Multifunctional Mode of Action of Substituted Nitrodiphenylethers in *Scenedesmus* Cells

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Nitrodiphenylethers substituted with Cl, CF_3, and other comparatively simple substituents are compared with respect to their primary effects on the photosynthetic apparatus. These are direct electron-transport inhibition, energy-transfer inhibition (of ATP synthesis), and pigment bleaching, the latter accompanied by decrease of electron transport activity. Certain substituents and their positions at the diphenylether skeleton can be correlated with a particular influence on these phytotoxic effects.

The unicellular alga, *Scenedesmus*, is an appropriate species to furnisch quantitative data on these primary herbicidal modes of action within a 24-h assay period.

Introduction

Inhibition of photosynthesis by nitrodiphenylethers apparently is caused by at least three independent primary effects of this herbicide class. Firstly, nitrodiphenylethers can directly inhibit photosynthetic electron transport between the two photosystems [1, 2] with a concurrent decrease of photophosphorylation. Secondly, as could be demonstrated in a recent investigation, presence of nitrofen (2,4-dichlorophenyl-4′-nitrophenylether) decreased ATP synthesis due to its influence on the phosphorylating (ATP synthetase) system [3]. Study of photosynthetic control and enzymological kinetics showed a competition between nitrofen and ADP, classifying this herbicide as an "energy-transfer inhibitor".

Thirdly, nitrodiphenylethers may cause light-induced destruction of the photosynthetic pigment and redox apparatus [4], as was established for oxyfluorfen (2-chloro-4-trifluoromethoxyphenyl-3′-ethoxy-4′-nitrophenylether), which triggers peroxidative reactions leading to decrease of fatty acids and pigments [5].

Apparently, due to their substituents, diphenylether herbicides have different primary targets at the photosynthetic electron transport system. Therefore, in this study various substituted nitrodiphenylethers were assayed for their phytotoxic mode of action on cultures of autotrophically growing microalgae. Attempts were made to assign various substituents at special positions of the molecule to the primary actions such as (1) electron-transport inhibition, (2) inhibition of ATP synthesis (energy-transfer inhibition), and (3) peroxidative degradation of the thylakoid.

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Materials and Methods

The green microalga, *Scendesmus acutus* (276-3a, Algae Culture Collection, University of Göttingen, Bundesrepublik Deutschland), was cultivated in a sterile mineral medium according to [6]. Growth was carried out in a growth apparatus (Kniese, Marburg, Bundesrepublik Deutschland) at 22 °C in 250 ml vessels as described [7]. Herbicides were added from a 10 mM methanolic stock solution to yield a final concentration of 10 µM, the cell density of the culture was 1 µl packed cell volume (PCV) per ml. Chlorophyll (Chl) was measured after hot methanol extraction [8]. Packed cell volume was determined in calibrated microcentrifuge tubes of 80 µl capacity. ATP was extracted from cells by boiling freshly harvested aliquots of the culture suspension for 10 min in 20 mM Tris-HCl buffer, pH 7.5, and monitoring the supernatant by the firefly-luciferase reaction in an LKB Luminometer, Mod. 1250 [9]. Photosynthetic oxygen evolution was measured at 20 °C with a Clark-type oxygen electrode under saturating-light conditions using an RG 610 red cut-off filter + KG 1 heat filter from Schott [10]. Electron transport activity with isolated spinach chloro-

plasts was determined according to [3] with the system water → methylviologen (1,1′-dimethyl-4,4′-dipyridylium dichloride) or ferricyanide. Chemicals were "pro analysi" from Merck AG, Darmstadt. Biochemicals were purchased from Boehringer, Mannheim, or Serva, Heidelberg, respectively.

