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Identification of alpha-terthienyl radical *in vitro*: A new aspect in alpha-terthienyl phototoxicity

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Alpha-terthienyl, the naturally occurring larvicide from tagetes species, exhibits unique free radical generating property. Besides generating singlet oxygen and superoxide anion radical, it generates alpha-terthienyl radical, when exposed to ultraviolet light. This free radical generating property of alpha-terthienyl can be utilized to understand the mechanism of action against insects; however this use also poses a threat to the non-target species exposed to it.

LIGHT-activated pesticides are recognized as a new technology with considerable promise in the area of pest control. Erythrosin B has already been registered for housefly control, and a promising photodynamic herbicide based on porphyrin metabolism is under development[1]. Among the most active biocides whose activity is enhanced by light are the naturally occurring thiophenes and biosynthetically-related polyacetylenes which are characteristic secondary plant metabolites of the plant family Asteraceae[1]. For example, alpha-terthienyl (α-T) (Figure 1) has been extensively studied and evaluated as a larvicide[2], fungicide[3] and nematocide[4]. Several attempts have been made to examine the phototoxicity of terthienyl and bithenyl analogs[5–8], which have been moderately successful in improving our understanding of the structure–activity relationship and for improving efficacy. α-T gives an absorption peak at 351 nm in ethanol (Figure 2) and generates singlet oxygen[6] and superoxide anion radical both *in vivo*[9,10] and *in vitro*[10,11] under ultraviolet (UV) light.

A stock solution of 2 µg/ml of α-T was prepared in absolute ethyl alcohol. Ten µl of α-T solution was added to 100 µl on *n*-*t*-butyl-*a* phenyl nitrone (PBN) (50 mM, final concentration). The total mixture of 100 µl was irradiated with ultraviolet light (320–400 nm) for different time intervals from 1 to 120 s (1, 2, 5, 10, 15, 30, 45, 60, 90 and 120 s). Another tube containing the same reagents was kept in total darkness for 1 h, which served as control. After incubation, 50 µl aliquots from each of the above samples were transferred to glass capillary tubes and one end of each capillary tube was flame-sealed.

Electron paramagnetic resonance (EPR) spectra of the PBN free radical adducts formed were then recorded on a Varian E-104 EPR spectrometer (Pal Alto, USA) equipped with a TM_{110} cavity, and were compared with the reference spectrum of superoxide radical generated by autoxidation of pyrogallol[12], which served as a control. Instrument settings were as follows: field set, 3237 G; temperature, 27 + 5°C; scan range, 10 × 10 G; time constant, 0.5 s; scan time, 4 min; microwave power, 5 mW; microwave frequency, 100 kHz; modulation amplitude, 2 G; modulation frequency, 9.01 GHz; and receiver gain, 1.25 × 104 × 10 (unless otherwise stated).

Figure 3 shows spin-trapping results of free radicals formed by exposure of α-T to UV light. The EPR signals obtained by ultraviolet irradiation of α-T for 1, 2, 5, 10, 15, 30 and 40 s (Figure 3 *a–g*) exhibited an EPR signal with three lines. The line intensities were 1:2:1 (aN = 16 G) which differed from the line intensities of

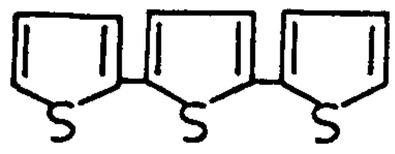

Figure 1. Chemical structure of alpha-terthienyl.

