BIOLOGIA PLANTARUM (PRAHA)

32 (1): 28–34, 1990

Preliminary Studies on the Mechanisms of Action of Phosphonic Analogues of Morphactins on Plants and Bacteria

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Abstract. Two classes of phosphonopeptides, those containing P-terminal 9-aminofluoren-9-ylphosphonic acid and those of dialkyl 9-aminofluoren-9-ylphosphine oxides, influence plant growth according to different mechanisms. The effect of these compounds on the growth of several bacterial species, including the photosynthetic bacterium *Rhodospirillum rubrum*, as well as on the activity of photosystems 1 and 2 in isolated *Pisum sativum* and *Spirodela oligorrhiza* chloroplasts was studied. The peptides of free, unblocked 9-aminofluoren-9-ylphosphonic acid acted in a morphactin-like manner, whereas those of dialkyl 9-aminofluoren-9-ylphosphine oxides influenced photosynthesis indirectly.

Additional index words: Pisum sativum L., *Spirodela oligorrhiza* (KURZ.) HEGELM., *Bacilllus subtilis* PCM 1949, *Escherichia coli* PCM 2057, *Micrococcus luteus* PCM 1944, *Serratia marcescens* PCM 549, *Rhodospirillum rubrum*, phosphonopeptides, photosynthesis.

Only few herbicides inhibit plant growth by directly suppressing the activity of enzymes involved in the major physiological processes. Roughly, herbicides fall into three categories: those acting as inhibitors of certain enzymes of the major metabolic pathways, those influencing photosynthesis or respiration, and those affecting specific processes of plant growth (hormonal regulation, defense system, *etc.*). The derivatives of 9-aminofluoren-9-ylphosphonic acid I (phosphonic analogues of morphactins) are newly designed, synthetic plant growth regulators (GANCARZ *et al.* 1985, KAFARSKI *et al.* 1985, LEJCZAK *et al.* 1985). Depending on the applied dose and the chemical structure, they cause morphological changes or even the death of plants. Among these compounds the peptides containing P-terminal derivatives of fluorene are of special interest. Peptides II and III (Fig. 1) of 9-aminofluoren-9-ylphosphonic and 1-aminocyclohexylphosphonic acids, respectively, exhibit typical morphactin-like activity, such as causing root elongation with simultaneous stem shortening (LEJCZAK *et al.* 1985), while the peptides IV and V containing dialkyl

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Received October 26, 1988; accepted March 15, 1989

9-aminofluoren-9-ylphosphonates show high herbicidal activity (KAFARSKI *et al.* 1985).

The object of this study was to probe the possible mechanism(s) of action of these two classes of peptides (compounds II, III and IV, V, respectively) using three model systems. Since in bacteria and plants the major metabolic pathways are similar, bacteria seem to be a useful tool for elucidation of the mode of action of herbicides (BAXTER *et al.* 1973, STEINRUCKEN and AMRHEIN 1980, SCHULZ *et al.* 1985, FISCHER *et al.* 1986, LEJCZAK and KIJAS-KACZANOWSKA 1987) which act as antimetabolites or inhibitors of certain enzymes. Thus, we have studied the influence of both classes of peptides containing P-terminal derivatives of fluorene on the growth of selected bacterial species. Herbicidal action of peptides IV and V is usually accompanied by chlorosis, and one may speculate that they may exert their effect through the photosynthetic apparatus. Therefore, we studied the influence of all

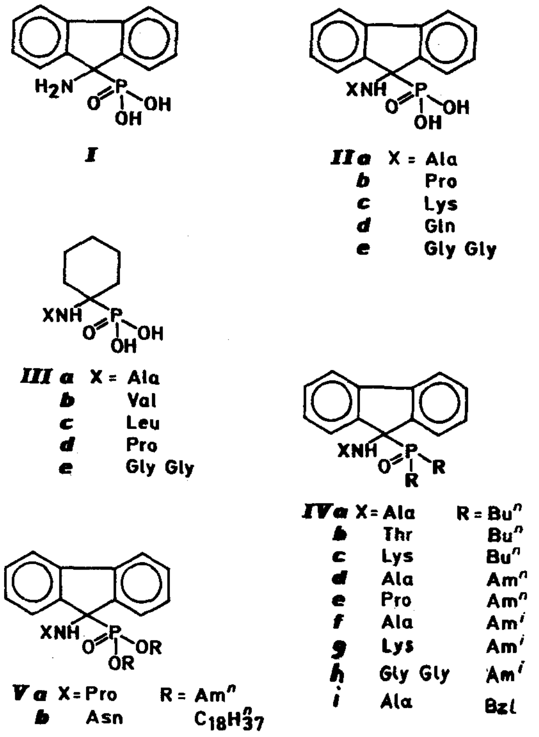

Fig. 1. 9-Aminofluoren-9-ylphosphonic acid (I); peptides (II) based on (I); peptides (III) of 1-aminocyclohexylphosphonic acid; peptides (IV) of 9-aminofluoren-9-ylphosphine oxides; and peptides (V) based on dialkyl 9-aminofluoren-9-ylphosphonates.

