Inhibition of Photosynthesis by 4-Nitro-6-alkylphenols: Structure-Activity Studies in Wild Type and Five Mutants of *Chlamydomonas reinhardtii* Thylakoids

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Photosynthesis Inhibition, 4-Nitro-6-alkylphenols, *Chlamydomonas reinhardtii*, D1 Protein, Mutants

Photosynthesis inhibition data were determined with thylakoids of the green alga *Chlamydomonas reinhardtii* on twenty-two 2-bromo-4-nitro-6-alkyl- and 2,4-dinitro-6-alkyl-phenols. Wild type and five different mutants of the algae were employed with defined changes in the *psb*A gene and a single amino acid substitution in the herbicide binding protein D1 of photosystem II. Their response to the various inhibitors was quite different from that of the wild type. Tolerance as well as enhanced inhibitory activity was found in the mutants, with tolerance in all phenols in the val219ile and of some in the phe255tyr mutant and supersensitivity of most phenols in the ala251val, ser264ala and leu275phe mutant. This implies that the phenols are bound specifically to the D1 protein. Structure-activity studies revealed parabolic relationships with all descriptors increasing with the alkyl chain length for wild type and most mutant inhibition values. Molecular modelling studies suggested that the position of the alkyl chains in different phenols in the Q_B binding niche of the D1 protein does not show into the same direction.

Introduction

Photosystem II is inhibited by numerous compounds among them many herbicides (for a review see [1–3]). They block photosynthetic electron flow by displacing the secondary electron acceptor Q_B, plastoquinone, from its binding site [2, 3]. The herbicide or binding protein has been identified as a peptide that binds not only Q_B but also the reaction center chlorophylls P_{680}. Inhibitory compounds, the classical triazine/urea herbicides and the phenol type group, both displace Q_B and each other from the D1 protein and therefore are assumed to have the same mode of action. But quantitative structure-activity studies revealed quite different relationships of the two groups. Support for a different binding behaviour came from observations that in herbicide tolerant and algae with mutations in the D1 encoding *psb*A gene, phenol type inhibitors displayed enhanced potency [4–11]. Only two amino acid substitutions in the D1 protein were reported (at val219 and asn266) that led to tolerance of a few phenol type herbicides like ioxynil.

Here we wish to report on a systematic study of bromonitro- and dinitrophenols with linear alkyl chains from C1 to C12 in 6-position of the phenyl ring. By testing them in several mutants of *Chlamydomonas reinhardtii* screened for tolerance to classical herbicides, we do observe tolerance to these phenols in mutants with amino acid substitutions at val219, and phe255. None of the phenols showed tolerance in the ser264ala mutant, to the contrary many were supersensitive.

Structure-activity studies have been helpful in the past for understanding the influence of substituent effects on phenols. Already in 1979 we had noted [12] that the efficiency of substituted nitrophenols in inhibiting photosystem II can be described by a regression equation using only the STERIMOL parameters developed Verloop [13, 14], though the groups in 2-position of the phenols were electronically different groups. Here we use only descriptors of the alkyl side chain and describe first results on the docking of phenolic inhibitors into the D1 protein.

Materials and Methods

Chemistry

Synthesis of 2-bromo-4-nitro-6-methylphenol

2-Methyl-4-nitrophenol (**1**)

To a cool solution of 20.8 g (0.2 mol) 2-methylphenol in 500 ml ligroin, a mixture 20 ml

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water and 15 ml nitric acid (65%) was added dropwise. The mixture was stirred at room temperature for 12 h and the ligroin decanted. The yellow liquid residue was taken up in ethyl acetate, washed with water and evaporated. Yield: 19.3 g. Purity: 80% (GC-MS).

2-Bromo-4-nitro-6-methylphenol (**2**)

19.3 g of the crude 2-methyl-4-nitrophenol were dissolved in 250 methanol and 50 ml water. 6.2 g bromine were added dropwise with stirring for 12 h. The methanol-water was decanted and the brown residue dissolved in ethyl acetate, washed with water and evaporated. The oily brown residue was chromatographed with a cyclohexane/ethyl acetate 5:1 to yield 2.9 g (10%) of pure (>98%, HPLC) **2.** M.p. 119 °C.

Synthesis of 2-bromo-4-nitro-6-decylphenol

2-Decanoylphenol (**3**)

A solution of 150 g (1.1 mol) aluminum chloride in 600 ml chlorobenzene was cooled to 5–10 °C. 94 g phenol in 200 ml chlorobenzene were added quickly. After the solution had warmed to room temperature, 190.5 g decanoylchloride (Aldrich) in 150 ml chlorobenzene were added dropwise. The mixture was heated to boiling temperature for 18 h. After cooling, 250 ml conc. hydrochloric acid in 2 l water were added. The mixture was separated, dried, evaporated and distilled. Boiling point 148–150 °C/0.3 mbar. Yield: 124.3 g (50%). Purity: 89% (GC-MS).

2-Decylphenol (**4**)

Amalgamated zinc was prepared by vigorous stirring of 163 g grated zinc in 175 ml water with 8 g mercuric chloride for 1 h. The granules were filtered and washed with water. A mixture of 124 g 2-decanoylphenol, 250 ml water and 125 ml conc. hydrochloric acid was added and heated to boiling. After 5 and 10 h, 55 ml fresh conc. hydrochloric acid were added and the solution mixture boiled for further 12 h. After cooling, methylene chloride was added, separated, dried, evaporated and distilled. Boiling point: 136–138 °C/0.1 mbar. Yield: 62.7 g (54%). Purity: 81% (GC-MS).

