# Hydrophilic Photosystem II Inhibitors: Cyanoacrylate Thiolate Salts

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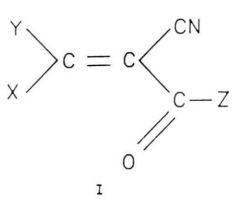
Z. Naturforsch. **45c,** 343–347 (1990); received November 15, 1989

PS II Inhibitors, Cyanoacrylate Thiolate Salts

Cyanoacrylate thiolate salts such as the sodium salt of ethoxyethyl-3-*p*-chlorobenzylthio-2-cyano-3-mercapto acrylate have been shown to be relatively slow binding, potent inhibitors of photosynthetic electron transport, that are equally active against thylakoids isolated from atrazine-susceptible and atrazine-resistant *Brassica napus* seedlings. It has been suggested that their mode of binding involves the substituted benzyl group interacting with the membrane lipids and the thiolate ion interacting in the region of the histidine$_{215}$ residue of the $D_1$ peptide, close to the non-heme Fe centre. This, together with their slow rate of binding, has led to the thiolate salts being classified as phenol type inhibitors.

## Introduction

Many compounds of diverse structure are known to inhibit photosynthetic electron transport (PET) in plant chloroplasts, most of them being neutral molecules in which increased PET inhibitory activity is associated with increased lipophilicity [1]. Such behaviour is consistent with these inhibitors acting by displacing a lipophilic plastoquinone molecule ($Q_B$) from its binding niche on the $D_1$ (32 kDa) peptide associated with the photosystem II (PS II) reaction centre (RC) in the thylakoid membrane [2].

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Alkylamino cyanoacrylates (1, X = (subst)alkylamino; Y = alkyl; Z = alkoxy) are one such group of neutral PS II inhibitors in which lipophilic and stereochemical factors have been shown to play an important role in determining activity [3]. This contribution is concerned with a related group of PS II inhibitors *viz.:* cyanoacrylate thiolate salts (1, X = (subst)alkylthio; Y = $S^- Na^+$; Z = alkoxy). Structure-activity relationships for these water-soluble, anionic inhibitors, have been determined and compared with analogous relationships for the neutral cyanoacrylates, with a view to understanding similarities and differences in their interaction with the $Q_B$ binding domain.

## Materials and Methods

Cyanoacrylate thiolate salts were prepared by first reacting an acetonitrile solution of a cyanoacetic acid ester with carbon disulphide and sodium hydride and then treating the product formed *in situ* with an alkyl or ara-alkyl halide. The compounds were characterized by proton NMR spectra using a Jeol FX-90Q NMR spectrometer.

The following illustrates a typical experimental procedure. 4.9 g (0.025 mol) of ethoxyethylcyanoacetate was dissolved in 75 ml of dry acetonitrile and 1.9 g (0.025 mol) of carbon disulphide and 2.0 g (0.05 mol) of sodium hydride (60% dispersion in oil) stirred in. The reaction was left stirring for 30 min until it returned to room temperature when 4.0 g (0.025 mol) of *p*-chlorobenzyl chloride, dissolved in 28 ml of acetonitrile, was added dropwise. After the addition was complete, the mixture was stirred for a further 30 min at 60 °C then cooled to room temperature and filtered. The filtrate was evaporated to a yellow brown powder which was washed with chloroform and chromatographed on silica gel with ethyl acetate to give 3.0 g of a yellow solid *viz.:* the sodium salt of ethoxyethyl-3-*p*-chlorobenzylthio-2-cyano-3-mercapto acrylate.

Compounds were assayed as inhibitors of the Hill reaction using thylakoids isolated from the leaves of 21 day old *Pisum sativum* (pea) or 21 day old *Brassica napus* seedlings, the latter being either