**Abbreviations used:* Am^i = isoamyl (isopentyl), Am^n = n-amyl (n-pentyl), Bu^n = n-butyl, Bzl = benzyl (phenylmethyl), chl = chlorophyll, MIC = minimal inhibitory concentration, PS 1 = photosystem 1, PS 2 = photosystem 2.

groups of peptides on photosynthetic electron flow in isolated chloroplasts, as well as their effect on the growth of the photosynthetic bacterium *Rhodospirillum rubrum*.

MATERIALS AND METHODS

Chemicals

The phosphonopeptides were synthesized according to the procedure described by KAFARSKI *et al.* (1985), and LEJCZAK *et al.* (1985).

The influence of phosphonopeptides on photosynthesis: As a testing material intact chloroplasts from pea (*Pisum sativum* L.) and duckweed [*Spirodela oligorrhiza* (KURZ.) HEGELM.] were used. They were isolated according to NAKATANI and BARBER (1977), suspended in 0.005 M Tris-HCl buffer (pH 7.5) and stored in liquid nitrogen. The influence of various concentrations of phosphonopeptides on chloroplasts was evaluated by measuring the activities of PS 1 and PS 2. PS 1 activity was expressed as O_2 uptake rate and measured according to BOSE *et al.* (1981), using an artificial donor-acceptor system (diaminoduren-methylviologen) and a Clark-type oxygen electrode equipped with a teflon membrane. O_2 release by PS 2 was followed spectrophotometrically using 2,6-dichlorophenylindophenol as artificial electron acceptor according to MILES (1982). IC_{50} values were determined as the concentration of the peptide required to reduce the activity of the respective photosystem by 50 % in relation to the control.

Microbiology

Four bacterial species: *Escherichia coli* PCM 2057, *Serratia mercescens* PCM 549, *Bacillus subtilis* PCM 1949 and *Micrococcus luteus* PCM 1944 were obtained as lyophilized preparates from the Polish Collection of Microorganisms (PCM). The MIC and IC_{50} values of the peptides for each strain were determined on a defined liquid medium according to ATHERTON *et al.* (1979).

Inocula of all strains were prepared by growing the test organism overnight in the liquid medium and diluting the cultures to approximately 10^6 cfu (colony forming units) per cm^3.

A doubling dilution series was prepared in the liquid medium for each compound. 0.5 cm^3 of each concentration was added to 9.4 cm^3 of the medium to give, after addition of 0.1 cm^3 of inoculum, a final concentration range from 0.0065 to 512.0 μg cm^{-3}. The incubation was carried out overnight at 37 °C for *Escherichia coli* or 72 h at 28 °C for other species. Turbidity of each culture was measured during the incubation time. IC_{50} values were defined from the growth curves as the concentration required to reduce the growth of the culture in the liquid medium to 50 % of the control value. The samples in which no turbidity was observed were used for MIC determination. MIC was defined as the lowest concentration of the peptide that either completely inhibited the growth or permitted 10 or fewer microcolonies to grow.

The effect of phosphonopeptides on the growth of *Rhodospirillum rubrum*: The bacterium *Rhodospirillum rubrum* was a kind gift from Prof. Peter von Felten (University of Zürich). MIC and IC_{50} values of the peptides were determined for this microorganism grown in darkness on the liquid medium (ATHERTON *et al.* 1979), chosen as the medium for heterotrophic growth, and for the bacterium grown under continuous illuminance (2000 lx) on the liquid medium for autotrophic microorganisms consisting of: 1.023 g $MgSO_4 \times 7\,H_2O$, 0.5 g $(NH_4)_2SO_4$, 1.5 g sodium succinate, and 5 g of yeast extract (final volume 1000 cm^3); pH 6.8–7.2 adjusted by addition of 1 M K_2HPO_4.

