Inhibition and Photoaffinity Labeling of Photosystem II by Thiazolyliden-ketonitriles

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Herbicides, Photoaffinity Label, Photosystem II, Q_B Protein, Thiazolyliden-ketonitriles, Thylakoid Membrane

A new group of photosynthetic inhibitors is described. Thiazolyliden-ketonitriles are blocking electron flow at the acceptor side of photosystem II. They displace radioactive metribuzin from the membrane. Functional properties indicate that the new inhibitors share the binding site on the herbicide binding protein D-1 identical to that of the classical triazinone/urea-type inhibitors. However, fine details in the functional inhibition pattern, like lag in the inhibition and no loss of activity in tris-treated membranes, group them more specifically among the phenol-type family of photosystem II inhibitors. By photoaffinity labeling and immunoblotting it is shown that an azido-derivative of the thiazolyliden-ketonitriles tags the 32 kDa polypeptide subunit of photosystem II. This shows that also the phenol group inhibitors interact with the D-1 protein.

MO calculations show that the predominant tautomeric form of the new ketonitrile inhibitors when bound to the membrane is the hydroxy-form, whereas cyanoacrylates with a comparable essential element are bound in the keto-form. As the first is a phenol-type, the second an urea-type inhibitor, this allows to define more clearly the essential chemical element in the phenol-type inhibitors responsible for effective binding to the D-1 protein, probably oriented in the binding niche towards histidine 215, rather than towards serine 264, as triazine/urea and the cyanoacrylate derivatives do.

Numerous compounds inhibit photosynthetic electron flow on the acceptor side of photosystem II [1, 2]. Although of quite different chemistry the compounds displace each other from the membrane indicating identical binding sites [3]. A concept of specific binding sites overlapping each other in a common binding area was developed [4–6]. It accommodates differences in chemistry of the compounds and details of inhibitory pattern in photosynthetic electron flow between two groups of inhibitors – a triazine/urea and a phenol/hydroxypyridine family.

By photoaffinity labeling with an azido-triazine [7], azido-triazinone [8] and azido-urea [9] derivative it was shown that these inhibitors interact with a 32 kDa polypeptide subunit of photosystem II, since then called the herbicide or Q_B binding polypeptide D-1. On the other hand, an azido-phenol derivative [10] acts less specific with the membrane and labels several polypeptide subunits of PS II depending on the membrane preparation used.

Recently a model for the folding of the D-1 herbicide binding polypeptide through the membrane has been presented [11] and the topology of the herbicide and Q_B binding niche on this polypeptide described [11, 12]. According to these and the paper in this issue [13] all inhibitors would fit into the binding niche (*i.e.* the common binding area) on the D-1 polypeptide, but with the triazine family oriented towards serine 264 and the phenoltype family towards histidine 215 of the amino acid sequence of the D-1 polypeptide (*i.e.* the specific binding sites overlapping with each other). The two groups of inhibitors were called the serine and the histidine family [13].

We wish to report here on a new group of efficient inhibitors supporting this concept. Thiazolyliden-ketonitrile derivatives interfere with photosynthetic electron flow on the acceptor side of PS II with an inhibitor pattern similar to the phenol/hydroxy-

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Abbreviations: DCMU, dichlorophenyldimethylurea; DCPIP, dichlorophenolindophenol; DPC, diphenylcarbazide; LHCP, light harvesting chlorophyll protein; LiDS, lithiumdodecylsulphate; MNDO, modified neglect of diatomic overlap; PS II, photosystem II.

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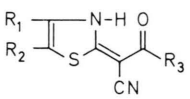
pyridine group. A radioactive and azido-photoaffinity derivative of these compounds binds to the 32 kDa polypeptide that is identified by immunoblotting, but also to other polypeptides of the membrane. This new series of compounds therefore connects the two families of inhibitors in its functional and chemical characteristics and allows to define more clearly the essential chemical elements responsible for inhibitor efficiency of the phenol-type inhibitors.