2-Decyl-4-nitrophenol (**5**)

Compound **5** was prepared in analogy to **2** from 46.8 g (0.2 mol) **4.** Yield: 55.8 g. Purity: 41% (GC-MS).

2-Bromo-4-nitro-6-decylphenol (**6**)

27.9 g (0.1 mol) crude 2-decyl-4-nitrophenol (**5**) were dissolved in 250 ml methanol and 50 ml water. 5.1 g bromine were added dropwise, the mixture stirred for 12 h, decanted, ethyl acetate added, washed with water, dried and evaporated. 33.3 g of a brown oil were obtained. This was chromatographed (cyclohexane/ethyl acetate 5:1) to yield 5.7 g (16%) **6.** Purity: 96% (HPLC), m.p. 35–38 °C.

Biochemistry

The *Chlamydomonas reinhardtii* mutants are described by Wildner *et al.* [9, 11]. The mutants MZ1, MZ2 and MZ4 were obtained by screening *Chlamydomonas reinhardtii* for metribuzin tolerance after mutagenizing wild type cells [12]. The changes in the *psb*A gene that encodes the D1 protein were identified by Johanningmeier *et al.* [11] as amino acid substitutions at ser264, ala251, and leu275. This means that MZ2 = ala251val, MZ1 = ser264ala and MZ4 = leu275phe. The Ar207+ (= phe255tyr) and Dr2 (= val219ile) mutants were kindly provided by Galloway and Mets [5] and the corresponding *psb*A genes sequenced by Rochaix *et al.* [5].

The wild type and mutants of *Chl. reinhardtii* were grown according to [9] under phototropic conditions without any inhibitor pressure. The cells were harvested, broken by sonication and the thylakoids centrifuged off. Photosynthetic activity in the thylakoids was measured spectrophotometrically be following dichlorophenolindophenol reduction without or in the presence of a concentration gradient of the inhibitor. The inhibitors were preincubated with the thylakoid membranes in order to compensate for the lag phase of about 2 min before maximal inhibition is obtained.

Structure-activity analyses

The plots of the pI_{50} values of wild type and mutant thylakoids *versus* the various descriptors were drawn by the program Cricket Graph 1.0 (Cricket software, Philadelphia, U.S.A.). Experimentally

based and calculated parameters were employed. Partition coefficients logP were obtained from HPLC retention data using a calibration program. CLOGP and CMR were calculated with the Pomona software [15]. The STERIMOL parameters L were taken from Verloop *et al.* [13, 14].

Molecular modeling and energy calculations

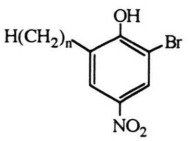
An ESV 30/33 RISC workstation was used for molecular modelling and energy calculations with the software package SYBYL (Tripos Ass., St. Louis, U.S.A.). The model of the D1 protein was restricted to the D1 binding niche consisting of 47 amino acids from gly207 to ser222 and asn247 to ala277. The structure was extrapolated from the X-ray analysis by Deisenhofer *et al.* [16].

Results

The pI_{50} values of eleven 2-bromo-4-nitrophenols and the parameters n, logP, CLOGP, CMR, and L are shown in Table I. The corresponding data of eleven 2,4-dinitrophenols are given in Table II. It is found that neither 2-bromo-4-nitro- nor 2,4-dinitrophenol derivatives are tolerant in the *Chlamydomonas reinhardtii* mutant MZ1 where serine264 is changed to alanine as already observed for bromonitrothymol [9]. This behaviour differs from the classical inhibitors whose high tolerance in the mutant is well documented [4–11]. Also in the mutants with the val219ile and phe255tyr substitution supersensitivity is observed. Again, the classical herbicides show differential tolerance in these mutants [4–11]. In the two mutants with the val219ile and phe255tyr substitution, however, there is tolerance towards phenols, in the val219 mutant for all phenols (except for one) and in the phe255tyr mutant for half of the phenols. The others show little change or supersensitivity.

A greater transparency of the same data is provided in Table III which gives the difference in pI_{50} values between the wild type and the respective mutants. A negative sign stands for supersensitivity, a positive sign for tolerance. The greatest sensitivity increase is shown in the ala251val mutant with compound 15 (2,4-dinitro-6-butylphenol) with a $\Delta pI_{50} = -1.4$, and in the ser264ala mutant with compound 1 (2-bromo-4-nitro-6-methylphenol) with a $\Delta pI_{50} = -1.9$. These logarithmic figures correspond to sensitivity increases of 25 and 75 respectively. As only linear alkyl derivatives of nitrophenols are tested here it should be mentioned that in an extended phenol series comprising also branched alkyl chains and apart from bromo also

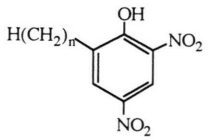
Table I. The inhibition of photosynthetic electron flow by eleven alkyl substituted 2-bromo-4-nitrophenols in wild type and five mutants of *Chlamydomonas reinhardtii*.



