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Photoactivation of the Nematicidal Compound α-Terthienyl from Roots of Marigolds (*Tagetes* Species)

A POSSIBLE SINGLET OXYGEN ROLE*

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The nematicidal compound α-terthienyl from roots of *Tagetes* species generates upon irradiation with near ultraviolet light reactive oxygen species on which the *in vitro* nematicidal activity depends. This system was studied by following the inhibition of glucose-6-phosphate dehydrogenase by photoactivated α-terthienyl and protection of the enzyme activity in the absence of oxygen and by various additions. Addition of mannitol, benzoate, superoxide dismutase or catalase did not have any effect nor did H_2O_2. This suggests that OH•, O_2^-, and H_2O_2 are not the reactive oxygen species involved. The enzyme was protected against photoactivated α-terthienyl in air-saturated solutions by singlet oxygen quenchers such as histidine, methionine, tryptophan, bovine serum albumin, and NaN_3. Furthermore, inactivation of the enzyme was about 3.5 times faster in D_2O than in H_2O. When α-terthienyl in CH_2Cl_2 was irradiated in the presence of the olefin adamantylideneadamantane, a stable dioxetane was formed which decomposed to adamantanone when heated above its melting point. These results indicate a singlet oxygen-mediated process.

The compound terthienyl (2,2′-5,2″-terthienyl) from *Tagetes* species (1) was described as a nematicidal principle from roots of these plants (2) together with the biogenetically related 5-(3-buten-1-ynyl)-2,2′-dithienyl (3). Thus, a chemical base was given to the suppressing effect of *Tagetes* on endoparasitic plant nematodes such as *Pratylenchus penetrans, Meloidogyne hapla, Meloidogyne incognita,* and *Meloidogyne arenaria* (2-5).

Further evidence for biological activity was found by screening other Compositae on their ability to suppress populations of *P. penetrans* in the soil. From the 16 tested plant species, distributed over the genera *Eclipta, Gaillardia, Flaveria, Tagetes, Berkheya,* and *Echinops* which contain α-terthienyl and the dithiophene derivative (6), 15 effectively suppressed *P. penetrans. Flaveria repanda* was the only species on which *P. penetrans* could breed (7).

It was shown that irradiation with near ultraviolet light strongly enhanced the nematicidal activity of α-terthienyl (8). *P. penetrans* which had been in the roots of *Tagetes* for up to 10 days was also rapidly killed when exposed to near ultraviolet light (9). This suggested that inside the plant roots, α-terthienyl or compounds with similar properties (or both) permeate into the nematodes and are involved in killing these endoparasitic nematodes.

In this study, it is demonstrated that α-terthienyl upon irradiation generates reactive oxygen species, probably singlet oxygen, on which the nematicidal activity *in vitro* depends.

MATERIALS AND METHODS

The nematode *Ditylenchus dipsaci* (onion race) for homogenates was reared in potato tubers at 15°C.

Glucose-6-P dehydrogenase (yeast), 6-phosphogluconate dehydrogenase, and glutamate dehydrogenase were purchased from Boehringer Mannheim Chemicals; catalase (beef liver), superoxide dismutase (bovine blood), bovine serum albumin, glucose 6-phosphate, $NADP^+$, and amino acids, from Sigma Chemical Co. Deuterium oxide, benzoate, mannitol, NaN_3, and most of the other chemicals used were from Merck.

Photooxidation in biochemical experiments was done at 21°C (room temperature) in disposable borosilicate glass culture tubes (10 × 75 mm) (absorbance, $\lambda < \pm 300$ nm) with 1 ml of medium and a Philips TL W/8 black light tube. Light intensity was determined with an EG and G spectroradiometer and was 3.25 watt m^{-2} with a maximum intensity at 352 nm of 0.087 watt m^{-2} nm^{-1}.

A typical medium contained 1 μg ml^{-1} of glucose-6-P dehydrogenase, 0.1 M triethanolamine buffer, pH 7.6, and mostly 32 μM α-terthienyl (m.p. 93.2-94.7°C; λ_{max} 350 nm; ε 23,600 M^{-1} cm^{-1}) emulsified in 1 g $liter^{-1}$ of Tween 20® or Tween only (control).

Other additions were as indicated. After different times of irradiation, 50 μl was removed from the reaction vessel and assayed for glucose-6-P dehydrogenase activity in 0.95 ml of 0.1 M triethanolamine buffer, pH 7.6, with 2.2 mM glucose 6-phosphate, 0.1 mM $NADP^+$, and 15 mM $MgCl_2$ in a Unicam Sp 1800 spectrophotometer at 340 nm.