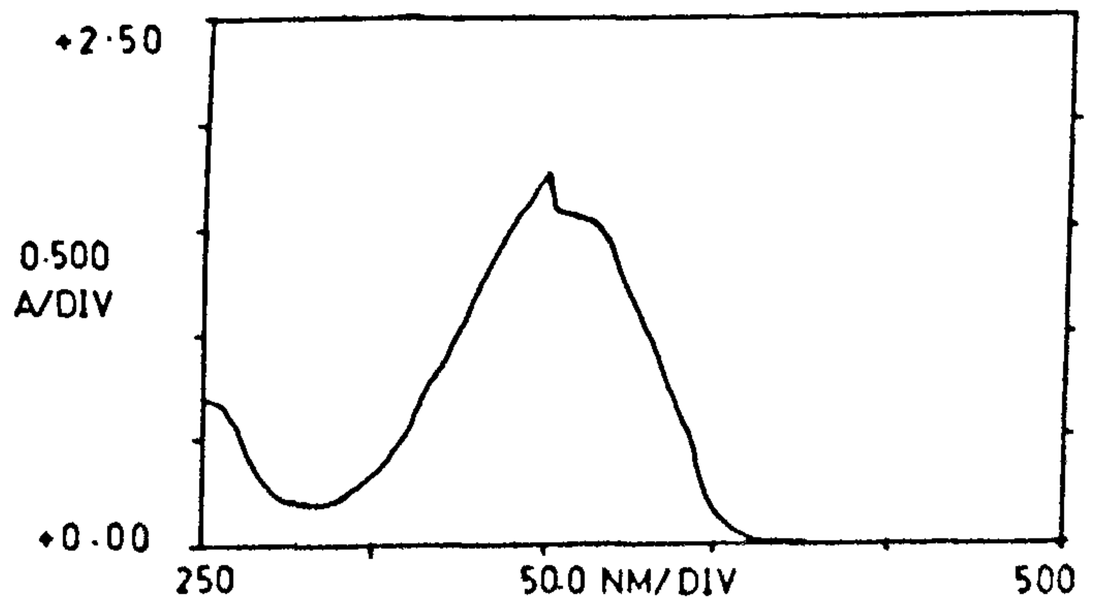

Figure 2. UV–VIS absorption spectrum of alpha-terthienyl showing a sharp absorption peak at 351 nm.