2-Bromo-4-nitrophenols

No.	*n*	WT	Mutants					logP	CLOGP	CMR	L
			val 219 ile	ala 251 val	phe 255 tyr	ser 264 ala	leu 275 phe				
1	1	4.4	4.6	4.7	5.2	6.3	5.3	2.29	3.23	4.81	2.87
2	2	4.6	4.2	5.6	4.6	5.7	5.6	2.70	3.76	5.27	4.11
3	3	6.0	4.7	6.4	5.5	6.6	6.8	3.15	4.29	5.74	4.92
4	4	6.5	5.8	7.6	6.6	7.3	7.5	3.59	4.82	6.20	6.17
5	5	7.0	5.8	7.6	7.0	7.4	7.7	4.03	5.35	6.66	6.97
6	6	7.3	6.4	8.0	7.2	7.6	8.0	4.48	5.88	7.13	8.22
7	7	7.5	7.0	8.1	7.8	8.0	8.5	4.95	6.41	7.50	9.03
8	8	8.0	6.8	8.4	7.7	8.4	8.3	5.44	6.94	8.06	10.27
9	9	8.1	7.6	8.6	7.8	8.0	8.7	5.87	7.47	8.52	11.26
10	10	8.1	7.4	8.7	7.8	8.3	8.6	6.39	8.00	8.98	12.33
11	12	7.6	7.1	8.0	7.2	7.8	8.0	7.30	9.05	9.91	14.38

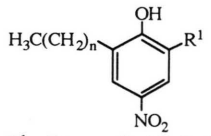
Table II. The inhibition of photosynthetic electron flow by eleven alkyl substituted 2,4-dinitrophenols in wild type and five mutants of *Chlamydomonas reinhardtii*.



2,4-Dinitrophenols

No.	*n*	WT	Mutants					logP	CLOGP	CMR	L
			val 219 ile	ala 251 val	phe 255 tyr	ser 264 ala	leu 275 phe				
12	1	4.2	4.1	4.8	5.3	4.8	5.1	2.29	2.56	4.76	2.87
13	2	4.7	4.5	5.8	4.8	5.3	5.8	2.77	3.09	5.22	4.11
14	3	5.1	4.7	6.0	5.1	5.7	5.5	3.25	3.62	5.68	4.92
15	4	5.6	5.4	7.0	5.8	6.4	6.5	3.71	4.15	6.15	6.17
16	5	5.7	5.2	6.8	6.0	6.4	6.1	4.19	4.68	6.61	6.97
17	6	6.7	6.0	7.3	6.3	6.7	7.0	4.54	5.21	7.08	8.22
18	7	7.0	6.5	7.8	7.0	7.4	7.7	5.09	5.74	7.54	9.03
19	8	7.4	6.7	8.0	7.0	7.7	7.8	5.57	6.27	8.00	10.27
20	9	7.7	7.4	8.5	7.2	8.0	8.3	6.01	6.80	8.47	11.26
21	10	7.7	7.3	8.1	7.3	8.0	8.3	6.49	7.33	9.03	12.33
22	12	7.4	7.3	8.2	7.3	8.0	8.1	7.36	8.38	9.86	14.38

Table III. ΔpI_{50} values of twenty-two 6-alkyl-4-nitrophenols. $\Delta pI_{50} = pI_{50\,wt} - pI_{50\,mut}$. A positive sign reflects tolerance, a negative sign supersensitivity.



R^1 = Br for No. 1 - 12
R^1 = NO_2 for No. 12 - 22

No.	*n*	WT	Mutants								
			val 219 ile	ΔpI_{50}	ala 251 val	ΔpI_{50}	phe 255 tyr	ΔpI_{50}	ser 264 ala	ΔpI_{50}	leu 275 phe	ΔpI_{50}
1	1	4.4	4.6	−0.2	4.7	−0.3	5.2	−0.8	6.3	−1.9	5.3	−0.9
2	2	4.6	4.2	+0.4	5.6	−1.0	4.6	0.0	5.7	−0.9	5.6	−1.0
3	3	6.0	4.7	+1.3	6.4	−0.4	5.5	+0.5	6.6	−0.6	6.8	−0.8
4	4	6.5	5.8	+0.7	7.6	−1.1	6.6	−0.1	7.3	−0.8	7.5	−1.0
5	5	7.0	5.8	+1.2	7.6	−0.6	7.0	0.0	7.4	−0.4	7.7	−0.7
6	6	7.3	6.4	+0.9	8.0	−0.7	7.2	+0.1	7.6	−0.3	8.0	−0.7
7	7	7.5	7.0	+0.5	8.1	−0.6	7.8	−0.3	8.0	−0.5	8.5	−1.0
8	8	8.0	6.8	+1.2	8.4	−0.4	7.7	+0.3	8.4	−0.4	8.3	−0.3
9	9	8.1	7.6	+0.5	8.6	−0.5	7.8	+0.3	8.0	+0.1	8.7	−0.6
10	10	8.1	7.4	+0.7	8.7	−0.6	7.8	+0.3	8.3	−0.2	8.6	−0.5
11	12	7.6	7.1	+0.5	8.0	−0.4	7.2	+0.4	7.8	−0.2	8.0	−0.4
12	1	4.2	4.1	+0.1	4.8	−0.6	5.3	−0.9	4.8	−0.6	5.1	−0.9
13	2	4.7	4.5	+0.2	5.8	−1.1	4.8	−0.1	5.3	−0.6	5.8	−1.1
14	3	5.1	4.7	+0.4	6.0	−0.9	5.1	0.0	5.7	−0.6	5.5	−0.4
15	4	5.6	5.4	+0.2	7.0	−1.4	5.8	−0.2	6.4	−0.8	6.5	−0.9
16	5	5.7	5.2	+0.5	6.8	−1.1	6.0	−0.3	6.4	−0.7	6.1	−0.4
17	6	6.7	6.0	+0.7	7.3	−0.6	6.3	+0.4	6.7	0.0	7.0	−0.3
18	7	7.0	6.5	+0.5	7.8	−0.8	7.0	0.0	7.4	−0.4	7.7	−0.7
19	8	7.4	6.7	+0.7	8.0	−0.6	7.0	+0.4	7.7	−0.3	7.8	−0.4
20	9	7.7	7.4	+0.3	8.5	−0.8	7.2	+0.5	8.0	−0.3	8.3	−0.6
21	10	7.7	7.3	+0.4	8.1	−0.4	7.3	+0.4	8.0	−0.3	8.3	−0.6
22	12	7.4	7.3	+0.1	8.2	−0.8	7.3	+0.1	8.0	−0.6	8.1	−0.7