Materials and Methods

Synthesis of 2-(2-thiazolyliden)-3-oxonitriles



1

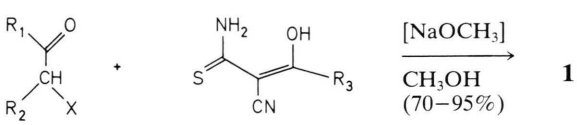
R_1 = H; alkyl, aryl
R_2 = H; halogen, alkyl, aryl
R_3 = alkyl, aryl; aralkyl, alkoxy, halogenalkyl

1a: R_1 = p-N_3-phenyl
R_2 = H
R_3 = CH_2-phenyl

Method A

Base-catalyzed condensation of C-acylated cyanothioacetamides (**3**) [14] with halogenoketones (**2**) (X = Cl, Br) results in thiazolyliden-oxonitriles (**1**) in good to very good yields ($NaOCH_3$, CH_3OH, 50 °C, 0.5 h).

Most of the oxonitriles **1** were synthesized by this method, specifically derivatives with R_1 = substituted phenyl and R_3 = benzyl.

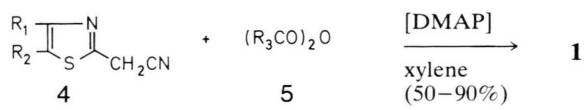


2 3 [$NaOCH_3$] / CH_3OH (70–95%) → **1**

Method B

2-cyanomethylthiazoles (**4**) [15] were reacted with acid anhydrides (**5**) (or acid chlorides) under catalysis of dimethylaminopyridine in boiling xylene (5 mol% DMAP, 140 °C, 1 h).

The yields of the oxonitriles (**1**) were in the range of 50–90%. Especially compounds **1** with R_3 = CF_3 were synthesized by this method.



4 5 [DMAP] / xylene (50–90%) → **1**

Method C

The base-catalyzed condensation of α-thiocyanato-ketones (**6**) with cyanoacetic esters (**7**) was used for synthesis of oxonitriles (**1**) with R_3 = alkoxy [18].

6 7 → **1**

Synthesis of 2-[4-(4-chlorophenyl)-5-ethyl-2,3-dihydrothiazol-2-yliden]3-oxo-4,4,4-trifluoro-butyronitrile

(according to method B)

A solution of 5.26 g (0.02 mol) 5-ethyl-4-(4-chlorophenyl)-2-cyanomethyl-thiazole [19], 0.2 g dimethylaminopyridine and 4.2 g (0.02 mol) trifluoroacetic-anhydrid in 5 ml xylene was heated for 1.5 h at 120 °C. After destillation of solvents *in vacuo* the residue was recrystallized from methanol. 6.19 g (86.4%), Fp. 210 °C.

Analysis

Calc.: 50.21% C 2.80% H 7.80% N 9.88% Cl
Found: 50.72% C 3.11% H 7.99% N 10.29% Cl
IR: $-C\equiv N$ 2210 cm^{-1}
>= 0 1605; 1625 cm^{-1} (KBr)
^1H NMR δ (in DMSO):
7.52–7.62 (m, 4 arom H.)
2.75 (q, J = 7.5; $-O\underline{CH_2}-CH_3$)
1.21 (t, J = 7.5; $-OCH_2\underline{CH_3}$)

Synthesis of 2-(4-phenyl-2,3-dihydrothiazol-2-yliden)-2-cyanoacetic-n-butylester

(according to method C)

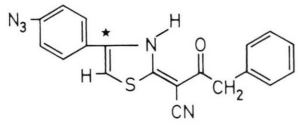
A solution of 1.77 g (0.01 mol) 2-thiocyanato-acetophenone, 1.84 g (0.013 mol) cyanoacetic-*n*-butylester and 4.11 ml (0.03 mol) triethylamine in 5 ml dimethylformamide was stirred over night at room temperature. After addition of 100 ml water

the mixture was acidified to pH 5 by addition of acetic acid followed by extraction with ethylacetate. The crude product was crystallized from methanol. 1.77 g (58.3%), Fp. 130–132 °C.

Analysis

Calc.: 63.97% C 5.37% H 9.33% N 10.68% S
Found: 63.89% C 5.58% H 9.20% N 10.39% S
IR: $-C\equiv N$ 2210 cm^{-1};
$-COOC_4H_9$ 1660 cm^{-1} (KBr)

*Synthesis of the radioactive azido-derivate (**1a**)*
according to method A
(by Dr. B. Acksteiner, Dep. Isotopenchemie der Schering AG, Berlin–Bergkamen)



1a

4-Azido-[carboxyl-^{14}C]benzoic acid

70 mg (0.5 mM) (ca. 925 MBq) 4-amino-[carboxy-^{14}C]benzoic acid (Amersham) were dissolved in 0.1 ml 10 N HCl and 0.25 ml water by heating to 50 °C for 10 min.