Results and Discussion

Scenedesmus cultures are quite appropriate for assaying herbicidal modes of action as far as their effect on the photosynthetic apparatus is concerned [7]. As the peroxidative effect of nitrodiphenylethers on the chlorophyll content develops not earlier than 4 or more hours after application [5], the *direct* inhibitory effect of *p*-nitrodiphenylethers on photosynthetic oxygen evolution and ATP formation can be determined about 3 h after application when chlorophyll and carotenoids are not yet bleached and the destruction of the photosynthetic apparatus is still negligible. As demonstrated in Table II, col. 1 (data in parentheses), the direct electron transport inhibition of uncoupled isolated spinach chloroplasts by the diphenylethers Nos. 2, 6, and 7 is in the same concentration range as observed with inhibition of photosynthetic oxygen evolution of *Scenedesmus* cells. Algae are somewhat more sensitive than isolated spinach chloroplasts.

Decrease of oxygen evolution after short-term treatment is due to inhibition of electron flow plus inhibition of the ATP-synthetase system. However, in most cases, *i. e.* when the ATP level is but slightly affected (see cols. 2 of Tables III, IV), a decrease of oxygen evolution after a 3-h treatment is due to direct inhibition of electron transport by the diphenylether. Data after a 24-h treatment (cols. 4 of Tables II-IV) demonstrate a combination of three herbicidal effects, *i. e.* inhibition of electron transport by direct interference of diphenylethers with the redox system, inhibition of photophosphorylation, as well as decay of electron flow due to herbicide-mediated membrane degradation. This latter effect is indicated by chlorophyll bleaching (col. 3).

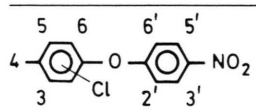
The decreased ATP level after treatment with certain derivatives of Table II is seemingly due to direct inhibition of the ATP-synthetase system. This mode

Table I. Assay of photosynthetic control with diphenylethers present*.

Diphenylethers (50 μM)	(−) ADP	(+) ADP	(+) NH_4Cl
a) Control, (−) diphenylethers	50	267	393
b) 4′-Nitrodiphenylether (No. 1)	50	195	390
c) 4-Chlorophenyl-4′-nitrophenylether (No. 4)	53	115	–
d) 2,6-Dichlorophenyl-4′-nitrophenylether (No. 5)	49	160	380

* System H_2O → methylviologen (0.5 mM) with isolated spinach chloroplasts. Data are μmol O_2 taken up per mg Chl and hour. ADP, 0.125 mM; inorg. phosphate, 10 mM; NH_4Cl, 2.5 mM.

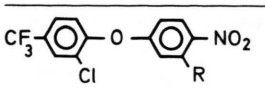
Table II. Inhibition of chloro-substituted 4′-nitrodiphenylethers on *Scenedesmus* cells after a 3- or 24-h treatment.



No.	Substituent	(1) Oxygen evolution after 3 h: μmol O_2 / mg Chl × h	(1) % of control	(2) ATP level after 3 h: nmol ATP / mg Chl	(2) % of control	(3) Chlorophyll content after 24 h: mg Chl / ml pcv	(3) % of control	(4) Oxygen evolution after 24 h: μmol O_2 / ml pcv	(4) % of control
	Herbicide-free control	189	100	5.85	100	11.72	100	2338	100
1	–	176	93	5.47	94	8.56	73	1463	63
2	2-chloro	113	60 [67]*	5.15	88	5.98	51	748	32
3	3-chloro	147	78	4.13	71	7.15	61	1051	45
4	4-chloro	157	83	4.39	75	6.33	54	956	41
5	2,6-dichloro	155	82	3.92	67	5.27	45	474	20
6	3,4-dichloro	125	66 [38]*	5.79	99	6.21	53	757	33
7	2,4-dichloro	79	42 [50]*	4.39	75	4.81	41	269	12

* Data in parentheses are % values of control assaying inhibition of a 50 μM herbicide concentration on photosynthetic electron flow with isolated spinach chloroplasts, system H_2O → ferricyanide (1 mM) including 2.5 mM NH_4Cl; control rate 400 μmol O_2/mg Chl × h. See text for dark control of ATP.

Table III. Inhibition of 3′-substituted 2-chloro-4-trifluoromethylphenyl-4′-nitrophenylethers on *Scenedesmus* after a 3- and 24-h treatment.



