Irreversibly Binding Photosynthetic Electron Transport Inhibitors II. Halogen-Substituted 1,4-Naphthoquinones and Halogenmethyl-1,4-quinones*

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Several halogen-substituted 1,4-naphthoquinones have been synthesized and found to be effective photosystem II inhibitors. Due to their properties as vinylogous acid halides they can react with nucleophiles under formation of a covalent linkage. In their presence other photosystem II herbicides show a decreased binding affinity. This decrease is dependent from the preincubation time. Halogenmethyl-1,4-quinones also turned out to be efficient photosystem II inhibitors and, in addition, possessed herbicidal *in vivo* activity. They function as "bioreductive alkylating agents" in a way that after reduction they can split off hydrogen halide under formation of a *o*-quinonemethide. This quinonemethide can react with nucleophilic groups in proteins.

Introduction

In photosynthetic electron transport the reducing power of photosystem II is transferred to the cytochrome b_6/f-complex *via* plastoquinone. Plastoquinone reduction and plastohydroquinone reoxidation can be inhibited specifically and selectively by a broad variety of compounds. In this respect, quinone type inhibitors are of special interest because they can undergo redox reactions like plastoquinone itself.

We have recently reported that halogen-substituted 1,4-benzo- and -naphthoquinones are powerful inhibitors of photosystem II electron transport [1, 2]. Halogenated 1,4-benzoquinones in addition inhibit electron transport through the cytochrome b_6/f-complex [1]. Due to their chemical nature as vinylogous acid halides, halogen-substituted 1,4-benzoquinones in a Michael type addition/elimination reaction can add onto nucleophilic groups in proteins under formation of a covalent linkage. In isolated thylakoids a [^{14}C]tetrabromo-1,4-benzoquinone (bromanil) labels a 20 kDa protein which comigrates with the Rieske iron-sulfur protein and, in addition, a 41 kDa protein [1].

We wish to report here that 1,4-naphthoquinones with one or two halogen substitutions in the 2- and/or 3-position(s) can react covalently in a similar fashion like halogenated 1,4-benzoquinones. The same is true for halogenmethyl-1,4-quinones. The latter, however, have to be transformed into a reactive state by reduction. This reduction to the hydroquinone can be achieved by the photosynthetic electron transport chain. After reduction, hydrogen halide can be split off and the resulting *o*-quinonemethide can react with a nucleophilic group ("bioreductive alkylation"). Due to the fact that an activation is required before a reactive species is formed, halogenmethyl-1,4-quinones can be considered as suicide substrates or mechanism based enzyme inactivators [3]. Besides their *in vitro* activity as inhibitors of photosynthetic electron transport, these compounds also show *in vivo* herbicidal activity.

* Part I of this series see ref. [1].

Abbreviations: asc, ascorbate; Chl, chlorophyll; DCIP, dichlorophenolindophenol; DAD, diaminodurene; DNP-INT, 2-iodo-2′,4,4′-trinitro-3-isopropyl-6-methyl-diphenylether; TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine.

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Materials and Methods

The 1,4-quinones as listed in Tables I–III were synthesized according to the methods or references in ref. [2] and [4]. [^{14}C]atrazine (spec. activity 5.9 mCi/mmol) was a generous gift from CIBA-GEIGY, Basle, Switzerland.

Chloroplasts from spinach were prepared according to [5] and stored in liquid nitrogen in the presence of 10% glycerol. NADP- and DCIP-reduction, the latter in the presence of 1 μM DNP-INT [6] to allow only for photosystem II dependent reduction were performed as described in [7]. First, the control rates were determined. Then the quinone was added and its inhibitory activity assayed immediately after addition.

For binding experiments, thylakoids corresponding to 100 μg Chl were incubated in 2 ml of a medium containing 20 mM tricine buffer, pH 8.0, and 20 mM $MgCl_2$ with 2-bromo-3-*n*-propyl-1,4-naphthoquinone for the time indicated. Then [^{14}C]-atrazine was added and incubation continued for another 10 min. Thylakoids were pelleted by centrifugation at 10000 × *g* for 10 min and the pellet and an aliquot of the supernatant assayed for radioactivity in a 1219 Rackbeta liquid scintillation counter (LKB-Wallac) with automatic quench correction.