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0341–0382/90/0500–0343 $ 01.30/0

susceptible (wild type) or resistant (mutant) to atrazine. The experimental procedure involved mixing 0.1 ml of thylakoid suspension (chlorophyll concentration ~10 μg/ml) with 4.8 ml of a pH 7.2 Tris-buffered solution of trichlorophenol indophenol (~$10^{-5}$ M) and 0.1 ml water in the case of the control or 0.1 ml of an aqueous solution of a thiolate salt of known concentration. The sample was then transferred to a 1 cm cuvette and the optical density at 648 μm ($OD_{648}$) determined against a blank containing 0.1 ml of thylakoid suspension and 4.9 ml of pH 7.2 Tris buffer. After a given equilibration time in the dark (30 sec to 10 min), the sample was illuminated for 45 sec by a 250 Watt Philips photolita lamp and the $OD_{648}$ remeasured. The ratio of the OD difference (pre- and post-illumination) in the presence of the inhibitor to that in the control represented the degree of inhibition of the rate of dye reduction associated with the particular concentration of inhibitor. The assay was repeated with different inhibitor concentrations and graphed to determine $I_{50}$ *i.e.* the molar concentration of compound giving 50% inhibition of the rate of dye reduction. Results in Tables 1–4 have been expressed as $pI_{50}$ *i.e.* as the negative logarithm of the $I_{50}$ value.

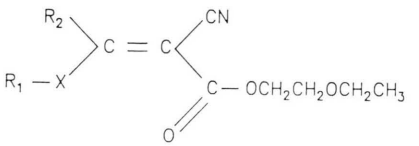
Compounds giving the same level of inhibition at different equilibration times were classified as fast binders, *i.e.* equilibrium was complete within 30 sec, whilst those showing a progressive change in the level of inhibition with time were classified as slow binders.

## Results

Table I compares $pI_{50}$ values using pea thylakoids for a series of sodium salts of ethoxyethyl-3-alkylthio-2-cyano-3-mercapto acrylates with values previously reported for an analogous series of ethoxyethyl-3-alkylamino-2-cyano-3-ethyl acrylates [4]. The direct relationship between chain length and $pI_{50}$ value for the 3-alkylthio thiolate salts is similar to that observed with 3-alkylamino cyanoacrylates which suggests that, in both series, the 3-substituent region of the molecule interacts with the membrane lipids.

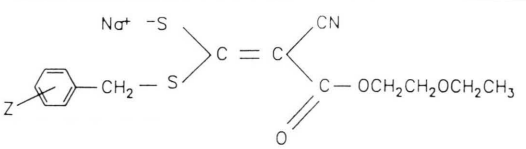
Table II A records $pI_{50}$ data (peas) for a series of sodium salts of ethoxyethyl-3-*p*-substituted benzylthio-2-cyano-3-mercapto acrylates. The spread of data is inadequate for a satisfactory Hansch regression analysis, although there are indications

Table I. The effect on $pI_{50}$ values (pea thylakoids) of varying the alkyl group in ethoxyethyl-3-alkylamino-2-cyano-3-ethyl acrylates and in the sodium salts of ethoxyethyl-3-alkylthio-2-cyano-3-mercapto acrylates.




| $R_1$ | $pI_{50}$ X = S, $R_2$ = $S^-$ $Na^+$ | $pI_{50}$ X = NH, $R_2$ = Et |
|---|---|---|
| $C_4H_9$ | 5.7 | 5.3 |
| $C_6H_{13}$ | 6.8 | 6.7 |
| $C_8H_{17}$ | 7.4 | 7.9 |

Table II. $pI_{50}$ values (pea thylakoids) for a series of sodium salts of ethoxyethyl-3-(substituted) benzylthio-2-cyano-3-mercapto acrylates.




| | Z | $pI_{50}$ |
|---|---|---|
| A | 4-H | 6.4 |
| | 4-F | 7.3 |
| | 4-Cl | 7.4 |
| | 4-Br | 7.2 |
| | 4-Me | 7.6 |
| | 4-Bu | 7.7 |
| | 4-Ph | 7.7 |
| | 4-$CF_3$ | 7.7 |
| | 4-OMe | 6.4 |
| | 4-CN | 6.8 |
| | 4-$NO_2$ | 7.4 |
| | 4-COOH | 4.0 |
| B | 2-Cl | 6.6 |
| | 3-Cl | 7.4 |
| | 4-Cl | 7.4 |
| | 2-F | 6.6 |
| | 3-F | 7.0 |
| | 4-F | 7.3 |
| | 2-$NO_2$ | 6.6 |
| | 3-$NO_2$ | 7.3 |
| | 4-$NO_2$ | 7.4 |