At room temperature 34 mg (0.5 mM) sodium nitrite in 0.125 ml water were added. After 20 min the solution was cooled to 5 °C and 32 mg sodium azide in 0.125 ml water were added. Stirring was continued for 5 min followed by the addition of 1.5 ml water. The crystals were collected and recrystallized from ethanol-water. 69 mg with a Fp. 181 °C (Dec.).

4-Azido-[carboxyl-^{14}C]benzoyl chloride

To the suspension of 69 mg 4-azido-[carboxyl-^{14}C]-benzoic acid in 0.2 ml thionyl chloride, 5 µl dimethyl formamid were added. After 0.5 h at 60 °C the excess of thionyl chloride was evaporated. The crude acid chloride was dissolved in 1 ml toluene and the crude product was used in the next reaction.

4-Azido-[carbonyl-^{14}C]chloroacetophenone

The crude acid chloride in 1 ml toluene was added to a solution of diazomethane (prepared from 0.3 g N-nitrosomethyl urea and 0.4 ml 40% KOH in 3 ml diethyl ether) with ice cooling. After 0.5 h 0.3 ml 10 N HCl were added. The reaction (transformation of diazoketone to the chloroketone) was monitored by TLC.

The solvents were carefully evaporated *in vacuo*, the residue was dissolved in ethyl acetate and washed for several times with sodium bicarbonate and sodium chloride solutions. After evaporation of the solvent 62 mg of nearly pure chloroketone (Fp. 64–65 °C) were obtained.

2-[4-(4-Azidophenyl)-2,3-dihydro-4-^{14}C-thiazol-2-yliden]3-oxo-4-phenyl-butyronitrile (**1a**)

To a suspension of 69.3 mg (0.32 mM) 2-cyano-3-hydroxy-4-phenyl-2-butenethioamide in 0.3 ml ethanol, 250 µl of a solution of 50 mg sodium in 2 ml ethanol were added, followed by the addition of 62 mg 4-azido-[carbonyl-^{14}C]chloroacetophenone, dissolved in 1.2 ml ethanol. After 2 h at 30 °C the crystals were collected, washed and recrystallized from ethanol and acetonitrile. Further purification is reached by NDC on silica ICN 30–100/methylen-chlorid.

Yield: 20 mg with Fp. 176 °C.
Radioactivity: 102 mBq 1835 MBq/mmol.
IR: $-C\equiv N$ 2200 cm^{-1}; $N=\overset{\oplus}{N}-\underline{\overset{\ominus}{N}}-$ 2080, 2120 cm^{-1}

1610 cm^{-1} (KBr)

^{1}H NMR (in DMSO): 7.73 (d, J = 8.4; 2 arom H)
(60 MHZ) 7.13 (d, J = 8.4; 2 arom H)
7.23 (s; 5 arom H)
7.4 (s; thiazol-H)
3.9 (s;)

Azido(ethyl-1-^{14}C)-atrazine and 4-azido(methyl-^{14}C)-diuron were obtained from Amersham.

Inhibitory potency in photosynthetic reactions

Spinach chloroplasts were prepared by homogenizing leaves in 0.4 M NaCl, 20 mM tricine-NaOH buffer, pH 8.0, and 20 mM $MgCl_2$. Tris-treated chloroplasts were prepared essentially according to [20]. Accordingly thylakoid membranes (5 mg chlorophyll) were suspended in 10 ml of 1 M Tris buffer, pH 9.0, and centrifuged down after 10 min at 0 °C. After washing in 20 mM MES buffer, pH 6.5, con-

taining 5 mM $MgCl_2$ and 15 mM NaCl they were resuspended in the same buffer. The photosystem II preparation was prepared according to Berthold *et al.* [21].

Photosynthetic activity was measured either spectroscopically with ferricyanide (420 nm) or DCPIP (600 nm) as acceptor or by an oxygen electrode with methylviologen as acceptor in uncoupled (10 μg gramicidin) thylakoid membranes (equivalent to 50 μg chlorophyll) in 30 mM MES buffer, pH 6.8, 10 mM $MgCl_2$ and 0.3 mM Na-azide. Inhibitors were added to the probes in 10 μl methanol that was also added to the controls.