Irradiation experiments in D_2O were carried out in 0.1 M triethanolamine buffer, pD 7.6, and after adding 1 μg ml^{-1} of glucose-6-P dehydrogenase, D_2O concentration was better than 98%. In this experiment and the control experiments in H_2O, the concentration of α-terthienyl was 7.2 μM.

Twenty milligrams of the olefin adamantylideneadamantane® (m.p. 185.6-186.8°C) in 25 ml of CH_2Cl_2 (in 25 ml of volumetric glass of borosilicate glass; absorbance, $\lambda < \pm 325$ nm) with 0.8 μM of α-terthienyl was irradiated in a Rayonet Photochemical Reactor with 16 Rayonet Photochemical Reactor Lamps (R.P.R. 3500 Å) without filter; O_2 flow, 2 ml/min (10). Detection was gas chromatographical with a Perkin Elmer F17 (flame detection), Carbowax; injection temperature, 250°C; column, 205°C; retention times: adamantanone, ±105 s; adamantylideneadamantane, ±465 s.

RESULTS

Activities of cholinesterase, glucose-6-P dehydrogenase, and malate dehydrogenase in homogenates of the plant-parasitic nematode *D. dipsaci* drastically decreased in the presence of α-terthienyl when irradiated with near ultraviolet light. Pure glucose-6-P dehydrogenase from yeast, 6-phosphogluconate dehydrogenase, and glutamate dehydrogenase were also inhibited by α-terthienyl (32 μM) upon irradiation. Details were studied with glucose-6-P dehydrogenase.

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Data in Fig. 1 compare the inhibition of glucose-6-P dehydrogenase by different concentrations of photoactivated α-terthienyl in air-saturated solutions. Concentrations of α-terthienyl higher than 32 μM hardly had additional effect because oxygen became a limiting factor. The inhibitory properties of photoactivated α-terthienyl were hardly affected in the pH range 5.9 to 9 or by changing from 0.1 M triethanolamine, pH 7.6, to 0.1 M Tris/HCl, pH 7.6, or 0.1 M phosphate buffer, pH 7.6. However, increasing the molarity of the triethanolamine buffer, pH 7.6, protected the enzyme as is shown in Fig. 2.

Good protection of the enzyme against photoactivated α-terthienyl was obtained with 10 mM dithionite and 150 mM mercaptoethanol. These concentrations were high enough to create anaerobic conditions in the media which was checked with an oxygen electrode. Support that O_2 is essential for inhibition of the enzyme by photoactivated α-terthienyl was found by irradiation in a nitrogen atmosphere. After 3 h of incubation in light, there was only a slight decrease in activity of the enzyme compared to the control. There was also protection of the enzyme in air-saturated solutions by 10 mg ml^{-1} of bovine serum albumin and by 10 mM of the amino acids histidine, tryptophan, and methionine as is shown in Fig. 3. Proteins and these amino acids are quenchers of singlet oxygen (11, 12). None of the other amino acids (10 mM) had this effect. In D_2O, there is an increase in $^1O_2$ reactions. The lifetime of $^1O_2$ has been determined to be 2 μs in H_2O and 20 μs in D_2O (13). This solvent deuterium effect was used as diagnostic test for $^1O_2$ (12–15). The presence of this D_2O activation effect as shown in Fig. 4 also supports the hypothesis that $^1O_2$ is generated by α-terthienyl upon irradiation.

Further evidence that $^1O_2$ is generated by photoactivated α-terthienyl was found by testing other quenchers of $^1O_2$ and inhibitors or potentiators of active oxygen species H_2O_2, O_2^- and OH·. Table I shows that the $^1O_2$ quencher N_3^- (16) protected the enzyme against photooxidation. Catalase did not affect the inhibition of glucose-6-P dehydrogenase nor did addition of H_2O_2. Therefore, H_2O_2 may be excluded as a mediator of the photooxidation. Addition of superoxide dismutase did not show any effect which makes mediation of O_2^- unlikely.

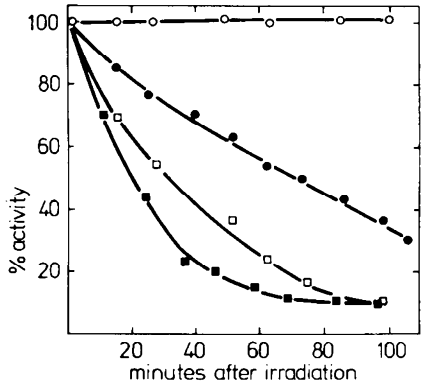


FIG. 1. Effect of 0 (○——○), 8 (●——●), 32 (□——□), and 64 (■——■) μM photosensitized α-terthienyl in air-saturated solutions on activity of glucose-6-P dehydrogenase. Experiments were carried out as described under "Materials and Methods."