A 14 d-old homogeneous culture of the microorganism was used as inoculum. The inoculated samples were cultured during two weeks at 25 °C in the presence of the peptide in the final concentration range from 0.0065 to 512.0 $\mu g\ cm^{-3}$ (a doubling dilution series). Dry mass was then determined and was taken as a measure of bacterial growth. Thus, the samples were filtered through Synpor membrane filter (pore size 0.40 μm; Ø 50 mm), washed with distilled water to remove any medium constituents and dried at 105 °C to a constant mass. IC_{50} values were defined as the concentration of peptide required to reduce dry mass of *Rhodospirillum rubrum* culture by 50 % in relation to the control. MIC was defined as the lowest concentration of the peptide that completely inhibited bacterial growth. The quantitative observations of the influence of the peptidyl derivatives of fluorene on the growth of *Rhodospirillum rubrum* were also carried out by growing the microorganism on solid media for heterotrophic and autotrophic microorganisms as described above.

RESULTS AND DISCUSSION

The peptides II and III were practically inactive towards all studied bacteria and did not influence photosynthetic activity in chloroplasts from *Spirodela oligorrhiza*. The only exception was 9-(N-alanylamino)fluoren-9-ylphosphonic acid (IIa) which inhibited slightly the growth of *Escherichia coli* (IC_{50} = 445.0 $\mu g\ cm^{-3}$) and *Rhodospirillum rubrum* in heterotrophic conditions (IC_{50} = 235.0 $\mu g\ cm^{-3}$). This compound also affected PS 2 of *Spirodela oligorrhiza* (40 % of inhibition at the concentration of 166.0 $\mu g\ cm^{-3}$). Moreover, there was no relation between the plant growth regulatory properties of the peptides II and III and their influence on any of the model systems studied. For example, the herbicidal 1-(N-valylamino)cyclohexylphosphonic acid (IIIb) exhibited a slight stimulatory effect on both photosystems of duckweed

On the basis of the literature data (OMURA *et al.* 1984, TACHIBANA *et al.* 1986, LEJCZAK and KIJAS-KACZANOWSKA 1987) and present observations we postulate that the mode of action of phosphonopeptides II and III may be similar to that observed for antibacterial phosphonopeptides (ALLEN *et al.* 1978, LEJCZAK *et al.* 1986), *i. e.* consisting of transport into plant tissues, intracellular hydrolysis of the peptide and release of the free aminophosphonic acid which influences the growth of the plant.

On the contrary, the herbicidal peptides IV and V were active in all three model systems used in this work. Most of them caused a continuous decrease of oxygen development by PS 2 of *Spirodela oligorrhiza*, while the effect on *Pisum sativum* chloroplast activity was less significant or even absent (Table 1). However, there was a relationship between their inhibitory and herbicidal effects. For example, the strongest herbicides di-*n*-butyl 9-(N-threonylamino)fluoren-9-ylphosphine oxide (IVb) and di-isoamyl 9-(N-glycylglycylamino)fluoren-9-ylphosphine oxide (IVh) exhibited the most significant inhibitory effect towards PS 2 in both tested plants. These phosphonopeptides also inhibited PS 1 in *Pisum sativum* chloroplasts (Table 1) and they exhibited the same relationship with herbicidal activity as in the case of PS 2. The kinetics of their action, however, was unusual because at inhibitory concentrations a non-linear oxygen uptake rate was observed (Table 1) and the inhibitory effect increased with time. Anyway, the peptides IV and V appeared to be very weak inhibitors of PS 1 and PS 2 and the observed IC_{50} values for photosynthetic electron transport inhibition in isolated chloroplasts were much higher than IC_{50} values for herbicidal activity towards duckweed. This may suggest an indirect effect on photosynthesis.

TABLE 1
The influence of phosphonopeptides IV and V on the activity of isolated *Spirodela oligorrhiza* and *Pisum sativum* chloroplasts

