicity of substituents at positions 2 and 5 are the most important for the activity of derivatives with a long side chain. The phenol-type inhibitors are known to bind preferentially to the peptide with M_r = 47 000 – a subunit of PS 2 and to interfere with plastoquinone reduction by PS 2 [2, 3]. As an additional factor, which increases the activity of 4-hydroxypyridines in the Hill reaction, the electron-attracting capacity of these substituents was expected. *Trebst et al.* [4] and *Asami et al.* [5] investigated inhibition of photosynthetic electron transport by halogenated 4-hydroxypyridines. The activity of 4-hydroxypyridines was markedly enhanced upon modifying their structure: introduction of halogens into both positions 3 and 5 of the pyridine ring and additional substitution at the α-position of the side chain at position 6 were effective in enhancing the activity. Insertion of a phenyl ring into the side chain at position 6 of the pyridine ring also increased the activity [5]. It was confirmed that the halogenated 4-hydroxypyridines belong to the group of phenol-type inhibitors [2, 5].

Nicotinamide, one of the most known derivatives of pyridine is an essential vitamin and the use of isonicotinic acid hydrazide, an efficient antituberculotic agent, as a standard at evaluation of antimycobacterial activity of new compounds is frequently applied [6, 7]. The 4-substituted derivatives of 2-alkyl or 2-alkylsulfanylpyridine belong to the group of biologically active compounds showing antifungal [7, 8], photosynthesis-inhibiting [9, 10], and antimycobacterial activity [7]. It has been found that the dependence of the antifungal and photosynthesis-inhibiting activity of 2-alkylsulfanylpyridine-4-thiocarboxamides on the alkyl chain length of the alkylsulfanyl substituent showed a quasi-parabolic course [8, 9]. These compounds inhibited the photosynthetic electron transport in plant chloroplasts due to their interaction with the intermediate $D^{\bullet}$, corresponding to tyrosine radical ($Tyr_D^{\bullet}$), which is situated in the 161st position in D_2 protein located on the donor side of PS 2 [9]. Similar site of inhibitory action in the photosynthetic apparatus of spinach chloroplasts, *i.e.* the intermediate $D^{\bullet}$, has been determined also for 2-alkylsulfanyl-substituted benzothiazole derivatives [11]. Some of 2,6-bis(alkylsulfanyl)pyridine-4-thiocarboxamides inhibited chlorophyll content in freshwater alga *Chlorella vulgaris* [6].

The aim of this paper is to investigate the inhibition of photosynthetic electron transport in spinach chloroplasts by 2,6-disubstituted pyridine-4-thiocarboxamides, to study the structure—activity re-

lationships and to determine the site of action of these inhibitors in the photosynthetic apparatus of spinach chloroplasts.

EXPERIMENTAL

The studied 2-R^1-6-R^2-pyridine-4-thiocarboxamides (R^1 = alkylsulfanyl, R^2 = alkylsulfanyl, Cl or Br) were prepared by the reaction of 2,6-disubstituted pyridine-4-carboxamides dissolved in anhydrous toluene with Lawesson's reagent; the reaction mixture was heated at reflux until thin-layer chromatography indicated a complete reaction. The compounds were recrystallized from aqueous ethanol. The structure of the synthesized compounds has been confirmed by elemental analysis and ^{1}H NMR spectra [12]. In biological tests the following compounds were used: R^2 = Cl and R^1 = SC_2H_5 (*I*), SC_3H_7 (*II*), and SC_6H_{13} (*III*); R^2 = Br and R^1 = SCH_3 (*IV*), SC_2H_5 (*V*), SC_4H_9 (*VI*), SC_7H_{15} (*VII*), and SC_8H_{17} (*VIII*); R^1 = R^2 = SCH_3 (*IX*), SC_2H_5 (*X*), SC_3H_7 (*XI*), and SC_5H_{11} (*XII*); R^1 = SC_6H_{13} and R^2 = SC_4H_9 (*XIII*) and SC_5H_{11} (*XIV*). Lipophilicity of the compounds was computed using a program ACD/Log {*P*} version 1.0 (Advanced Chemistry Development Inc., Toronto).

Chloroplasts were prepared from market spinach according to the procedure described in [13]. The inhibitory activity of the studied compounds concerning oxygen evolution rate (OER) in spinach chloroplasts was investigated spectrophotometrically (Specord UV VIS, Zeiss, Jena, Germany) in the presence of the electron acceptor 2,6-dichlorophenolindophenol (DCPIP). The rate of photosynthetic electron transport, which is proportional to OER, was monitored as photoreduction of DCPIP according to the method described in [14]. The chlorophyll (Chl) content in these experiments was 30 mg dm^{-3}. This photochemical assay was carried out under saturating irradiance of white light from a 250 W halogen lamp ($\approx$ 900 μmol m^{-2} s^{-1} PAR). For low solubility of the studied compounds in water, these were dissolved in dimethyl sulfoxide (DMSO). The applied solvent content (up to 4 %) did not affect the photochemical activity in spinach chloroplasts. IC_{50} values were calculated from OER inhibition at 6—8 concentrations and tests in all concentrations were triplicated.

