

## Photodynamic action on *Escherichia coli* of natural acetylenic thiophenes, particularly 5-(buten-1-ynyl)-2,2'-bithienyl

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### Summary

Naturally occurring acetylenic photosensitizers, including  $\alpha$ -terthienyl, 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl, 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl, and 5-(buten-3-ynyl)-2,2'-bithienyl, from species of *Tagetes* displayed photodynamic killing of *Escherichia coli*, *Pseudomonas aeruginosa* and the yeast, *Saccharomyces cerevisiae* in near UV light. The last compound affected rec mutant strains of *E. coli* to varying degrees suggesting that cellular DNA may be a molecular target for the photo-activated process. This is in contrast to  $\alpha$ -terthienyl for which cellular membranes have been shown to be primary targets.

$\alpha$ -terthienyl; 5-(buten-3-ynyl)-2,2'-bithienyl; *Escherichia coli*; rec mutants; photodynamic killing

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### Introduction

The phototoxic nature of thiophenes has become an active area of research in the past few years [1].  $\alpha$ -Terthienyl ( $\alpha$ T), a photosensitizer synthesized by many mem-

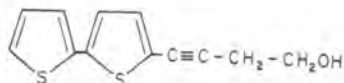
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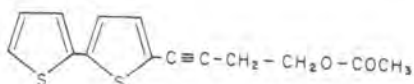
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bers of the plant family Asteraceae, has been studied in the most detail. Bakker et al. [2] reported that photoinactivation of glucose-6-phosphate dehydrogenase by  $\alpha$ T was oxygen-dependent and that the presence of various quenchers of singlet oxygen

BBT-OH



BBT-OAc



BBT

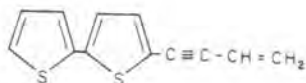
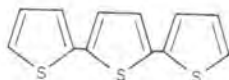
 $\alpha$ T

Fig. 1. Chemical structures of four phototoxic thiophenes which are derived from various plants of the family Asteraceae [10]. BBT-OH, 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl; BBT-OAc, 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl; BBT, 5-(buten-1-ynyl)-2,2'-bithienyl;  $\alpha$ T,  $\alpha$ -terthienyl.

( $^1\text{O}_2$ ) protected the enzyme from inactivation. They concluded that  $\alpha$ T was a photodynamic or type II photosensitizer. These findings were confirmed by subsequent studies which demonstrated the oxygen requirement for  $\alpha$ T phototoxicity both in vitro [3] and in vivo [4–6]. Questions concerning the in vivo mechanism of sensitization by  $\alpha$ T, however, have been raised. Kagan et al. [7] suggested that  $\alpha$ T could covalently bind to calf thymus and *Candida utilis* DNA. MacRae et al. [8], on the other hand, found that  $\alpha$ T/UV-A did not induce sister chromatid exchange or chromosomal aberrations in cultured Syrian hamster cells, in contrast to 8-methoxypsoralen/UV-A. Nor was evidence of DNA damage found by Downum et al. [6] using recombination deficient mutants of *E. coli* K12 irradiated in the presence of  $\alpha$ T.

Other thiophenes which are biosynthetically related to  $\alpha$ T also occur in the Asteraceae [9]. Structures of four of these which were used in these studies are shown in Fig. 1. An acetylenic monothiophene [1] and 5-(buten-1-ynyl)-2,2'-bithienyl (BBT) [10] are the only other natural thiophenes which have been reported to cause phototoxic responses in addition to  $\alpha$ T. Recently, however, several synthetic derivatives were also shown to cause photoinduced killing of the nematode *Aphelenchus avenae* [5].

In the present study, photosensitization of the bacterial species *E. coli* and *Pseudomonas aeruginosa* and the yeast, *Saccharomyces cerevisiae*, by two previously unstudied thiophenes, 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH) and 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc), was examined. In addition, the *in vivo* phototoxic effect of BBT was investigated in greater detail for comparison with what is presently known of the mechanism of its biosynthetic product,  $\alpha$ T.

## Materials and Methods

### Sources of thiophenes

$\alpha$ -Terthienyl ( $\alpha$ T) was prepared by Dr. F. Garcia, Chemistry Department, National University of Mexico, Mexico, D.F. Samples of 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH) and 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc) were kindly supplied by Dr. R. Sütfield, University of Münster, F.R.G. BBT (5-(buten-3-ynyl)-2,2'-bithienyl) was isolated from several species in the Asteraceae including *Tagetes patula*, *T. erecta* and *Dyssodia papposa*. The identity and purity of each compound were confirmed prior to use by spectral and chromatographic methods (UV, IR, NMR, GC-MS, HPLC).