Peptide	Herbicidal activity[a] IC_{50} [µg cm^{-3}]	Activity of PS 2 IC_{50} [µg cm^{-3}] *S. oligorrhiza*	Activity of PS 2 IC_{50} [µg cm^{-3}] *P. sativum*	Activity of *P. sativum* PS 1 IC_{50} [µg cm^{-3}]
IVa	22.7 (0.117)*	n.d.	s.	286.3 (0.136)*
IVb	< 2.2 (0.141)	< 2.2 (0.102)*	79.6 (0.141)*	24.3 (0.167)
IVc	37.5 (1.304)	234.5 < (0.316)	s.	450.2 < (0.133)
IVd	24.2 (0.063)	220.0 < (0.063)	396.0 (0.810)	136.4 (0.206)
IVe	18.6 (0.102)	233.0 (0.632)	419.4 > (0.102)	447.4 < (0.141)
IVf	26.4 (0.102)	n.d.	n.d.	143.0 (0.133)
IVh	< 2.4 (0.141)	4.8 (0.063)	101.4 (0.133)	33.8 (0.102)
Va	19.9 (0.141)	24.9 (0.063)	288.8 (0.172)	209.2 (0.141)
Vb	562.6 (0.141)	43.9 (0.075)	791.1 < (0.117)	n.d.

[a] Expressed as reduction of *Spirodela oligorrhiza* dry mass increase by 50 % in relation to control. Data taken from KAFARSKI *et al.* (1985).
n.d., not determined.
s., stimulatory effect.
*, standard deviation (δ) values calculated with LOTUS software.

The peptides IV and V did not influence the growth of *Serratia marcescens* and *Bacillus subtilis* while their effect on the growth of *Escherichia coli* and *Micrococcus luteus* was negligible (Table 2). *Rhodospirillum rubrum* was more sensitive to the action of these peptides than other bacterial species studied (Table 2). Peptides IV influenced more significantly the heterotrophically growing bacterial cultures than the autotrophically growing ones. However, also in this case there was a relationship between herbicidal activity against *Spirodela oligorrhiza* and inhibitory effect towards *Rhodospirillum rubrum.*

When *Spirodela oligorrhiza* was cultivated in the presence of sublethal doses of di-*n*-butyl 9-(*N*-threonylamino)fluoren-9-ylphosphine oxide IVb (*i.e.* concentrations lower than 2.21 µg cm^{-3}), the decrease of photosynthetic activity (60–70 % of control activity), as well as of chlorophyll (chl) content in isolated chloroplasts were observed, while the chl *a/b* ratio remained unchanged.

TABLE 2
The influence of phosphonopeptides IV and V on the growth of bacteria

Compound	Herbicidal activity^a IC$_{50}$ [µg cm^{-3}]	IC$_{50}$ [µg cm^{-3}] for *E. coli*	IC$_{50}$ [µg cm^{-3}] (MIC [µg cm^{-3}]) for *M. luteus*	IC$_{50}$ [µg cm^{-3}] (MIC [µg cm^{-3}]) for *R. rubrum*	
				heterotroph.	autotroph.
IVa	22.7 (0.063)*	256.0 (0.632)*	512.0 (0.126)*	20.0 (0.800)* [512.0] (1.414)	n.d.
IVf	26.4 (0.063)	512.0 < (0.080)	350.0 (0.632) [512.0] (0.063)	96.0 (1.166) [256.0] (1.327)	n.d.
IVg	17.4 (0.133)	512.0 (0.261)	66.0 (0.261) [128.0] (0.190)	55.0 (1.414) [128.0] (1.414)	26.0 (0.316) [64.0] (0.316)
IVh	2.4 (0.141)	512.0 (0.253)	150.0 (1.414) [256.0] (1.600)	55.0 (0.632) [512.0] (0.748)	8.0 (0.141) [32.0] (0.063)
IVi	307.2 (0.063)	512.0 < (0.253)	512.0 (0.063)	512.0 (632)	380.0 (0.632) [512.0] (0.632)
Vb	562.6 (0.063)	512.0 < (0.126)	512.0 < (0.063)	512.0 < (0.632)	512.0 < (0.632)

^{a}Expressed as the reduction of *Spirodela oligorrhiza* dry mass by 50 % in relation to the control (KAFARSKI *et al.* 1985).
n.d. – not determined. These peptides in concentrations over 64 µg cm^{-3} significantly inhibited the growth of *Rhodospirillum rubrum* on the solid medium.
* standard deviation (δ) values calculated with LOTUS software.

The effect on photosynthesis may occur through inhibition of photosynthetic electron transport, decrease of chl or carotenoid contents or by disturbing the membrane transport. Our results suggest that herbicidal phosphonopeptides IV and V may have only an indirect effect on the photosynthetic activity of plants.

The results of the present studies showed that the observed inhibition of photosynthesis by herbicidal phosphonic analogues of morphactins is a secondary or tertiary effect. Thus, the exact molecular mechanism of action of these compounds still remains to be determined.

Acknowledgement

This work was supported by the CPBR 03.13 Programme.

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