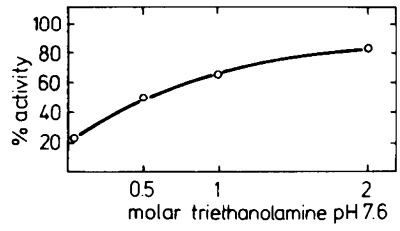


FIG. 2. Loss of activity of glucose-6-P dehydrogenase by 32 μM terthienyl in 0.05, 0.5, 1, and 2 M air-saturated triethanolamine buffer, pH 7.6. Activities of the enzyme were assessed after 40 min of irradiation with near ultraviolet light. Other conditions were as described under "Materials and Methods."

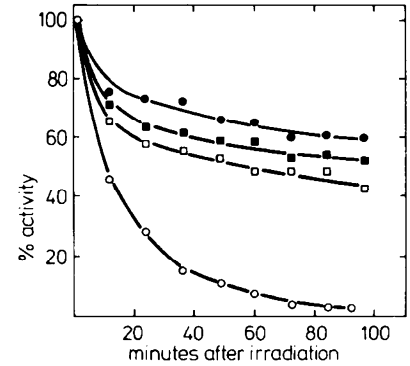


FIG. 3. Protection of glucose-6-P dehydrogenase activity against 32 μM photosensitized α-terthienyl in air-saturated solutions (○——○) by 10 mM histidine (●——●), tryptophan (□——□), and methionine (■——■). These separate experiments were carried out as indicated under "Materials and Methods."

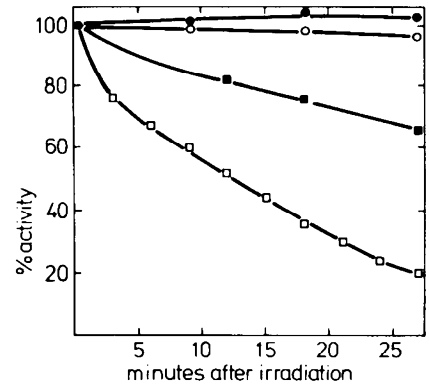


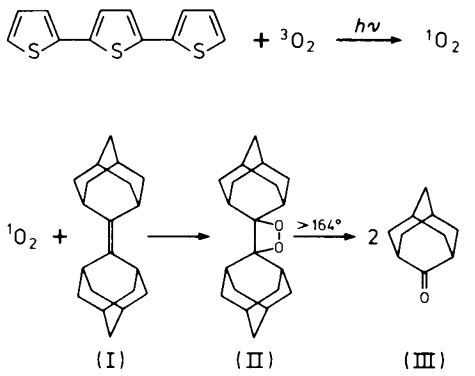
FIG. 4. Deuterium effect of photosensitized α-terthienyl on the activity of glucose-6-P dehydrogenase in air-saturated solutions, enzyme in D_2O (○——○), in H_2O (●——●), in D_2O with 7.2 μM α-terthienyl (□——□), in H_2O with 7.2 μM α-terthienyl (■——■). Other conditions were as indicated under "Materials and Methods."

TABLE I

Effects of a number of compounds on the inhibition of glucose-6-P dehydrogenase by photoactivated α-terthienyl

Experimental conditions were as described under "Materials and Methods." Values from reaction vessels without α-terthienyl (Experiment 1) served as a control and these were arbitrarily designated as 100%. In Experiment 2, 32 μM α-terthienyl was added and in the others, additives were added as indicated.

Experiment	Addition	Activity of glucose-6-P dehydrogenase as percentage of control	
		After 36-min irradiation	After 90-min irradiation
1	None	100	100
2	α-Terthienyl, 32 μM	24	3
3	+NaN_3, 10 mM	89	82
4	+Catalase, 417 units	26	2
5	+H_2O_2, 0.3 mM	20	2
6	+Superoxide dismutase, 0.01 mg	22	2
7	+Bovine serum albumin, 0.01 mg	23	0
8	+Bovine serum albumin, 10 mg	55	29
9	+Mannitol, 10 mM	20	1
10	+Sodium benzoate, 10 mM	18	3
11	+EDTA, 0.5 mM	24	9



Mannitol and benzoate are scavengers of the hydroxyl radical OH·. Both compounds had no effect on the glucose-6-P dehydrogenase inhibition, indicating that OH· is not the oxygen species mediated by irradiated α-terthienyl.