EPR measurements were carried out with an instrument ERS 230 (WG, Akademie der Wissenschaften, Berlin, Germany) operating in X band at 5 mW of microwave power and 0.5 mT modulation amplitude. EPR spectra were recorded in the dark and in the light. The chlorophyll content in the samples was 4 g dm^{-3}. The irradiation of the chloroplast samples was carried out directly in the resonator cavity with a 250 W halogen lamp ($\approx$ 400 μmol m^{-2} s^{-1} PAR) through 5 cm water filter. The studied compounds were dissolved in DMSO. The 10 vol. % DMSO content in the chloroplast suspension had not observable effect on EPR spectra of spinach chloroplasts.

All experiments were carried out at 25 °C.

RESULTS AND DISCUSSION

The studied 2,6-disubstituted pyridine-4-thiocarboxamides inhibited OER in spinach chloroplasts monitored by DCPIP photoreduction (Fig. 1). The OER-inhibiting activities were expressed by IC_{50} values corresponding to the molar concentration of the compound causing a 50 % decrease of photochemical activity of untreated spinach chloroplasts. The IC_{50} value could not be determined for compound *XII* due to its very low aqueous solubility.

From the dependence of log {$1/IC_{50}$} *vs.* log {*P*} (Fig. 1) it is obvious that the inhibitory activity was affected by the total lipophilicity of the compound (expressed by log {*P*} value) as well as by the substituent in position 6 (alkylsulfanyl or halogen). For compounds with R^2 = Cl or Br the inhibitory activity increased with the increasing lipophilicity of the compounds up to log {*P*} = 3.27, further increasing of the compound lipophilicity resulted in a sharp decrease of the activity. For compounds with R^1 = R^2 = alkylsulfanyl the dependence of the biological activity on the lipophilicity of the compounds showed a quasi-parabolic course, however, the differences between activities of these compounds were smaller than in the set with halogen substituent in position 6.

The presence of —CONH— or —CSNH— group in the molecule of the compound enables interaction of such compounds with proteins present in biological cells by forming hydrogen bonds. Due to these

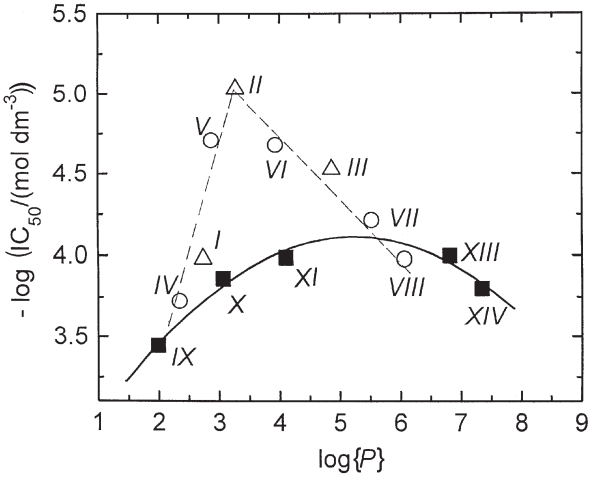

Fig. 1. Dependence of OER-inhibiting activity of the studied 2,6-disubstituted pyridine-4-thiocarboxamides on the logarithm of partition coefficient of the compounds (open triangles – compounds with R^2 = Cl (*I*—*III*); open circles – compounds with R^2 = Br (*IV*—*VIII*); full squares – compounds with R^2 = alkylsulfanyl (*IX*—*XI*, *XIII*, *XIV*)).

interactions the conformation of the proteins can be changed resulting in different biological effects [7, 9, 15—18]. All studied compounds contained —$CSNH_2$ group in their molecule, hence the differences in inhibitory activities were connected with the physicochemical properties of the substituents in positions 2 and 6. The total lipophilicity of the 14 investigated compounds varied in the range of $1.99 \leq \log \{P\} \leq 7.34$. From Fig. 1 it is evident that in the range of $2.5 \leq \log \{P\} \leq 5.5$ the presence of halogen atom in the molecule of inhibitor (Cl or Br) contributed to the enhancement of biological effectiveness. The 2,6-dialkylsulfanyl derivatives with comparable lipophilicity exhibited lower photosynthesis-inhibiting activity than the halogen-containing derivatives. Hence, it can be assumed that for inhibitory activity of less lipophilic 2-alkylsulfanylpyridine-4-thiocarboxamides the electron-attracting substituent in position 6 is more suitable than the electron-withdrawing one. The contribution of halogen substituent to the enhancement of photosynthesis-inhibiting effects in the set of substituted 4-hydroxypyridines was observed also in [5]. The decisive effect of electronic properties of the substituents on the antimycobacterial activity has been confirmed for example for a set of 3- and 4-fluorothiobenzanilides [19]. However, due to the lipophilicity increase of the studied compounds caused by prolongation of the alkylsulfanyl substituent in position 2, the solubility of the compounds decreased, which was reflected in their lower photosynthesis-inhibiting activity. By decreased solubility resulting in limited passage through the hydrophilic regions of thylakoid membranes can be explained also the lower photosynthesis-inhibiting activity of 2,6-dialkylsulfanyl derivatives, mainly of those with 2,6-bis(alkylsulfanyl) substituents ($R^1 = R^2$). The IC_{50} value for compound *XII* could not be determined due to its very low solubility.