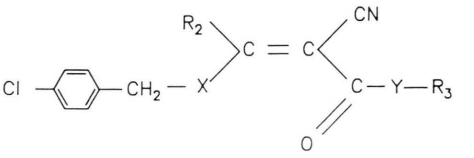
that the more lipophilic substituents *e.g.* $Bu^t$, $CF_3$, $CH_3$, F, Cl and Br are associated with higher activity ($pI_{50}$ >7.2) and the less lipophilic ones *e.g.* OMe and CN, with lower activity ($pI_{50}$ <6.8). The low activity of the *p*-carboxy derivative ($pI_{50}$ = 4.0) can

be attributed to it existing as the carboxylate ion under the conditions of the assay (pH = 7.2). The parent compound and its *p*-nitro derivative both behave anomalously, the former being less active ($pI_{50}$ = 6.4) and the latter more active ($pI_{50}$ = 7.4) than expected on the basis of lipophilicity. Similar anomalies have been observed with neutral benzylamino cyanoacrylates [5].

In general there is little difference in the activity of substituents in the *meta*- or *para*-positions of the benzyl moiety, although there is a 2–5-fold loss of activity with *ortho*-substituents (Table II B), suggesting some steric overlap between the *ortho*-position and the binding site. A similar trend, although the effect is of greater magnitude, has also been noted with neutral benzylamino cyanoacrylates [5].

Table III shows the influence of different ester groups on $pI_{50}$ (pea) in both a thiolate salt and a neutral cyanoacrylate. The activities of the thiolate salts appear to be relatively insensitive to variations in the chain length of the alkyl ester or to replacement of the alkyl by an alkoxyethyl group. This is in contrast to the behaviour of the neutral cyanoacrylates, where increasing the chain length of an alkyl ester led to a progressive loss of activity and replacement of an alkyl by an alkoxyethyl group led to a significant enhancement of activity [6]. This would indicate that the ester group in neutral cyanoacrylates is closely associated with the binding peptide and that the ether oxygen in the alkoxyethyl ester interacts specifically with that peptide [6]. Thiolate salts, on the other hand, behave as if the ester group were located in an aqueous environment out of contact with both the membrane and the binding peptide.

Table III. The effect on $pI_{50}$ values (pea thylakoids) of varying the ester group in 3-*p*-chlorobenzylamino-2-cyano-3-methylmercapto acrylates and in the sodium salts of 3-*p*-chlorobenzylthio-2-cyano-3-mercapto acrylates.



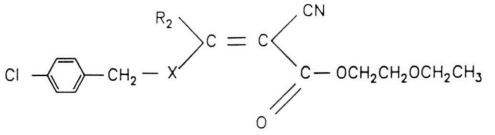

$pI_{50}$

| $YR_3$ | X = S, $R_2$ = $S^- Na^+$ | X = NH, $R_2$ = SMe |
|---|---|---|
| OEt | 7.0 | 7.0 |
| OPr | 7.2 | 6.6 |
| OBu | 7.3 | 6.3 |
| OEtOMe* | 7.3 | 7.8 |
| OEtOEt** | 7.4 | 8.5 |

* Methoxyethyl.
** Ethoxyethyl.

Table IV records $pI_{50}$ values and the relative rate of binding with atrazine-susceptible (wild type) and atrazine-resistant (mutant) *B. napus* thyla-

Table IV. $pI_{50}$ values and the rate of binding with atrazine-susceptible (wild type) and atrazine-resistant (mutant) *Brassica napus* thylakoids for atrazine, diuron, picric acid, ethoxyethyl-3-*p*-chlorobenzylamino-2-cyano3-methylmercapto acrylate and the sodium salt of ethoxyethyl-3-*p*-chlorobenzylthio-2-cyano-3-mercapto acrylate.




| Compound | Wild type** *B. napus* Rate of binding* | $pI_{50}$ | Mutant*** *B. napus* Rate of binding* | $pI_{50}$ | $\Delta pI_{50}$ |
|---|---|---|---|---|---|
| X = S; $R_2$ = $S^- Na^+$ | Slow | 7.4 | Slow | 7.4 | 0 |
| X = NH; $R_2$ = SMe | Fast | 8.4 | Fast | 7.0 | +1.4 |
| Atrazine | Fast | 6.5 | Fast | 3.5 | +3.0 |
| Diuron | Fast | 7.4 | Fast | 7.2 | +0.2 |
| Picric acid | Fast | 6.0 | Fast | 6.6 | −0.6 |