Results and Discussion

1. Halogen-1,4-naphthoquinones

Table I shows the inhibitory activity of various halogen-substituted 1,4-naphthoquinones on electron transport through photosystem II. This table includes 2-bromo-3-isopropyl-1,4-naphthoquinone (compound **8**) which has been recognized as the most active naphthoquinone type photosystem II inhibitor so far [8]. However, several other naphthoquinones exceed the latter in their inhibitory potency (compounds **9**–**11**). In general, activity increases if an alkyl side chain is present and from chlorine to bromine to iodine. Contrary to halogen-substituted 1,4-benzoquinones, halogen-substituted 1,4-naphthoquinones are only weak inhibitors of electron transport through the cytochrome b_6/f-complex (data not shown).

Table I. pI_{50}-values for inhibition of photosystem II-dependent DCIP-reduction (in the presence of DNP-INT) for various halogen-substituted 1,4-naphthoquinones.

Compound	R^2, R^3	pI_{50}-value
1	Br, H	4.72
2	Cl, CH_3	5.08
3	Br, CH_3	5.44
4	Cl, Cl	5.48
5	Br, $C_6H_5CH_2$	5.63
6	Br, *n*-C_4H_9	5.71
7	Br, $(CH_3)_2CH$	5.73
8	Br, *n*-C_7H_{15}	5.74
9	Br, Br	5.89
10	Br, *n*-C_3H_7	5.96
11	I, I	6.16

We had recently demonstrated that halogenated 1,4-benzoquinones in a Michael type addition/elimination can react with nucleophilic compounds, for instance β-mercaptoethanol [1]. The replacement of one or more bromine atoms in tetrabromo-1,4-benzoquinone (bromanil) by the thio-(2-hydroxy)-ethyl moiety will lead to new compounds which are characterized by a lower pI_{50}-value as compared to the parent compound. In an actual experiment this looked like a reversal of bromanil inhibition by β-mercaptoethanol [1]. A similar experiment using 2-bromo-3-*n*-propyl-1,4-naphthoquinone (compound **10**, Table I) is demonstrated in Fig. 1. Here photosynthetic NADP-reduction was monitored because β-mercaptoethanol chemically reduces DCIP. In addition, DNP-INT [6] is present in the assay mixture and TMPD, to bypass the DNP-INT inhibition [9]. This was done to overcome a slight inhibition of electron transport by the naphthoquinone in the

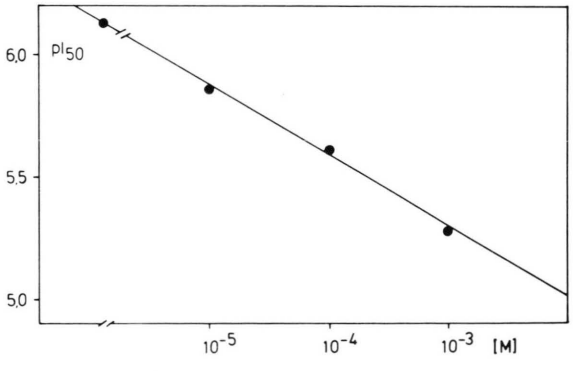

Fig. 1. Inhibition of photosynthetic NADP-reduction (in the presence of 1 μM DNP-INT and 100 μM TMPD) by 2-bromo-3-*n*-propyl-1,4-naphthoquinone after addition of varying amounts of β-mercaptoethanol. The incubation time with β-mercaptoethanol was 2 min.

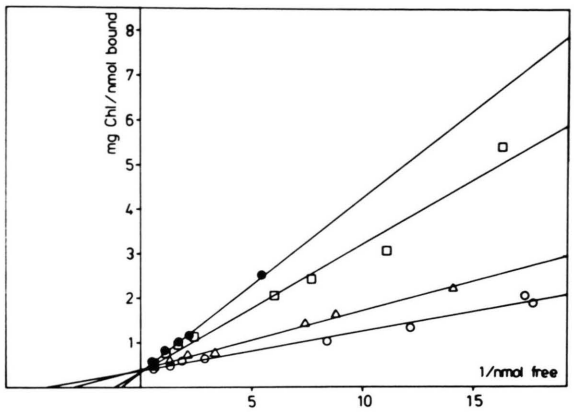

Fig. 2. Lineweaver-Burk plot for [^{14}C]atrazine binding to isolated thylakoids after various preincubation times with 2-bromo-3-*n*-propyl-1,4-naphthoquinone. (○———○) control, (△———△) 10 min, (□———□) 30 min, (●———●) 40 min. Chl = chlorophyll; free = unbound labeled atrazine.

cytochrome b_6/f-complex. Under these conditions the pI_{50}-value of 2-bromo-3-*n*-propyl-1,4-naphthoquinone drops from 6.1 to 5.3 in the presence of 1 mM β-mercaptoethanol. This indicates that indeed β-mercaptoethanol can react chemically with the naphthoquinone in a covalent fashion.