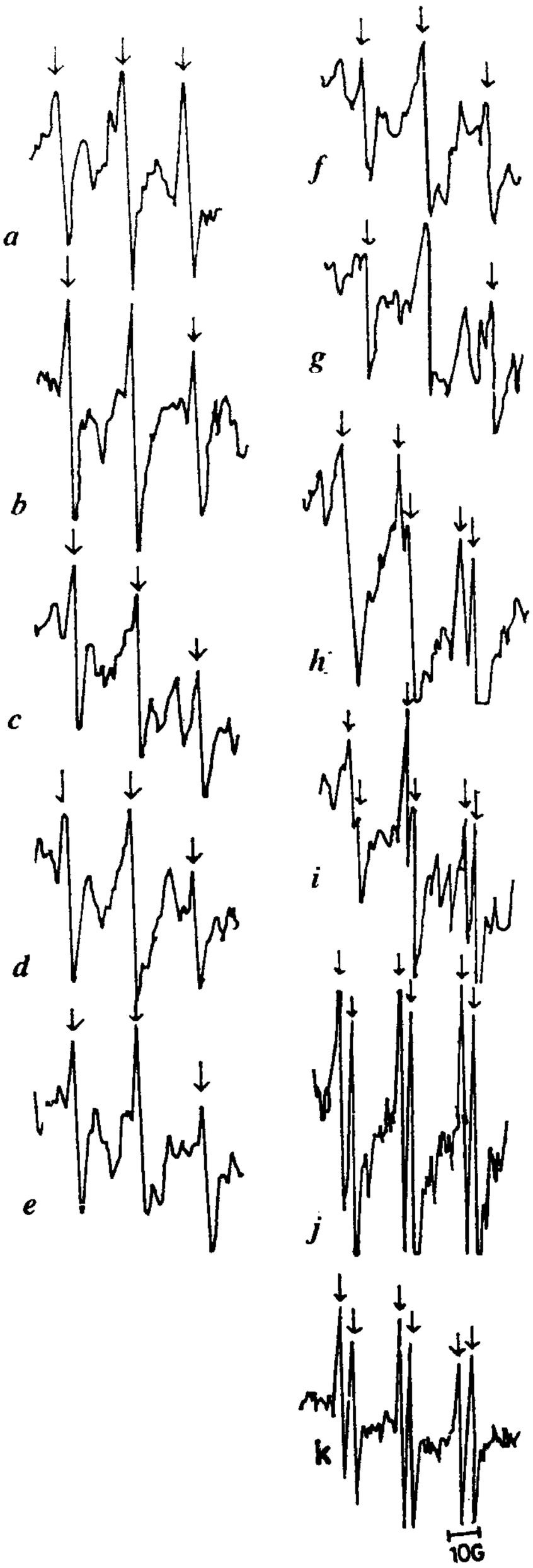

Figure 3. *a–g*, Formation of a three-line spectrum of alpha-terthienyl radical-PBN adduct; (indicated by arrows) after illumination of alpha-terthienyl with long wave ultraviolet light for 1, 2, 5, 10, 20, 30 and 45 s; *h–j*, Emergence of three more lines on further illumination of alpha-terthienyl for 60, 90 and 120 s, which is similar to superoxide anion radical; *k*, Reference spectrum obtained using artificially generated superoxide anion radical by pyrogallol autoxidation system (control).

the superoxide anion radical which exhibits a six line adduct with PBN (aN = 14.81 G). An increase in the illumination to 60, 90 and 120 s (Figure 3 *h–j*) showed the emergence of 3 more lines and the spectrum thus obtained corresponded to the superoxide-PBN adduct (Figure 3 *j*); this can then be compared with a reference spectrum of superoxide anion radical (Figure 3 *k*), the control. No signal was obtained from the control samples.

Triplet thiophenes are excellent electron donors both in homogeneous and micellar solutions. Electron transfer from triplet thiophenes to suitable electron acceptors leads to strongly absorbing, rather stable thiophene radical cations, which in nanosecond–microsecond time scale are insensitive to oxygen. The main decay pathways of these radical cations appear to be second-order diffusion controlled reactions, and quenching of thiophene triplet by oxygen leads to only minor yields of the corresponding cation radicals[13].

Our results provide evidence from EPR studies of the formation of an α-T radical and its further conversion to a superoxide anion radical (Scheme 1). Studies concerning the cellular target(s) of α-T have yielded conflicting data. MacRae *et al.*[14] were unable to detect chromosomal aberrations in cultured Syrian hamster cells following treatment with α-T and UV-A (ref. 14). Kagan *et al.*[15], on the other hand, reported that both calf thymus DNA *in vitro* and *Candida utilis* DNA *in vivo* were targets of α-T (ref. 15).

$$\alpha\text{-T} \xrightarrow{h\nu} \alpha\text{-T}^{\bullet}$$

$$\alpha\text{-T}^{\bullet} + O_2 \longrightarrow O_2^{\bullet -} + \alpha\text{-T}$$

Scheme 1. Formation of α-T and superoxide anion radical.

The substantial effect of this thiophene and UV-A on the inactivation of membrane-bound cholinesterase in human erythrocyte[16] and on *E. coli* membrane proteins[17], demonstrates the importance of these membrane components as the target of photodynamic attacks. Other reports have also indicated the damage of membranes in the photodynamic action of α-T. For example, Wat *et al.*[18] demonstrated lesions in the cytoplasmic membrane of human erythrocytes irradiated in the presence of α-T. This membrane damage might reflect involvement of either the lipid or protein components of the membrane. Experiments with liposomes entrapped with glucose as a membrane model system showed enhanced permeability to glucose and introduced high degree of unsaturation in liposomes involving lipid peroxidation in the presence of this thiophene and UV-A (ref. 19). Thus, a free radical role in these phototoxic effects is suggested.