No.	Substituent	(1) Oxygen evolution after 3 h		(2) ATP level after 3 h		(3) Chlorophyll content after 24 h		(4) Electron transport after 24 h	
		μmol O_2 / mg Chl × h	% of control	nmol ATP / mg Chl	% of control	mg Chl / ml pcv	% of control	μmol O_2 / ml pcv	% of control
	Herbicide-free control	141	100	5.68	100	8.18	100	1715	100
8	R = H	66	47	4.99	88	1.88	23	205	12
9	R = $NHCH_3$	80	57	5.57	98	1.88	23	43	3
10	R = CF_3	82	58	5.68	100	1.96	24	92	5
11	R = COONa	102	72	5.79	102	4.17	51	701	41
12	R = OCH_3	85	60	4.99	88	1.72	21	98	6
13	R = OC_2H_5	79	56	5.74	101	1.55	19	172	10
14	R = CN	66	47	4.03	71	2.70	33	338	20

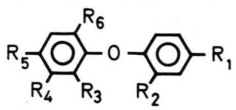
of action (energy-transfer inhibition) was amply documented [3] for nitrofen (no. 7) and is further shown by data of Table I. The increase of electron flow of isolated chloroplasts by ADP in the presence of inorganic phosphate (the so-called "photosynthetic control") is strongly suppressed by compounds No. 4 and 5 (lines c, d), which are also effective in lowering the ATP level in intact *Scenedesmus* while analog No. 1 has a small effect. Uncoupling speeds up the rates to about identical values, regardless of whether the diphenylethers are present in the assay or not (last column; comp. [3]). Without ADP in the assay all diphenylethers have no effect.

In Table II, the inhibitory effects of different chloro-substituted nitrodiphenylethers are listed. Looking separately at either the short-term effects on photosynthesis (cols. 1, 2), chlorophyll content (col. 3), or at the long-term decrease of oxygen evolution (col. 4), it is seen that the dichloro compounds are the most effective ones. It is the 2,6-dichloro derivative (No. 5) that is the best energy-transfer inhibitor, followed by the 3-chloro (No. 3) and the 4-chloro (No. 4) derivatives together with the 2,4-dichlorophenyl-4′-nitrophenylether (No. 7, nitrofen). Among the compounds listed in Table II, nitrofen exhibits the strongest electron transport inhibition (col. 1) as well as the strongest bleaching activity (col. 4). However, in the case of the compounds following nitrofen in the activity scale, bleaching does not match with inhibition of oxygen evolution. The 2,6-dichlorophenyl-4′-nitrophenylether (No. 5) as the second best bleaching compound is a rather poor electron transport inhibitor. Taking together the compounds of Tables II and III, it is evident that the mono- and dichlorophenyl-nitrophenylethers in general represent the best energy-transfer inhibitors with the exception of compound No. 14 having quite different substituents. Some ATP-synthesis inhibition is observed with compound No. 16, a derivative similar to No. 8, since the Cl at carbon-2 of compound No. 8 has been substituted for by an (additional) NO_2 group. In case of efficient inhibition of photophosphorylation the ATP level will not decrease to zero [9]. The dark level is about 50% of the light control.

The bleaching activity of compound No. 7, the best bleaching agent among the herbicides listed in Table II, is reached or surpassed by nearly all 2-chlorophenyl-4′-nitrophenylethers further having a trifluoromethyl group in 4 position (Table III). Electron transfer and ATP level are but slightly affected. The major part of the total herbicidal effect measured as inhibition of oxygen evolution after 24 h apparently is attributable to the bleaching activity. Bleaching is best with all lipophilic substituents at position 3′, whereas CN or COONa are not so effective in this respect. The highest bleaching of all *p*-nitrodiphenylethers assayed in this study can be found when an OC_2H_5 substituent is placed at position 3′ (No. 13, oxyfluorfen; comp. [5, 11]).

In Table IV, substituted diphenylethers are compared having the basic structures of the compounds listed in Tables II and III. However, they allow a conclusion about the influence of other substituents (like Br, CN and NO_2) in different positions. Compound No. 15 is related to No. 8 (nitrofluorfen) by

Table IV. Inhibition of various diphenylethers on *Scenedesmus* after a 3- and 24-h treatment.