For the binding and displacement studies, thylakoid membranes with 100 μg chlorophyll were incubated with 0.1 μM [^{14}C]metribuzin at pH 8.0 [22]. The photoaffinity labeling was performed according to the protocol by Oettmeier *et al.* [8, 23]. Spinach thylakoids with 0.5 mg/ml chlorophyll were illuminated with the radioactive label (10 nmol/mg chlorophyll) for 10 min under nitrogen with a mercury lamp at 0 °C. Membranes were then dissolved in 3% LiDS + $NaHCO_3$ + saccharose at room temperature, separated on polyacrylamide gel (11–15%) at 4 °C for 16 h. They were either directly exposed to a X-Omat film (XAR-5) or first blotted (Western blot [24]) for 3 h and 0.6 Å at 10 °C on nitrocellulose in 25 mM Tris, 192 mM glycine and 20% methanol. After saturation with 3% gelatine in Tris buffer pH 7.5 the first antibody was allowed to react overnight at room temperature in 1% gelatine. After washing in Tris and 0.05% 'tween 20 the second antibody (horseradish peroxidase conjugated) was allowed to react in 1% gelatine for 1 h, dilution 1:3000 and developed with HPR colour development and 0.005% H_2O_2. They were then laid on X-Omat film for spotting the radioactivity.

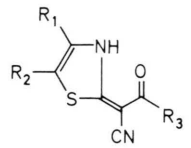
The antibody to the D-1 herbicide binding polypeptide was kindly provided by J. Hirschberg, Jerusalem, obtained from a fusion protein with part of the psbA gene [25].

MO calculations were done according to [26].

Results

The thiazolyliden-ketonitriles were synthesized and tested in photosynthetic reactions of isolated chloroplast thylakoid membranes. The pI_{50}-values for several derivatives are given in Table I showing a range of inhibitory potency from below 5 to above 7 depending on substituents. One of the most effective inhibitors – compound **19** in Table I – was checked in more detail in various part reactions and properties of photosynthetic electron flow. Besides inhibiting ferricyanide photoreduction it also inhibits methylviologen photoreduction. In this the compounds show a distinct lag of several minutes depending on concentration before inhibition starts and becomes constant. This is similar to the behaviour noted before for phenol inhibitors [27, 28] and is not observed with metribuzin or DCMU [6]. The thiazolyliden-ketonitrile derivative 19 displaces efficiently ^{14}C-labeled metribuzin from the membrane as well as DCMU does (Fig. 1). This displacement tech-

Table I. Inhibitory potency of substituted thiazolyliden-3-ketonitriles in photosynthetic electron flow. pI_{50} = negative logarithm of the concentration that inhibits photosynthetic electron flow in isolated thylakoid membranes. All keto-(= oxo-)nitriles are derivatives of compound **1** in methods, No. 21 is the same as **1a** in methods.



No.	R_1	R_2	R_3	pI_{50}
1	phenyl	H	OCH_3	4.8
2	phenyl	H	OC_4H_9	4.52
3	phenyl	H	phenyl	5.0
4	phenyl	H	CH_2-phenyl	5.22
5	phenyl	H	CF_3	5.48
6	phenyl	H	$-CH_2-$4Cl-phenyl	4.70
7	phenyl	H	$-CH_2-$2,4-diCl-phenyl	4.52
8	CH_3	H	$-CH_2-$4Cl-phenyl	4.52
9	CH_3	H	$-CH_2$-phenyl	4.52
10	t-butyl	H	$-CH_2$-phenyl	6.15
11	2-naphtyl	H	$-CH_2$-phenyl	6.70
12	4Cl-phenyl	H	$-CH_2$-phenyl	6.10
13	4Cl-phenyl	C_2H_5	$-CH_2$-phenyl	6.10
14	4Cl-phenyl	C_3H_7	$-CH_2$-phenyl	5.15
15	4Cl-phenyl	C_2H_5	CF_3	6.22
16	4Cl-phenyl	C_2H_5	CH_3	5.30
17	4Cl-phenyl	C_3H_7	CF_3	5.52
18	4Cl-phenyl	C_6H_{13}	CF_3	5.28
19	2,4-diCl-phenyl	H	CH_2-phenyl	7.20
20	3,4-diCl-phenyl	H	CH_2-phenyl	6.70
21	4-azido-phenyl	H	CH_2-phenyl	7.10