Free radicals are well-known causes of major damage to biological membranes, resulting in inactivation of membrane-bound proteins, membrane lysis and lipid peroxidation; in turn this damage decreases the membrane fluidity and increases leakiness of the membrane[20]. Formation of free radicals from α-T may also expose a number of non-target species to a free radical threat. Thus, some irreversible damage to those non-target species seems to be inevitable. Further studies are required to assess the role of α-T on the non-target species, with special reference to free radicals.

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Erratum

Crystal structure of the peanut lectin – T-antigen complex. Carbohydrate specificity generated by water bridges

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In the crystal structure of the complex, it was noticed that O4 in the GalNAc moiety of T-antigen (Galβ1-3GalNAc) was inappropriately positioned. This happened on account of the inadequacy of the geometrical restraints applied to this part of the molecule during refinement. O4 was refixed geometrically and 140 cycles of conjugate gradient refinement was carried out using XPLOR[1]. The final R-factor and R-free are 0.175 and 0.251, values identical to those obtained in the earlier refinement. The re-refined coordinates have been deposited in the PDB (code: 2TEP).

Expectedly, there is no significant change in the structure except in the position of O4. The protein carbohydrate interactions in the re-refined structure are listed in Table 1. The only change in them is an additional possible interaction between GalNAc O4 and Leu 212 N. Thus the main difference in interactions between the T-antigen and lactose complexes remains the additional water bridges in the former. Efforts are on to assess the effect of the possible additional interaction.

We thank Dr Remy Loris of Vrije Universiteit Brussel for pointing out this error which is deeply regretted.

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Table 1. Peanut lectin–T-antigen interactions. Lengths in the PNA-lactose interactions are in paranthesis. Distances are in Å

A. Hydrogen bonds

Sugar atom	Protein atom	Subunit 1	Subunit 2	Subunit 3	Subunit 4
Gal O3	Asp83 OD1	2.76 (2.67)	2.62 (2.43)	2.72 (2.58)	2.63 (2.49)
	Gly104 N	3.22 (3.08)	2.99 (2.92)	2.83 (3.07)	2.88 (2.81)
	Asn127 ND2	2.76 (2.86)	2.92 (3.00)	2.76 (3.12)	2.96 (3.02)
Gal O4	Asp83 OD2	2.73 (2.59)	2.66 (2.68)	2.57 (2.64)	2.91 (2.55)
	Ser211 OG	2.92 (2.62)	3.24 (2.82)	2.82 (2.66)	2.55 (2.76)
Gal O5	Ser211 OG	3.03 (3.12)	2.99 (3.34)	2.73 (3.09)	2.96 (3.16)
Gal O6	Asp80 OD2	2.85 (3.33)	2.95 (3.39)	3.48 (3.36)	3.13 (2.98)
Glycosidic O	Ser211 OG	3.24 (3.38)	3.34 (3.86)	3.20 (3.70)	3.25 (3.81)
GalNAc O4	Ser211 OG	3.09 (3.34)	2.78 (3.31)	3.07 (3.58)	3.07 (2.98)
	Gly213 N	2.73 (2.92)	2.88 (2.98)	2.78 (3.29)	2.76 (3.28)
	Leu212 N	3.34 (4.04)	3.21 (4.08)	3.01 (4.65)	3.08 (4.06)

B. Water-mediated interactions (distances averaged over four subunits)

GalO2--W1--Glu129 OE1	[O2--W1 = 3.03 (3.07); W1--OE1 = 3.30 (2.98)]
GalO2--W2--Gly104 N	[O2--W2 = 2.91 (2.67); W2--N = 2.91 (3.15)]
GalNAcO7--W3--Ile101 O	[O7--W3 = 3.06; W3--O = 2.95]
GalNAcO7--W4--Leu212 N	[O7--W4 = 2.78; W4--N=2.83]
GalNAcO7--W4--Asn41 ND2	[W4--ND2 = 2.65]

Water-mediated interactions involving W3 and W4 do not exist in the Lactose complex

C. Residues less than 4 Å from any sugar atom

Asp80, Ala82, Asp83, Gly103, Gly104, Tyr125, Asn127, Ser211, Leu212, Gly213, and Gly214.