Substituents							(1) Oxygen evolution after 3 h		(2) ATP level after 3 h		(3) Chlorophyll content after 24 h		(4) Oxygen evolution after 24 h	
No.	R_1	R_2	R_3	R_4	R_5	R_6	µmol O_2 / mg Chl × h	% of control	nmol ATP / mg Chl	% of control	mg Chl / ml pcv	% of control	µmol O_2 / ml pcv	% of control
	Herbicide-free control						168	100	6.29	100	11.5	100	2088	100
15	CN	H	Cl	H	CF_3	Cl	101	60	6.28	100	6.1	53	275	13
16	NO_2	H	NO_2	H	CF_3	H	57	34	4.90	78	5.3	46	106	5
17	H	NO_2	H	Cl	Cl	H	127	76	6.03	96	5.2	45	302	15
18	NO_2	H	Cl	H	Br	H	98	58	6.15	98	5.5	48	237	11
19	NO_2	H	H	H	NO_2	H	125	74	7.16	114	7.9	69	790	38

carrying an additional Cl at position 6 and a cyano group instead of NO_2. This substitution leads to a decrease of all three herbicidal effects (cols. 1–3), *i.e.* the percent values for No. 15 increased by 13, 12, and 30 as compared with the corresponding percent figures of compound No. 8. A similar behaviour was observed when the nitro group of compound No. 6 located at position 4′ was moved to position 2′ (compound No. 17), indicating a requirement of a nitro group in para position for optimum phytotoxic activity. On the other hand, when in compound No. 16 (fluorodifen) a nitro group (R_3) replaces the chloro substituent at position 2 of compound No. 8, this results in a stronger inhibition of electron transport and ATP formation, but not in an increase of chlorophyll bleaching. The 2-nitro, 4-trifluoromethyl, and the 4′-nitro substituents make fluorodifen the most effective electron transfer inhibitor of all compounds tested. Furthermore, together with nitrofen (No. 7), fluorodifen is the best multifunctional nitrodiphenylether, showing good activities of all three herbicidal effects assayed here. These three modes of action can be influenced gradually by replacing the chloro by a bromo substituent in compound No. 7. As compared to the 2,4-dichloro analog (No. 7) the resulting 2-chloro-4-bromophenyl-4′-nitrophenylether (No. 18) shows decreased bleaching and energy-transfer inhibition, the percent figures of No. 18 are increased by 6, 16 and 23, respectively (comp. cols. 3, 2, 1 of Tables II and IV). The 4-nitrophenyl-4′-nitrophenylether (No. 19) derived from compound No. 4 by replacing the chloro group at position 4 by a nitro substituent is an example that different herbicidal modes of action can be simultaneously enhanced and decreased by substitution. As compared to the 4-chlorophenyl-4′-nitrophenylether (No. 4), the 4-nitro-4′-nitro compound shows improved electron transfer inhibition, a smaller peroxidative (bleaching) effect, and no energy-transfer inhibition.

All diphenylethers with "bleaching" activity presented here exhibit a light-induced ethane production with intact *Scenedesmus* cells. This indicates peroxidation of unsaturated fatty acids initiating pigment and membrane degradation (comp. [5]). Data on peroxidative hydrocarbon production, inhibition constants of energy transfer and electron transport derived from cell-free systems will be presented elsewhere (Lambert *et al.*, this laboratory, unpubl.).

Except for ref. [5], the phytotoxic effect of nitrodiphenylethers was tested so far with intact higher plants ([12–14; see also [5, 11] for refs.). The only parameters reported were either dry weight or killing rate. Such data do not indicate the primary targets of diphenylethers, and consequently no possible relation between activity and chemical constitution can be established. Although the three effects on photosynthesis overlap to a certain extent as mentioned above, our data using microalgae apparently yield more specific and quantitative parameters for herbicidal influences on the photosynthetic apparatus. They allow for an approach to structure/activity correlations of primary modes of action.

Another advantage of the algal assay is the reliable application of the herbicide to a liquid culture and the fast expression of the phytotoxic effects within one day. Furthermore, electron transport inhibition, energy-transfer inhibition, and bleaching activity can be measured in the same culture at the proper physiological stage during herbicide treatment.

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