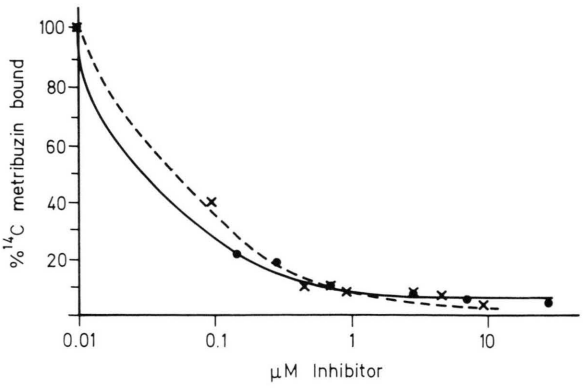


Fig. 1. Displacement from the thylakoid membrane of radioactive [^{14}C]metribuzin by a thiazolyliden-ketonitrile in comparison with DCMU. ●———● = DCMU; x....x = thiazolyliden-ketonitrile compound **19**.

Table II. Difference in inhibitory efficiency of two groups of inhibitors in control thylakoid *vs.* Tris-treated thylakoid membranes (testsystem: H_2O to DCPIP *vs.* DPC to DCPIP).

	pI_{50}	
	Thylakoid membranes	Tris-treated thylakoid
Thiazolyliden-ketonitrile 19	7.5	7.45
Bromonitrothymol	7.9	7.9
DCMU	7.2	6.7
Cyanoacrylate	7.4	6.4
Phenisopham	7.3	5.3

Cyanoacrylate = ethoxyethyl-2-cyano-3-*n*-decylaminoacrylate [37] kindly provided by John Phillips, Canberra.

nique [3, 22] indicates identical binding sites for the three compounds involved.

In Tris-treatment of the photosynthetic membrane the peripheral polypeptides on the donor side of photosystem II are displaced from the membrane [29] and oxygen evolution is lost [20]. The photosynthetic reaction has now to be measured by using an artificial electron donor DPC. As reported before [30] Tris-treatment has not only an effect on the donor side of photosystem II, but also on the acceptor side (now easily understood because the polypeptides involved span the membrane several times [13]). Therefore Tris-treatment changes the inhibitory efficiency of some compounds affecting the acceptor side of PS II. But in that it discriminates between the two families of inhibitors. As Table II shows DCMU, atrazine, phenisopham and a cyanoacrylate loose inhibitory potency in Tris-treated membranes. But the phenol-type inhibitors, like bromonitrothymol (and a hydroxypyridine [30]) do not. The thiazolyliden-ketonitrile derivative 19 also does not loose its inhibitory potency and therefore belongs to the phenol-type family.

A photoaffinity labeled radioactive derivative of the thiazolyliden-ketonitriles (**21** in Table I) was synthesized. It is a very effective inhibitor and in addition binds covalently to the membrane when on illumination by strong light the reactive nitren is formed. After covalent labeling (of a PS II preparation in Fig. 2, but with identical results with thylakoid membranes) the membrane was dissolved in detergent and the individual membrane polypeptides