### Irradiation sources

Two types of fluorescent UV-A sources (320–400 nm) were used during these studies and consisted of either four horizontal Sylvania F20T12/BLB lamps ( $\lambda_{max}$  at 360 nm;  $7.2 \text{ W} \cdot \text{m}^{-2}$  at 10 cm) or two vertical Sylvania F15T8/BL lamps ( $\lambda_{max}$  at 350 nm;  $3.2 \text{ W} \cdot \text{m}^{-2}$  at 15 cm). Total irradiance between 320 and 400 nm was measured with a Research Radiometer (International Light Inc.).

TABLE I

STRAINS OF MICROORGANISMS USED DURING THESE STUDIES AND THEIR RELEVANT CHARACTERISTICS

Strain	Parent	Genotype	Source
<i>P. aeruginosa</i> PA01	—	wild	Dr. A.M.B. Kropinski
<i>E. coli</i> B	—	wild	Laboratory stock
<i>E. coli</i> K12 <sup>a</sup>			
AB1157	—	wild	Dr. A.J. Clark
JC 2926	AB 1157	rec A <sup>-</sup>	Dr. A.J. Clark
JC 3881	AB 1157	rec A,B,C <sup>-</sup>	Dr. A.J. Clark
JC 5519	AB 1157	rec B,C <sup>-</sup>	Dr. A.J. Clark
JC 5547	AB 1157	rec B,C,F <sup>-</sup>	Dr. A.J. Clark
JC 9239	AB 1157	rec F <sup>-</sup>	Dr. A.J. Clark
<i>S. cerevisiae</i>	—	wild	Laboratory stock

<sup>a</sup> All *E. coli* K12 strains used were isogenic derivatives of AB 1157 differing only by the indicated characteristics. The genotype of AB 1157 is F<sup>-</sup>, *strA*-31, *tsx*-33, *sup*-37, *proA*-2, *his*-4, *argE*-3, *galK*-2, *ara*-14, *xyl*-5, *mtl*-1, *thr*-1, *leu*-6, *thi*-1, *supE*-44, *lacY*-1,  $\lambda$ <sup>-</sup>.

### *Microorganisms*

The bacterial strains and their relevant characteristics are listed in Table I. Cultures were grown to stationary phase at either 30°C (*S. cerevisiae*) or 37°C (all bacteria) with shaking at 250 rpm. *E. coli* was grown in nutrient broth (Difco) or BM2-glucose [6]. *P. aeruginosa* was grown in proteose peptone 2 (Difco), while *S. cerevisiae* was grown in Sabouraud's dextrose broth (Difco). Cells were plated on appropriately enriched agar for subsequent counting of colonies or for disc assays.

### *Phototoxicities of thiophenes*

Relative biological activities of BBT-OH, BBT-OAc, BBT and  $\alpha$ T were bioassayed by the method of Chan et al. [10]. Stationary phase cultures of *E. coli* B, *P. aeruginosa* and *S. cerevisiae* were diluted 1:100 with fresh media and 0.1 ml was spread onto enriched agar plates. Whatman No. 1 filter paper discs (7 mm in diameter) which had been previously loaded with 5–10  $\mu$ g of each thiophene were placed onto the bacterial or yeast lawns. The plates were incubated for 30 min in the dark and then irradiated for 1 h with four Sylvania F20T12/BLB lamps. Following irradiation, the plates were incubated for either 18 h (*E. coli* and *P. aeruginosa*) or 48 h (*S. cerevisiae*) at 37°C. Inhibitory zones were scored after incubation. Dark control plates were assayed simultaneously for comparison.

### *Fluence-response curves*

Cells were irradiated in sterile physiological saline by four Sylvania F20T12/BLB lamps following the method of Arnason et al. [11]. Samples (0.1 ml) were removed at various times between 2 and 10 min. After dilution and plating, plates were incubated for approximately 18 h at 37°C at which time colonies were scored. Control cultures with BBT in the dark or UV-A in the absence of the sensitizer were assayed simultaneously.

### *Oxygen experiments*

A 3-necked, borosilicate reaction flask (Pierce Chemical Co.) with a 10-ml capacity, equipped with stir bar, gas inlet, and luer-lok sampling port was used to determine whether oxygen was required for the expression of BBT phototoxicity toward *E. coli* B. Cells growing in BM2-glucose were irradiated with two vertically mounted Sylvania F15T8/BL lamps as described previously [6].

### *Assay of rec mutants*

Mutants of *E. coli* K12 were grown in nutrient broth. Overnight cultures were diluted 1:100 and 0.1 ml was spread onto nutrient agar plates. Sterile Whatman No. 1 filter paper discs (7 mm in diameter) which had been previously loaded with 8-MOP or BBT (10  $\mu$ g per disc) were placed onto replicate plates containing one of the rec strains. The plates were incubated for 1 h in the dark and subsequently irradiated for 1 h by four Sylvania F20T12/BLB lamps at 37°C. The plates were then incubated in the dark for 48 h at 37°C. Inhibitory zones were measured in millimeter.