chloro and iodo substituents in 2-position, tolerance in observed in the ala 251 val and leu 275 but never in the ser 264 ala mutant [17].

In Tables I and II also the descriptors n, logP, CLOGP, CMR, and L are given. Inspection of the data from Tables I and II suggests that there might be a parabolic relationship between the pI_{50} values and these parameters. That this is indeed the case is shown by Fig. 1 and 2 which present correlations of the second or third power of the partition coefficient logP, an experimental parameter. In the graphs, only the relationships for the eleven 2-bromo-4-nitro-6-alkylphenols are shown. The 2,4-dinitrophenols give similar pictures, and the same holds true with the other calculated descriptors in Tables I and II: the number of carbon atoms in the alkyl chain n, the parameters CLOGP and CMR from the Pomona database obtained by increment addition, and the STERIMOL parameter L. All these descriptors have in common that they increase homogeneously with the number of carbon atoms. The problem of colinearity in most of these parameters has been discussed in literature [18, 19]. Since only linear chains from $n = 1$ to $n = 12$ are reported here, the results are not contaminated by branching effects. These second or third power relationships are summarized in Tables IV and V. The decision whether the second or the third power was employed depended on the correlation coefficient. If the third power provided no improvement, the second power is given. The statistical quality of the relationships varies to some extent with the squared correlation coefficient r^2 ranging between 0.90 and 0.98.

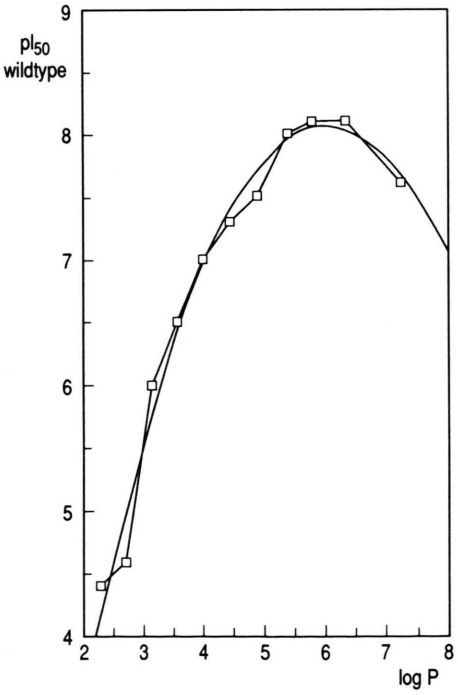


Fig. 1. Parabolic relationship of the wild type pI_{50} values of eleven 2-bromo-4-nitro-6-alkylphenols with the partition coefficient logP:
$pI_{50\,wt} = -1.79 + 3.24\,\log P - 0.267\,(\log P)^2$ ($r^2 = 0.98$).

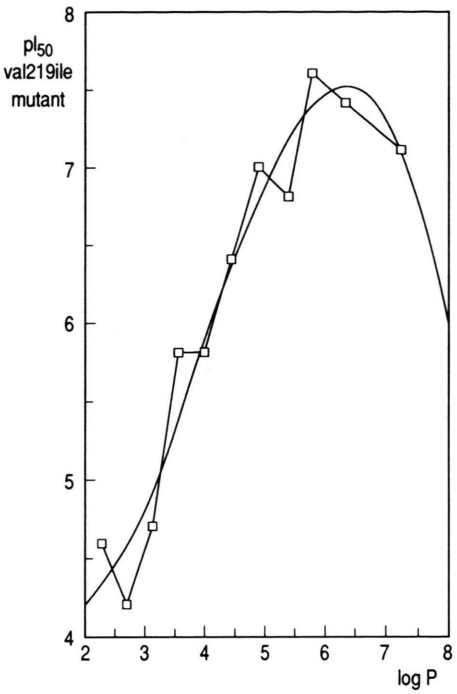
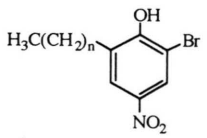


Fig. 2. Third power relationship of ala 251 val mutant pI_{50} values of eleven 2-bromo-4-nitro-6-alkylphenols with the partition coefficient logP:
$pI_{50\,mutant} = 5.65 - 2.00\,\log P + 0.768\,(\log P)^2 - 0.064\,(\log P)^3$
($r^2 = 0.96$).