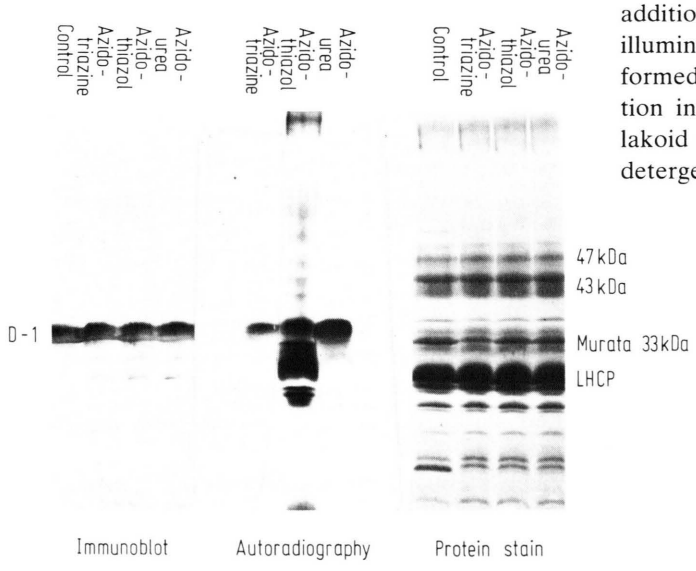


Fig. 2. Photoaffinity labeling of photosystem II particles with a radioactive azido-thiazolyliden-ketonitrile (No. 21) compared with an azido-triazine and an azido-urea. After photolabeling the membranes were dissolved in detergent and the polypeptides separated on agar gel electrophoresis. Column left: western blot with antisera against the D-1 protein; column middle: autoradiography; column right: protein stain of the polypeptide pattern. Lanes in the three columns from left to right: control, azido-triazine, azido-thiazol, azido-urea.

separated by agar gel electrophoresis. The position of the major radioactive band obtained comparing three photoaffinity labels is at the 32 kDa polypeptide (Fig. 2). This is well known for azido-triazine [7] and has been reported also for azido-urea derivatives [9, 31] and for azido-triazinone [8]. In case of the azido-thiazolyliden-ketonitrile derivative **21** further radioactive bands appeared on the autoradiography. There is in particular radioactivity on the LHCP polypeptides and below. There is also some radioactivity above in the 40 kDa region. This is only slightly so with azido-urea, but not with azido-triazine. In these additional bands in the labeling with compound **21** it reflects earlier reports on the labeling of spinach thylakoids or reaction center preparations from a blue-green algae with an azido-phenol derivative [10, 31] where there are bands in the 40 kDa region (41, 43, 47 kDa) depending on the membrane preparation used.

The identity of the 32 kDa label was checked by immunoblotting. A specific antibody to the D-1 polypeptide prepared by J. Hirschberg [25] blotted specifically the radioactive 32 kDa band in all three preparations (Fig. 2). There is little indication that the tagging with the photoaffinity label shifts the position of the polypeptide on the gel or gives rise to another band of different conformation of the D-1 polypeptide, as there is no further band in the immunoblots.

MNDO calculations [26] were used to determine the charge distribution and to calculate the tautomeric equilibrium between the keto- and the hydroxy-form of a thiazolyliden-ketonitrile in comparison with a cyanoacrylate. We had earlier [32] pointed to the possible significance of a positive or negative π-charge on a particular atom equivalent to C2 in the structures in Table II. There is, however, no marked difference between the forms calculated here (Fig. 3), but there is a marked difference in the tautomeric equilibrium. According to the data in Table III where a negative energy indicates a more stable, a positive energy a more labile structure than the reference, the thiazolyliden-ketonitriles prefer *in vacuo* (apolar environment) the hydroxy-form C and in a polar solvent $[\Delta H(\mathrm{f}) + (1\text{-}1/_{\varepsilon})\Delta H(\mathrm{s})]$, ε = dielectricity constant] both hydroxy- and keto-form. The substituted cyanoacrylate from a series of very efficient inhibitors, introduced by Huppatz and Phillips [37] will, however, prefer *in vacuo* both the keto- and hydroxy-form and the keto-form A in a polar environment. Assuming the binding of inhibitors to