If halogen-naphthoquinones as inhibitors of photosystem II are binding covalently in the photosystem II reaction center core complex in the vicinity of the Q_B binding site, this should affect the binding properties of another photosystem II inhibitor. Covalent binding is a time-dependent process and, hence, the binding of another photosystem II inhibitor should be more affected with increasing time. In the experiment as shown in Fig. 2, 2-bromo-3-*n*-propyl-1,4-naphthoquinone (100 nmol/mg Chl) was allowed to react with thylakoids for different time intervals and subsequently the binding of [^{14}C]-atrazine has been studied. For increasing time intervals, the affinity of atrazine decreased (ordinate intercepts) whereas the number of binding sites remained constant (abscissa intercepts). If the binding sites for atrazine and 2-bromo-3-*n*-propyl-1,4-naphthoquinone were identical, one would expect no change in the atrazine affinity but a decrease in the number of binding sites. Obviously, the binding sites for the two compounds are different. A similar observation was reported by Vermaas *et al.* [10]. They have used a covalently binding azido-quinone and studied atrazine and ioxynil binding as well.

2. *Halogenmethyl-1,4-quinones*

Halogenmethyl-1,4-quinones are possible candidates for functioning as "bioreductive alkylating agents", *i.e.* that they become potent alkylating agents after they undergo a reduction (for reviews, see [11, 12]). The underlying basic mechanism is depicted in Fig. 3. After reduction to the hydroquinone, hydrochloride may be split off under formation of a *o*-quinonemethide. This quinonemethide is prone to an attack by a nucleophile (Nu) and a new covalent bond is formed. The nucleophile may be a suitable amino acid within the sequence of the enzyme which catalyzes the quinone reduction. In this case the enzyme may be inactivated by the formation of the covalent bond. It has been reported indeed that 1,4-benzo- and naphthoquinones equipped with suitable leaving groups can function as inhibitors of ubiquinone mediated mitochondrial enzyme systems and possess inhibitory activity against adenocarcinoma and sarcoma ascites cells [13]. Quinone reductions take place in photosynthetic systems and, therefore, we have investigated the inhibitory properties of halogenmethyl-substituted 1,4-benzoquinones (Table II) and 1,4-naphthoquinones (Table III). They indeed proved to be potent photosystem II inhibitors with maximal pI_{50}-values of 5.3 and 5.9, respectively. In a series of homologous 3-alkyl-2-chloromethylnaphthoquinones, an optimal chain length of five carbon atoms was found; below and above this number, inhibitory activity decreased (Table III). Besides their *in vitro* activity, both types of quinones showed herbicidal *in vivo* activity and

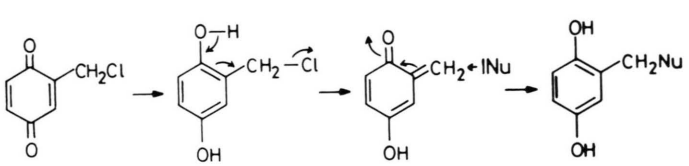

Fig. 3. Mechanism of reaction for formation of a *o*-quinonemethide from a chloromethyl-1,4-quinone and its reaction with a nucleophile (Nu).

Table II. pI_{50}-values (H_2O–DCIP) and herbicidal activity (% damage three weeks after postemergence application of 4 kg/ha) of halogenmethyl-substituted 1,4-benzoquinones.

No.	...1,4-benzoquinone	pI_{50} value	*Beta vulg.*	*Amaranthus retrofl.*	*Sinapis alba*	*Panicum miliac.*	*Gossypium hirsut.*	*Phaseolus vulg.*
1	2,5-Bis(chloromethyl)-	4	0	100	100	0	0	0
2	2-Chloro-5-chloromethyl-	4.40	30	100	90	70	0	0
3	2-Chloromethyl-6-t.butyl-	4.66	0	100	40	0	0	0
4	2-Chloromethyl-3-methyl-6-isopropyl-	5.18	70	70	80	90	70	90
5	2,3-Bis(chloromethyl)-5,6-dimethyl-	5.22	10	90	0	95	10	40
6	2-Bromo-3,6-bis-(chloromethyl)-	5.30	0	80	0	0	0	50
7	2,5-Dibromo-3,6-bis-(chloromethyl)-	5.30	0	100	10	0	0	50
8	2-Chloromethyl-3,5,6-trimethyl-	5.31	0	30	80	30	30	80

Table III. pI_{50}-values (H_2O–DCIP) and herbicidal activity (% damage three weeks after postemergence application of 4 kg/ha) of halogenmethyl-substituted 1,4-naphthoquinones.