The regressions of pI_{50} values on the second or third power of the various descriptors that are correlated with chain length, are not surprising since chain length is the only variable in the two sets of phenols. It is in line, however, with our earlier finding [12] on a more complex set of phenols that only steric parameters suffice to describe photosynthesis inhibition. More modelling data and QSARs on a larger set of phenols will be described in an

Table IV. Coefficients of the relationships in the regression analyses between wild type and five mutant pI_{50} values with five parameters: eleven 2-bromo-4-nitro-6-alkylphenols. The coefficients refer to second or third power relationships of parameters increasing homogeneously with chain length, to wild type and mutant pI_{50} values.
The parameters: logP, octanol/water partition coefficient calculated from HPLC retention time; *n*, number of carbon atoms in the alkyl chain; L, STERIMOL parameter, see [13], [14]; CLOGP, calculated octanol/water partition coefficient; CMR, 1/10 of the calculated molar refraction; CLOGP and CMR see [15].



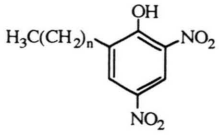
Mutant	X	$pI_{50} = a + b \cdot X + c \cdot X^2 + d \cdot X^3$				
		a	*b*	*c*	*d*	r^2
WT	logP	− 1.79	+3.24	−0.267		0.98
WT	*n*	+ 3.23	+1.01	−0.053		0.98
WT	L	+ 1.26	+1.15	−0.049		0.98
WT	CLOGP	− 3.33	+2.95	−0.19		0.98
WT	CMR	−11.10	+4.39	−0.25		0.98
Val219Ile	logP	+ 5.65	−2.00	+0.768	−0.064	0.96
Val219Ile	*n*	+ 4.27	−0.03	+0.102	−0.007	0.96
Val219Ile	L	+ 4.77	−0.49	+0.13	−0.006	0.96
Val219Ile	CLOGP	+ 7.98	−3.03	+0.735	−0.046	0.96
Val219Ile	CMR	−18.69	+7.89	+1.342	−0.067	0.94
Ala251Val	logP	− 1.57	+3.47	−0.296		0.98
Ala251Val	*n*	+ 3.55	+1.24	−0.089		0.98
Ala251Val	L	+ 1.59	+1.25	−0.056		0.98
Ala251Val	CLOGP	− 3.31	+3.19	−0.215		0.98
Ala251Val	CMR	−11.73	+4.79	−0.282		0.98
Phe255Tyr	logP	+ 2.98	+0.15	+0.369	−0.043	0.92
Phe255Tyr	*n*	+ 4.36	+0.36	+0.049	−0.005	0.92
Phe255Tyr	L	+ 4.09	+0.04	+0.081	−0.005	0.92
Phe255Tyr	CLOGP	+ 4.50	−1.03	+0.454	−0.034	0.92
Phe255Tyr	CMR	+ 9.04	−3.92	+0.869	−0.050	0.92
Ser264Ala	logP	+ 5.47	−0.46	+0.384	−0.038	0.90
Ser264Ala	*n*	+ 5.76	−0.17	+0.051	−0.004	0.90
Ser264Ala	L	+ 5.84	−0.13	+0.078	−0.004	0.90
Ser264Ala	CLOGP	+ 6.80	−1.29	+0.414	−0.029	0.90
Ser264Ala	CMR	+11.99	−4.05	+0.787	−0.043	0.90
Leu275Phe	logP	− 1.17	+3.55	−0.355	+0.006	0.98
Leu275Phe	*n*	+ 4.26	+0.96	−0.051	−2.335	0.98
Leu272Phe	L	+ 2.29	+1.13	−0.056		0.96
Leu275Phe	CLOGP	− 2.23	+2.92	−0.198		0.98
Leu275Phe	CMR	− 9.95	+4.39	−0.259		0.98

accompanying paper [20]. The properties of further substituted nitrophenols and the effect on photosynthesis in the *Chlamydomonas reinhardtii* mutants is reported elsewhere [17].

Discussion

Herbicides displace the secondary electron acceptor plastoquinone from its binding protein in photosystem II [2, 3]. This protein has been identi-

Table V. Coefficients of the relationships in the regression analyses between wild type and five mutant pI_{50} values with five parameters: eleven 2,4-dinitro-6-alkylphenols. The coefficients refer to second or third power relationships of parameters increasing homogeneously with chain length, to wild type and mutant pI_{50} values.
The parameters: see Table IV.