Direct evidence for the production of $^1O_2$ by irradiation of α-terthienyl in the presence of air was obtained. When α-terthienyl was irradiated by ultraviolet light of 325 to 400 nm in CH_2Cl_2 solution in the presence of the olefin adamantylideneadamantane (I), the dioxetane (II) formed could be detected (10). After about 7 h of irradiation, no more adamantylideneadamantane could be found. When the experiment was repeated under identical conditions but omitting α-terthienyl, no decomposition of the olefin could be detected and no dioxetane or adamantanone was formed. The detection of the decomposition product of II, adamantanone (III), was done quantitatively using gas chromatography. Clearly, α-terthienyl acts as a sensitizer in the conversion of $^3O_2$ to $^1O_2$.

DISCUSSION

Dye-sensitized photooxidation of alcohols, carbohydrates, lipids, amino acids, and proteins or the bases in nucleic acids is considered to be a basis for photodynamic action such as inactivation of viruses, distortion of membranes, inactivation of enzymes, and killing of cells, unicellular and multicellular organisms (17–20). It is well established that $^1O_2$ in aqueous solutions is an intermediate in a number of these photodynamic reactions since it reacts rapidly with histidine, tryptophan, methionine, and proteins (11, 12), with the specific quencher N_3^- (16) and by enhancement of its effects in a deuterated solvent which increases the lifetime of $^1O_2$ (13). Over 400 compounds have been reported to sensitize biological systems to light (21). Typical classes of photodynamically active dyes for proteins are representatives of the acridines (acridine orange, proflavine), anthraquinones, azins (safranins), flavins, porphyrins, thiazins (methylene blue, thionin), thiopyronin, and the xanthenes (eosin Y, rose bengal, etc.) (20–22).

The terthienyl does not belong to one of the aforementioned classes and may therefore be the first of a new species of sensitizers. Also, a great number of dithienyls, which seems to be an essential group, exerted excellent nematicidal activity *in vitro* as did α-terthienyl (23).

The necessity of $^3O_2$ for biological activity of α-terthienyl upon irradiation was demonstrated by carrying out experiments in the absence of $^3O_2$. The results mitigate against mediation of other activated oxygen species than $^1O_2$. By using various quenchers, inhibitors, or potentiators, involvement of O_2^-, OH·, and H_2O_2 could be excluded. The absence of protection of enzyme activity by EDTA indicates that also metal ion·oxygen complexes are probably not involved. On the other hand, the specific $^1O_2$ quenchers histidine, tryptophan, methionine, and N_3^- effectively protected glucose-6-P dehydrogenase against the combination of α-terthienyl and near ultraviolet light. There was also a clear deuterium solvent effect and direct evidence for $^1O_2$ production was obtained because of the specific reaction with the olefin adamantylideneadamantane (10).

Apparently, dispersion of the insoluble α-terthienyl with Tween 20 did not prevent diffusion of $^1O_2$ through the micelles into the aqueous phase. This is consistent with a recent kinetic study (24).

To be solved remains the problem of the nematicidal activity of α-terthienyl *in vivo*, if active at all. Exoradicular effects of growing *Tagetes* plants hardly exist. Populations of saprozoic soil-inhabiting nematode species are not affected by growing *Tagetes* and there are ectoparasitic plant nematodes that use *Tagetes* as a host plant. This agrees with the findings that root diffusates of marigolds do not affect nematodes (5, 25, 26). Moreover, in the soil, in absence of light, photoactivation of α-terthienyl, which was shown to be necessary for nematicidal activity (8), does not take place. There is good evidence that only endoparasitic nematodes can be suppressed by growing *Tagetes* (5) and it may be postulated that inside the living root systems α-terthienyl or related compounds (or both) are activated through other mechanisms than light. The endoparasitic nematode *P. penetrans*, was rapidly killed by near ultraviolet light when it had been in *Tagetes* roots (9). Recently, it was shown that extracts from roots, leaves, and flowers of many Compositae contain phototoxic activity against the yeast *Candida albicans*. The active components from *Chrysanthemum* and *Cirsium* were tentatively identified as a C_{13}- and a C_{14}-triyn-diene-acetate (27). Two other acetylenes (C_{13}-triyn-enes) with excellent nematicidal activity against the leaf nematode *Aphelenchoides besseyi* were isolated from the aerial parts of safflower (*Carthamus*) (28). These phenomena together with the fact that most of the Compositae that suppress populations of *P. penetrans*, nearly 70 plant species divided over 27 genera, contain α-terthienyl or a number of biogenetically related compounds (terthienyls, dithienyls, dithioacetylenes, and acetylenes) (6, 7), makes a chemical base for suppressing effects likely.

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