Chloroplasts of higher plants exhibit EPR signals (the so-called signal I and signal II) in the region of free radicals ($g \approx 2.00$) belonging to both photosystems, which can be affected by compounds causing inhibition of photosynthetic electron transport. Signal I belongs to the chlorophyll dimer in the reaction centre of PS 1 (P700) [20]. Signal II consists of two constituents (signal II_{slow} and signal $II_{very\ fast}$) belonging to the intermediates $Z^\bullet/D^\bullet$, which are situated on the donor side of PS 2 and secure the electron transfer from the oxygen evolving complex to the primary donor of PS 2 (P680). Fig. 2 presents EPR spectra of the untreated chloroplast suspension (A) as well as those in the presence of compound 2-bromo-6-ethylsulfanylpyridine-4-thiocarboxamide (B) in the dark and in the light. Signal II_{slow} corresponds practically to the whole EPR signal registered in the dark at $g = 2.0046$ with the line width $\Delta B = 2$ mT (Fig. 2A, full line). It is stable in the dark during several hours and it belongs to the intermediate $D^\bullet$, *i.e.* to the radical of tyrosine 161 (Tyr_D), which is situated in D_2 protein on the donor side of PS 2 [21]. EPR signal induced by light (the difference of the signal intensity in the light and in the dark in Fig. 2A) corresponds approximately to 20 % of the signal $II_{very\ fast}$. The signal $II_{very\ fast}$ belongs to the intermediate $Z^\bullet$, *i.e.* to the radical of tyrosine 161, which is located in D_1 protein (Tyr_Z) on the donor side of PS 2 [21]. From Fig. 2B it is evident that the intensity of EPR signal II, mainly the intensity of its constituent signal II_{slow}, (full line) has been decreased by the studied compound. That means that the studied compounds interacted predominantly with $D^\bullet$ intermediate, the photosynthetic electron transport between PS 2 and PS 1 was impaired and consequently a pronounced increase of signal I intensity in the light (dashed line; $g = 2.0026$, $\Delta B \approx 0.7$ mT) belonging to the cation radical of the chlorophyll dimer in the reaction centre of PS 1 could be observed. It was found that the intermediate $D^\bullet$ is situated in more hydrophobic environment than the intermediate $Z^\bullet$ [21]. Whereas the studied compounds interact predominantly with the intermediate $D^\bullet$ it can be assumed that the lipophilicity of the substituents in positions 2 and 6 contributed to more effective approach of the inhibitor to this intermediate. These results are in accordance with those obtained in [9] for 2-alkylsulfanylpyridine-4-thiocarboxamides as well as with the results obtained for 2-alkylsulfanyl-substituted benzothiazoles [11].

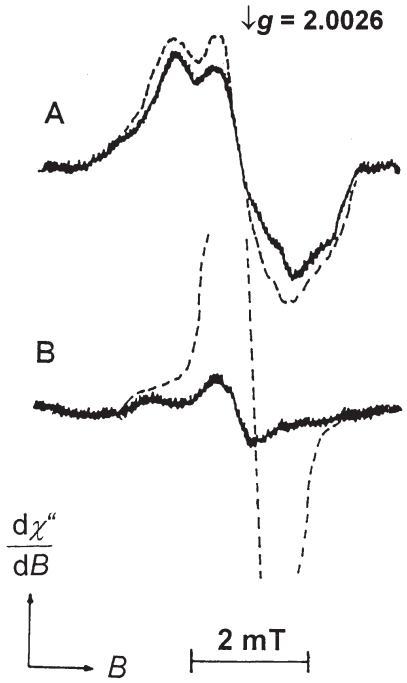

Fig. 2. EPR spectra of untreated spinach chloroplasts (A) and of chloroplasts treated with 0.05 mol dm^{-3} of 2-bromo-6-ethylsulfanylpyridine-4-thiocarboxamide (B). The full lines correspond to chloroplasts kept in the dark, the dotted lines to the illuminated chloroplasts.

The 1,5-diphenylcarbazide (DPC) is an artificial electron donor of PS 2 acting in the intermediate $Z^{\bullet}/D^{\bullet}$ on the donor side of PS 2 [22]. If the OER in plant chloroplasts is inhibited by compounds, which do not impair the photosynthetic transport chain from P680 to plastoquinone, the photosynthetic electron transport can be restored by the addition of DPC to the chloroplast suspension. After addition of DPC (0.5 mmol dm^{-3}) to spinach chloroplasts treated with the studied 2,6-disubstituted 4-thiocarboxamides (causing inhibition of OER up to 90 %) the oxygen evolution rate was practically completely restored. Consequently, it can be assumed that in the presence of the studied compounds the primary donor of PS 2 (P680) remains intact.

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