Mutant	X	$pI_{50} = a + b \cdot X + c \cdot X^2 + d \cdot X^3$ a	b	c	d	r^2
WT	logP	− 0.123	+2.12	−0.144		0.96
WT	*n*	+ 3.27	+0.74	−0.031		0.96
WT	L	+ 1.81	+0.83	−0.029		0.96
WT	CLOGP	− 0.036	+1.86	−0.112		0.96
WT	CMR	− 6.30	+2.86	−0.151		0.96
Val219Ile	logP	+ 5.81	−2.00	+0.685	−0.052	0.98
Val219Ile	*n*	+ 4.12	−0.008	+0.085	−0.005	0.98
Val219Ile	L	+ 2.67	−0.34	+0.103	−0.005	0.98
Val219Ile	CLOGP	+ 0.79	−1.67	+0.514	−0.035	0.98
Val219Ile	CMR	+15.32	−6.16	+1.046	−0.051	0.98
Ala251Val	logP	+ 0.64	+2.23	−0.163		0.96
Ala251Val	*n*	+ 4.20	+0.76	−0.035		0.96
Ala251Val	L	+ 2.67	+0.86	−0.033		0.96
Ala251Val	CLOGP	+ 0.79	+1.93	−0.125		0.96
Ala251Val	CMR	− 5.85	+3.04	−0.164		0.96
Phe255Tyr	logP	+ 8.28	−3.04	+0.863	−0.074	0.96
Phe255Tyr	*n*	+ 5.18	−0.22	+0.106	−0.006	0.96
Phe255Tyr	L	+ 5.99	−0.61	+0.130	−0.006	0.96
Phe255Tyr	CLOGP	+ 7.96	−2.46	+0.628	−0.041	0.96
Phe255Tyr	CMR	+21.04	−8.04	+1.274	−0.061	0.96
Ser264Ala	logP	+ 1.32	+1.73	−0.109		0.98
Ser264Ala	*n*	+ 4.13	+0.62	−0.024		0.98
Ser264Ala	L	+ 2.92	+0.68	−0.022		0.98
Ser264Ala	CLOGP	+ 1.41	+1.51	−0.085		0.98
Ser264Ala	CMR	− 3.65	+2.28	−0.111		0.98
Leu275Phe	logP	+ 7.86	−2.76	+0.862	−0.066	0.96
Leu275Phe	*n*	+ 5.29	−0.115	+0.106	−0.006	0.96
Leu272Phe	L	+ 5.73	−0.48	+0.124	−0.006	0.96
Leu275Phe	CLOGP	+ 5.67	−2.30	+0.643	−0.043	0.96
Leu275Phe	CMR	+20.48	−8.01	+1.318	−0.064	0.96

fied as the D1 protein subunit of photosystem II by labelling with azidoatrazine [21] and the correlation of its trypsin sensitivity to that of the DCMU sensitive "rapidly turning over" protein [22]. From homology in sequence and folding to the L-protein in the bacterial photosynthetic reaction center, the main function of the D1 protein as a reaction center polypeptide of PS II has been proposed [22–25], and this is accepted now. From the known X-ray structure of the bacterial reaction center, a three-dimensional model for the folding of the amino sequence in the Q_B and herbicide binding niche of photosystem II was extrapolated [1, 24, 26]. This model could be well rationalized by amino acid substitutions that led to herbicide tolerance [1, 4–10, 24].

Inhibitors of the triazine/urea type display cross resistance in numerous mutants that are now available by mutagenesis of the *psb*A gen that encodes for the D1 protein [1, 4–11]. The depend-

ence of herbicide tolerance on the substituents in the various mutants provided the basis for docking of some of these compounds into the Q_B binding niche [27]. Members of the phenol type family as tested so far, however, have in most cases shown no tolerance or became even supersensitive (= negative cross resistant) [4–11]. It appeared therefore that phenol type inhibitors might not bind into the Q_B binding niche or that the compounds might even be attached to another protein. Tolerance to ioxynil was observed by Astier *et al.* [10] in two asn 266 mutants of *Synechocystis,* and Wildner *et al.* [9] found resistance in a val 219 mutant of *Chlamydomonas reinhardtii.* Böger communicated tolerance to certain phenol inhibitors in a not yet published leu 218 mutant of *Bumilleriopsis filiformis* (Xanthophyceae). These were the first evidences for phenols binding indeed in the Q_B binding niche of the D 1 protein. But it was shown that the tolerance to ioxynil in the asn 266 thr mutant, constructed by site directed mutagenesis in *Chlamydomonas reinhardtii* [28], could not be generalized in the sense that asn 266 specifically interacts with phenols. Moreover, nitrophenols can act on the donor side of PS II as well (see [2, 3]). Therefore, the concept of overlapping sites for phenol and classical type inhibitors or their allocation to a serine and a histidine family [29] appeared not to be sufficient to remove all doubts on a multiple interaction of phenols with the thylakoid membrane.

The data in this paper now show clearly that the principal effect of phenolic inhibitors on photosystem II is due to binding to the Q_B site on the D 1 protein. This follows from the response of the inhibitory potency of the phenols in a D 1 protein mutant with a specific amino acid substitution. The response can be positive (tolerance) or negative (supersensitivity). Tolerance may seem more convincing for the conclusion that the primary reaction of phenols is due to binding to the D 1 protein, but also supersensitivity indicates a specific interaction of the inhibitor with the changed amino acids.

All bromonitro- and dinitrophenols (except for one) get tolerant in the val 219 ile mutant. For example, the I_{50} value of 2-bromo-4-nitro-6-propylphenol (No. 3) is about 15-fold lower in the val 219 ile mutant than in the wild type. The highest tolerance of dinitrophenols is that of the hexyl- and octyl derivatives (No. 17, 19). In the other mutants tolerance is observed for nitrophenols only in the phe 255 tyr mutant. The highest value is measured for 2,4-dinitro-6-nonylphenol. In the ala 251 val and leu 275 phe mutants only supersensitivity is observed with a highest I_{50} value increase of a factor of 25 for 2,4-dinitro-6-butylphenol (No. 15) in the ala 251 val mutant. It should be pointed out that other derivatives of bromonitro- and dinitrophenols do get tolerant in these mutants as described in [17, 20, 30] with derivatives which are substituted by a second alkyl group in 3-position or a branched alkyl chain in 6-position. Supersensitivity is the response of all phenols in the ser 264 ala mutant. It should be remembered that ser 264 is considered to be of prime importance in the binding of classical inhibitors which is directly shown by the X-ray structure in the L-subunit of *Rps. viridis* [31] where there is hydrogen bonding to ser 223 which is equivalent to ser 264 in the D 1 protein. Substitution of this serin to alanine in D 1 or proline in L of *Rps. capsulatus* leads to strong tolerance for this type of herbicides and inhibitors in the homologous reaction centers of plants and purple bacteria.