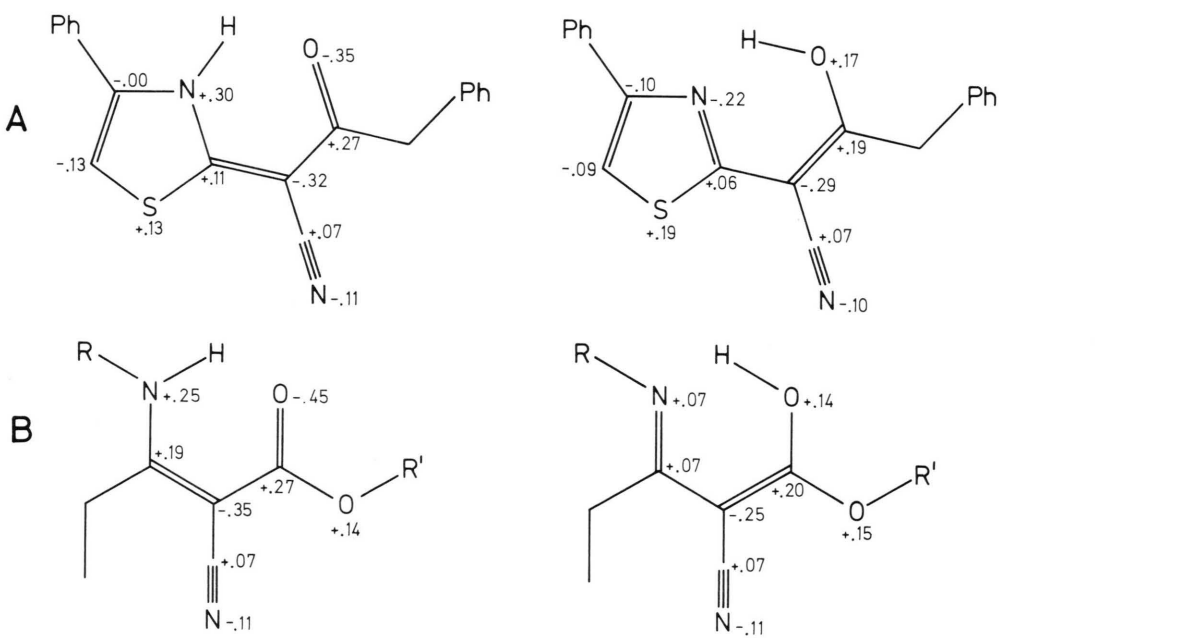
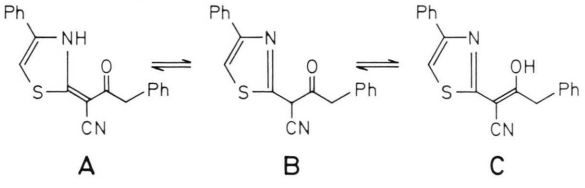


Fig. 3. π-charge distribution of a thiazolyliden-ketonitrile = A and a cyanoacrylate = B in the keto- and the hydroxy-form, calculated by MNDO [26].

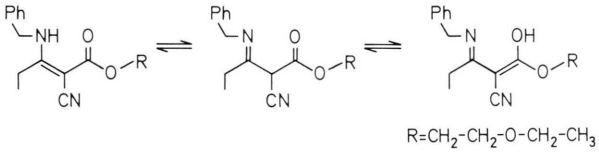
Table III. The equilibrium between the keto- and hydroxy-form of a thiazolyliden-ketonitrile, compared with a cyanoacrylate.

Phenyl-thiazolyliden-oxobutyronitrile



A B C

Ethoxyethylester of 2-cyano-3-ethyl-3-(4-chlorbenzyl)-aminoacrylate (R = ethoxyethyl)



A′ B′ C′

	Relative heat of formation [kcal/mol] (Hf)	+	Relative heat of solvation [kcal/mol] (Hs)	= sum	Ionization potential [eV] I	Dipol momen μ [Deb
A	5.3		−5.1	0.2	8.8	5.5
B	10.1		2.8	12.9	9.5	1.9
C	0		.0	.0	9.2	3.2
A′	.4		−8.4	8.0	9.3	3.9
B′	4.5		.9	5.3	9.4	4.6
C′	.0		.0	.0	9.2	.4

The bond length and angles were optimized, except for rigid p rings. The structures were assumed to be planar, except for perpendicular phe-rings.
A problem in the calculation is that the thiazolyl ring is aromati B and C, but not in A.

occur in a hydrophobic domain of the D-1 polypeptide [12, 13] the binding niche prefers the hydroxy-form of the thiazolyliden inhibitor. The functional properties would be in agreement with the calculation that on the other hand the cyanoacrylate prefers the keto-form.