No.	...1,4-naphthoquinone	pI_{50} value	*Beta vulg.*	*Amaranthus retrofl.*	*Sinapis alba*	*Panicum miliac.*	*Gossypium hirsut.*	*Phaseolus vulg.*
1	2-Chloromethyl-3-methyl-	5.24	0	100	100	70	0	60
2	2-Chloromethyl-3-ethyl-	5.26	50	100	95	40	50	40
3	2,3-Bis(chloromethyl)-	5.39	30	50	100	100	30	0
4	2-Bromomethyl-3-methyl-	5.39	0	100	90	0	0	30
5	2-Bromomethyl-3-ethyl-	5.43	0	100	80	95	30	50
6	2-Chloromethyl-3-*n*-octyl-	5.57	0	0	50	0	0	0
7	2-Chloro-3-chloromethyl-	5.57	0	100	100	0	0	90
8	2-Chloromethyl-3-*n*-butyl-	5.58	30	40	80	0	30	0
9	2-Chloromethyl-3-*n*-heptyl-	5.78	0	30	60	30	0	20
10	2-Chloromethyl-3-*n*-pentyl-	5.92	50	80	80	30	0	30

plant specifity (Tables II and III). In the homologous series of 3-alkyl-2-chloromethyl-1,4-naphthoquinones in the *in vivo* system the maximal biological activity was reached at a length of only two carbon atoms (Table III). It should be noted, however, that the *in vivo* effects are not necessarily connected to an inhibition of photosynthetic electron transport. At any place within the whole plant where a quinone reduction may occur the resulting quinonemethide can attack and inactivate the participating enzyme system.

So far no direct proof for a covalent binding of a halogenmethylquinone to a protein component of the thylakoid membrane could be demonstrated because no radiolabeled compound is available yet. An indirect proof for covalent binding of a halogenmethyl-1,4-benzoquinone was obtained in the following way. Thylakoids were incubated with 10 μM 2,3-bis(chloromethyl)-5,6-dimethyl-1,4-benzoquinone (compound **5**, Table II) for 10 min in the light or in complete darkness. After that the samples were washed twice and centrifuged twice to remove non-covalent bound material. Both samples were then assayed for their capability in performing light-induced NADP-reduction. The sample which has been preincubated in the dark exhibited a normal rate of NADP-reduction which was slightly stimulated by addition of DAD/asc (Fig. 4, left). However, the sample which was preincubated in the light showed only little activity in light-induced NADP-formation,

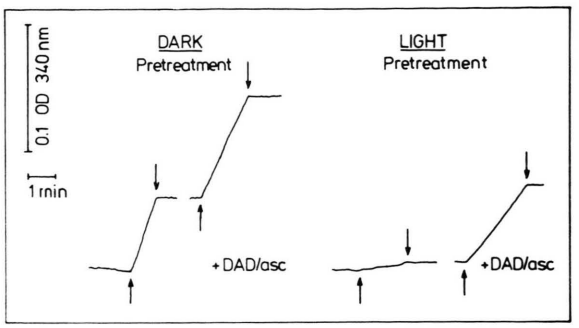

Fig. 4. Photosynthetic NADP-reduction with isolated thylakoids pretreated in the dark (left) or in the light (right) with 10 μM 2,3-bis(chloromethyl)-1,4-benzoquinone for 10 min. Upward arrow means light on; downward arrow light off. For details, see text.

but this low rate was drastically stimulated by DAD/asc addition (Fig. 4, right). This indicates that a covalent attachment of the halogenmethyl-1,4-benzoquinone obviously takes place, but predominantly in photosystem II because photosystem I dependent NADP-reduction at the expense of DAD/asc is not impaired. However, further experiments have to be performed to assure a covalent binding and to localize this presumable binding site within the photosystem II complex.

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