Structure-activity studies of the substituted nitrophenols described here show a dependence of inhibitory activity on the chain length and other descriptors that increase homogeneously with length like lipophilicity, CLOGP, and CMR. Again, as reported earlier [12] the STERIMOL parameter L [13, 14] correlates very well with all pI_{50} values. (Added in proof): Recently molecular orbital calculations of new alkyl-substituted dinitrophenols studied in the inhibition of electron flow have been reported [33].

Molecular modelling studies were carried with two compounds of this series: the 2-bromo-4-nitro-6-methylphenol (No. 1) and the 2,4-dinitro-6-heptylphenol (No. 18). These compounds were chosen because of their ΔpI_{50} values. In the ser 264 ala mutant $\Delta pI_{50} = -1.9$ for No. 1, and in the ala 251 val mutant $\Delta pI_{50} = -0.8$ for No. 18. Figs. 3 and 4 show compound **1** in D 1 binding niche of the wild type and the ser 264 ala mutant, and Figs. 5 and 6 show compound 18 in the D 1 binding niche of the wild type and the ala 251 val mutant. In these figures the compounds are sitting in the D 1 protein binding niche. For clarity, only the amino acid backbone with the marked mutation sites is displayed. One can take from them

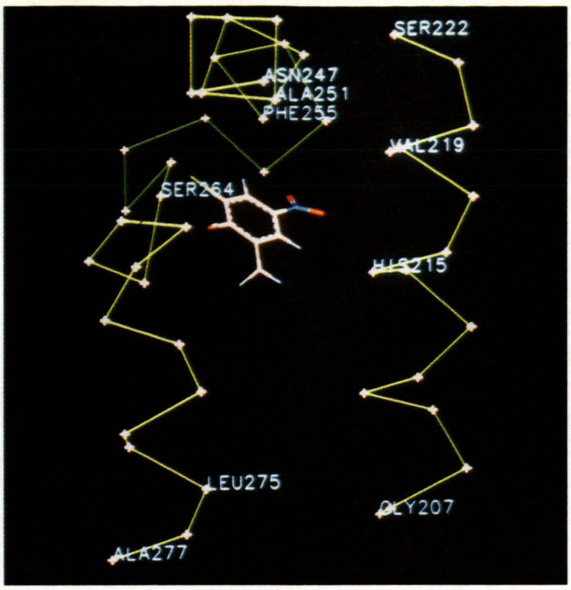


Fig. 3. 2-Bromo-4-nitro-6-methylphenol in the binding niche of wild type *Chlamydomonas reinhardtii* D 1 protein.

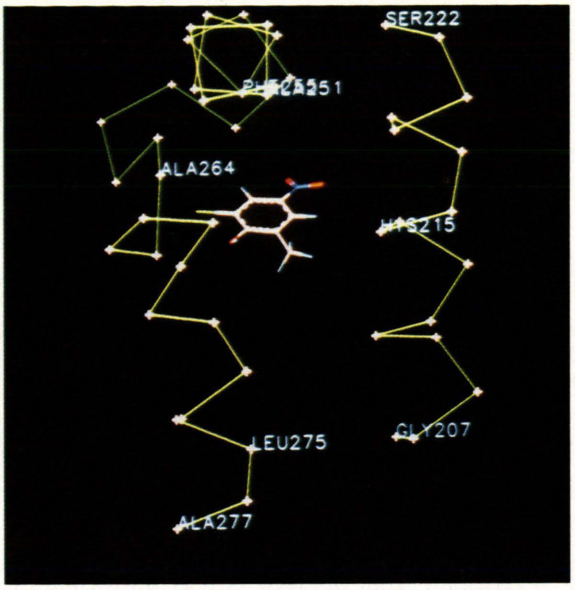


Fig. 4. 2-Bromo-4-nitro-6-methylphenol in the binding niche of the ser264ala mutant of *Chlamydomonas reinhardtii* D 1 protein.

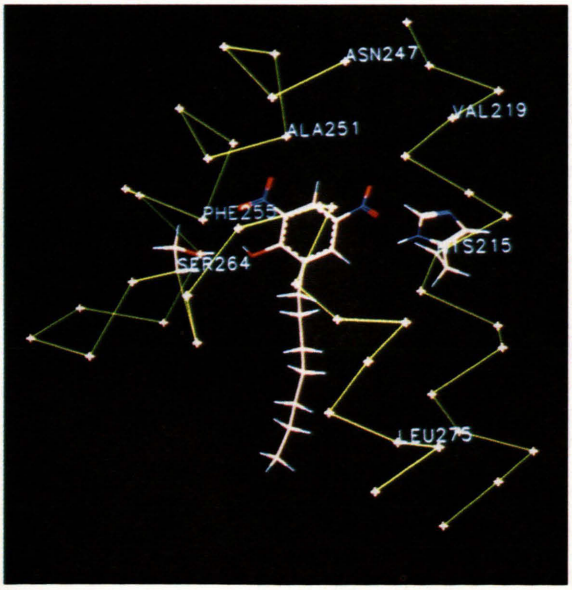


Fig. 5. 2,4-Dinitro-6-heptylphenol in the binding niche of wild type *Chlamydomonas reinhardtii* D 1 protein.