Discussion

The thiazolyliden-ketonitrile compounds described here are potent inhibitors of photosystem II. The structure activity correlation of the derivatives tested indicates that maximal inhibition is obtained with compounds that carry a benzyl group next to the carbonyl (or hydroxyl) and a strong lipophilic group at the thiazolyliden ring at its 4 position. They inhibit photosynthetic electron flow whether an acceptor for photosystem I or photosystem II is used. Not documented in this paper is that the compounds have no effect on either photosystem I, nor the cytochrome b_6/f-complex. The new groups of inhibitors displace [^{14}C]metribuzin from the membrane indicating an identical binding site. In this functional behaviour the new compounds are similar to all the other specific inhibitors of the acceptor side of photosystem II, *i.e.* at the Q_B site on the 32 kDa polypeptide subunit [3]. In showing a lag (in minutes) before the inhibition of electron flow gets constant, in photoaffinity labeling and in their behaviour in Tris-treated thylakoid membranes the new compounds follow specifically that subgroup of inhibitors, called the phenol-type family [4, 6, 12, 32]. As reported [30] in Tris-treated thylakoid membranes the compounds of the "classical"-type – diuron/triazine, biscarbamates, cyanoacrylates – loose inhibitory potency, whereas those of the phenol-type family including hydroxypyridines are as effective as in control thylakoids. Tris-treatment of the membrane has also no effect on the thiazolyliden compounds described here. As discussed [12, 13] Tris-treatment mimics very much the effect of the serine 264 change in triazine tolerant mutants [33, 34] as far as cross-resistance of the different compounds is concerned because the classical inhibitors loose binding affinity, whereas the phenol compounds may actually increase in potency [5, 33–36]. It was suggested [13] that those compounds that loose inhibitory potency in Tris-treatment or in a serine mutation may primarily gain affinity to the membrane polypeptides by a hydrogen bridge towards their carbonyl group from a peptide group close to serine 264 in the amino acid sequence of the D-1 protein, whereas the other family with an OH-group reacts towards histidine 215 with either a nitro or free electron pair on a heteroatom.

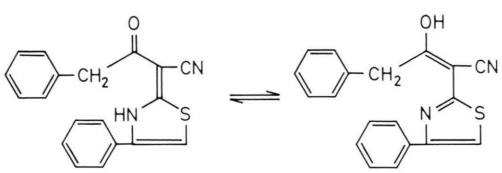
The photoaffinity labeling experiments with different compounds support the grouping of the new compounds. The photoaffinity labeling with an

azido-derivative, followed by immunoblotting, showed clearly that the thiazolyliden-ketonitriles bind to the 32 kDa herbicide binding polypeptide. By comparison with the photoaffinity labeling and the results in the literature [7–10, 31] they follow the pattern of an azido-dinoseb labeling in that additional membrane polypeptides are tagged, whereas the azido-triazines, -triazinone and -urea derivatives are more specific [7–9].

The new compounds described here allow to define more clearly at the molecular level the differences in the two groups of inhibitors (triazines *vs.* phenols) that bind to the 32 kDa polypeptide and how they orient in the binding niche.

The thiazolyliden-ketonitriles can be described in two tautomeric forms. Calculating the energy and electron density charges it appears that the keto-form is more stable in polar and the hydroxy-form predominantly in apolar media, like the membrane. If they are oriented in the hydrophobic binding niche in the 32 kDa polypeptide in the hydroxy-form with the lipophilic side chain arresting it like it does Q_B, the hydroxy group cannot form the hydrogen bridge to the peptide bond in the stretched amino acid sequence close to the serine 264, whereas the triazines, triazinones, ureas, cyanoacrylates and the other members of this group do, as the latter contain a carbonyl – or equivalent suitable group. Also the cyanoacrylates [37] follow this later group, as the tautomeric equilibrium is shifted in an apolar medium towards the carbonyl-form. This explains easily the functional difference in the two nitrilo compounds – thiazolyliden-ketonitrile and cyanoacrylate, discussed here, where the essential element appears quite similar, but is shifted in the binding niche to different tautomeric forms. Whereas the keto-group of the cyanoacrylates approach the peptide bond close to serine 264, the thiazolyliden-ketonitriles may be pushed towards histidine 215 and form a hydrogen bridge there *via* the heteroatom of the thiazol ring.

This interaction with a histidine that at the same time is involved in metal binding – a Fe in the case of his 215 – might be responsible for the unspecific binding of this type of inhibitors to other polypeptides of the membrane in the photoaffinity labeling experiments, as is characteristic for the phenol-type inhibitors. In particular, such histidines occur in the chlorophyll containing polypeptides of the LHCP and 43/47 kDa subunits of photosystem II where likely histidines are involved in chlorophyll binding.



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