* See Materials and Methods.
** Atrazine-susceptible.
*** Atrazine-resistant.

koids for a thiolate salt (ethoxyethyl-3-*p*-chlorobenzylthio-2-cyano-3-mercapto acrylate-sodium salt), and a neutral cyanoacrylate (ethoxyethyl-3-*p*-chlorobenzylamino-2-cyano-3-ethyl acrylate). Data for atrazine, diuron and picric acid have also been included. As the Table shows, the thiolate salt is unable to discriminate between wild type and mutant *Brassica* thylakoids ($\Delta pI_{50} = 0$), indicating that it interacts with a region of the $D_1$ peptide away from the site of mutation *i.e.* $serine_{264}$. This is in contrast to the highly discriminatory behaviour of the neutral cyanoacrylate ($\Delta pI_{50} = +1.8$) and atrazine ($\Delta pI_{50} = +3.0$) and more like that of diuron ($\Delta pI_{50} = +0.2$) and picric acid ($\Delta pI_{50} = -0.6$). The thiolate salt, however, differs from the other PS II inhibitors in Table IV in reacting only slowly with the binding site possibly due to a slow conformational change in the binding peptide.

## Discussion

The structure of the thiolate salts and the data presented in Tables I–IV provide a basis for speculation regarding the orientation of these inhibitors within the $Q_B$ binding domain and the nature of their interaction with the receptor in terms of the Trebst model of the herbicide binding site [7]. This model locates the PS II RC between *trans*-membrane helices 4 and 5 of both the $D_1$ and $D_2$ peptides which are cross-linked by a non-heme iron atom (Fe) coordinating four histidine residues 215 and 272 ($D_1$); 215 and 262 ($D_2$). PS II inhibitors are assumed to bind in the region of the $D_1$ peptide between the non-heme Fe and the amino acid residues, extraneous to the membrane, which bridge helices 4 and 5.

The effects of varying the alkylthio (Table I) and benzylthio (Table II) substituents in the 3-position of the thiolate salts imply that this region of the molecule interacts with a lipophilic environment. On the other hand, varying the structure of the ester group has little effect on activity (Table III), indicating that the ester moiety is not in close proximity to either the binding peptide or the lipid membrane. The lack of discrimination between wild type and mutant *Brassica* thylakoids shown by the thiolate salts is also consistent with these compounds being out of contact with the binding peptide, at least in the $serine_{264}$ region. It would appear, therefore, that these thiolate inhibitors are oriented within the $Q_B$ binding domain, so that the 3-substituent lies towards the interior of the membrane and the ester group towards the outside – possibly in the aqueous environment.

The anionic character of the thiolate salts is a feature which distinguishes them from most types of PS II inhibitors except phenolic compounds. The bicarbonate anion [$HCO_3^-$] is known to influence the rate of photosynthetic electron transport and other small anions, such as formate and nitrite, have been shown to inhibit the [$HCO_3^-$] effect, presumably by competitive displacement [8]. Recently it has been reported that nitrous oxide [NO] can bind to the non-heme Fe in the PS II RC and that [$HCO_3^-$] can displace the NO [9]. This would suggest that [$HCO_3^-$] acts in the vicinity of the non-heme Fe, possibly coordinating to it or interacting with the adjacent $histidine_{215}$ residue of the $D_1$ peptide. It seems reasonable to suppose that the thiolate anion may act in the same region but, being a larger moiety, is also able to overlap the $Q_B$ binding site and thus inhibit photosynthetic electron transport.

Trebst [7] has classified PS II inhibitors into two broad categories, typified by urea/triazines on the one hand and phenols on the other and proposed that the former, which include the neutral cyanoacrylates, interact with a peptide bond close to the $serine_{264}$ ($D_1$) whilst the latter interact with $histidine_{215}$ ($D_1$). Cyanoacrylate thiolate salts fit into the phenol category because of their lack of discrimination between wild type and mutant thylakoids in which $serine_{264}$ is modified and their suggested interaction in the region of $histidine_{215}$. Such a classification is further supported by their slow binding behaviour which is similar to that observed with some phenolic inhibitors [10].

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