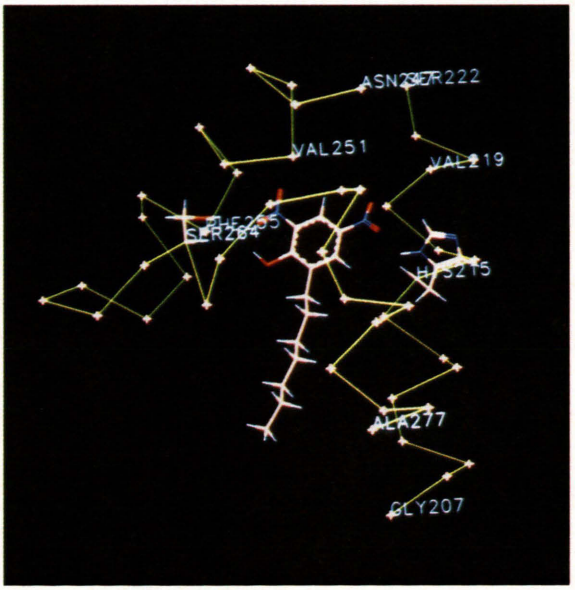


Fig. 6. 2,4-Dinitro-6-heptylphenol in the binding niche of the ala251val mutant of *Chlamydomonas reinhardtii* D 1 protein.

that his215 interacts *via* hydrogen bonding with the nitro group, whereas there is no interaction with ser264 as in the classical inhibitors. This confirms the rough separation into the two families [29]. Impossible it is, however, to discern from Fig. 3 to 6 which of the compounds show enhanced or decreased activity in the mutants. Therefore we carried out simplified energy calculations which have some shortcomings. These are: 1. Molecular dynamics was not taken into account, therefore the given ΔE are not ΔG values. 2. Entropy was not accounted for. 3. The energy minimizations were broken off before full convergence was reached (after 2000 iterations). Table VI shows the outcome of the calculations. The terms used are

E = steric energy;
ΔE_{wt} = gain of steric energy on formation of the complex $P_{wt} \cdot I$;
ΔE_{mut} = gain of steric energy on formation of the complex $P_{mut} \cdot I$.

The dimension of the energy data is kcal · mol^{-1}.

The last column of Table VI seems to indicate that the calculations point into the right direction despite the mentioned simplifications.

In the modelling studies, the extended loop in the D1 protein between the transmembrane helix IV and the parallel helix was omitted. In [30] the effect of a series of substituted bromonitrophenols on the rapid turnover of the D1 protein in *Spirodela* and on the trypsination of the D1 protein at arginine238 in spinach thylakoids is presented, both cleavage sites being on the extended loop. Particularly the tolerance in the val219ile mutant towards a specifically substituted phenol parallels its protection from trypsin. In that paper the role of the extended loop of the D1 protein in herbicide mode of action is discussed.

Conclusions

Photosynthetic electron flow determinations on eleven 2-bromo-4-nitro-6-alkylphenols and eleven 2,4-dinitro-6-alkylphenols with wild type and five mutants of *Chlamydomonas reinhardtii* demonstrated that the phenols enter the Q_B binding niche of the reaction center polypeptide D1 of photosystem II. The dependance of the pI_{50} values of the compounds in the different mutants shows that each mutant has an individual response. This could be rationalized by parabolic or cubic equations relating inhibitory activity to various parameters which homogeneously increase with the number of carbon atoms. Computer modelling studies of two compounds give an indication for the position of the phenols in the binding niche of the D1 protein. Energy calculations were made to support the behaviour of the inhibitors in the mutants.

The data presented here show that tolerance of phenol type inhibitors can be achieved by amino acid substitution in the D1 protein. This is the hitherto missing support that the mode of action of phenol inhibitors points indeed to the displacement of Q_B from its binding site on the D1 protein as with the classical herbicides and inhibitors. Secondary interactions with membranes like binding to other polypeptides or interference with the donor side of PS II do not contribute significantly to their inhibitory potency, for then tolerance by a single amino acid substitution should not be possible. One should note that the data with phenols were obtained with thylakoid membranes *in vitro* and not by *in vivo* screening. *In vivo* the high uncoupling activity of many nitro- and cyanophenols could well contribute to their complex inhibition pattern. Although uncoupling requires higher concentrations than inhibition of electron flow [3] this would limit the possible tolerance by amino acid changes in the D1 protein in an intact organism.

Acknowledgements

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Table VI. Energy calculations of 2-bromo-4-nitro-6-methylphenol (**1**) and 2,4-dinitro-6-heptylphenol (**18**). Both inhibitors display supersensitivity, thus the gain in steric energy ($\Delta E_{wt} - \Delta E_{mut}$) should be positive.

Mutant	Inhibitor	$pI_{50\,wt}$	$pI_{50\,mut}$	ΔpI_{50}	ΔE_{wt}	ΔE_{mut}	$\Delta E_{wt} - \Delta E_{mut}$	Expected result?
Ser264Ala	1	4.4	6.3	−1.9	−15.0	−19.0	+1.0	yes
Ala251Val	18	7.0	7.8	−0.8	−23.6	−24.0	+0.4	yes

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