## Results

*E. coli* B, *P. aeruginosa* and *S. cerevisiae* were exposed to BBT-OH, BBT-OAc, BBT and  $\alpha$ T and irradiated with UV-A. Growth of the microorganisms was inhibited by the photoinduced antibiotic action of each of these biosynthetically related thiophenes (Table II).  $\alpha$ T and BBT, which have previously been shown to kill bacteria during UV-A irradiation [10], were the most effective growth inhibitors. BBT-OH and BBT-OAc were less toxic, but still caused inhibition in the light. Microorganisms exposed to each of the sensitizers, but not irradiated showed no growth inhibition.

The synergistic action of BBT and UV-A irradiation on the survival of *E. coli* B is demonstrated in Fig. 2. Exponential declines in cell viability were observed with  $0.35 \mu\text{g} \cdot \text{ml}^{-1}$  during the first 4 min of irradiation. Exponential killing was also evident at lower sensitizer concentrations, although the inactivation rates were considerably lower. Higher concentrations of BBT (above  $0.35 \mu\text{g} \cdot \text{ml}^{-1}$ ) did not increase the rates of cellular inactivation substantially.

The photoinduced antibiotic activity elicited by BBT/UV-A under aerobic and anaerobic conditions is shown in Fig. 3. Cells were least affected by treatment with BBT under anaerobic conditions, but were quite sensitive to inactivation in the presence of air. This may suggest that photoactivated BBT kills cells via a photodynamic mechanism similar to that reported for  $\alpha$ T [4].

In a previous paper, we reported that mutants of *E. coli* K12 deficient in various *rec* genes could be used as sensitive indicators of compounds which react with DNA *in vivo* [6]. In that study, 8-methoxypsoralen (8-MOP), a photosensitizer which reacts with DNA [12], and bleomycin, a non-photoactivated antibiotic which damages DNA [13], were shown to cause increased growth inhibition of *rec*<sup>-</sup> strains. Wild type cells, while still susceptible to the effects of these compounds, were affected to a much lesser extent. Photoactivated  $\alpha$ T, on the other hand, caused inhibition of both wild type and *rec*<sup>-</sup> strains to the same extent which suggested that growth inhibition probably resulted from damage to cellular components other than DNA.

*Rec* mutants were employed in this investigation for the purpose of determining whether DNA might be a molecular target of photoactivated BBT (Table III). The sensitizer 8-MOP served as a control. Strains deficient in the *rec A* gene were most

TABLE II  
RELATIVE 'PHOTOTOXICITIES' OF THIOPHENES TOWARD MICROORGANISMS

Compound	<i>E. coli</i> B	<i>P. aeruginosa</i>	<i>S. cerevisiae</i>
BBT-OH	+	+	+
BBT-OAc	+	+	+
BBT	+++	++	+++
$\alpha$ T	++	++	++

<sup>a</sup> Inhibitory zone (edge to edge) between 7 and 12 mm in diameter.

<sup>b</sup> Inhibitory zone (edge to edge) between 12 and 17 mm in diameter.

<sup>c</sup> Inhibitory zone 17 mm or larger.

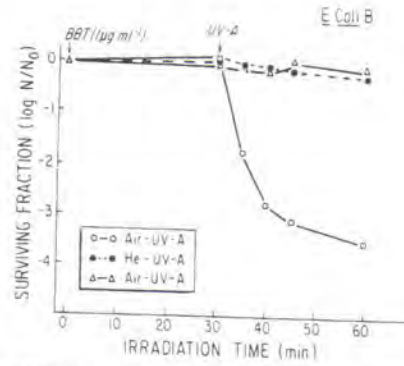
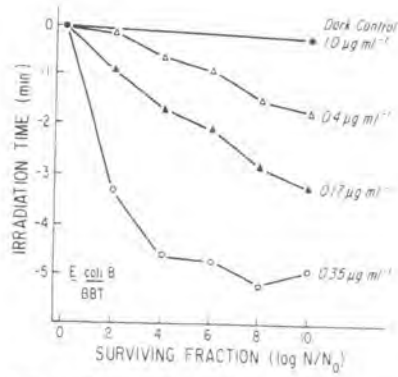


Fig. 2. Fluence-response for different concentrations of BBT on *E. coli* B. Irradiation source consisted of four horizontal F20T12/BLB Sylvania lamps ( $\lambda_{max}$  at 360 nm;  $7.2 \text{ W} \cdot \text{m}^{-2}$  at 10 cm).

Fig. 3. The survival of aerobically grown *E. coli* B irradiated with UV-A in the presence of BBT under aerobic (○) and anaerobic (●) conditions. BBT was added to cultures at 0 min and irradiation was started at 30 min. Δ, BBT added, but culture not irradiated.

susceptible to 8-MOP/UV-A treatment. Rec B, C and F deficient cells were also inhibited by treatment, but less than cells deficient in the rec A gene. Differences between wild type cells and rec<sup>-</sup> cells treated with BBT/UV-A were somewhat smaller than those exposed to 8-MOP, but cells with impaired recombination mechanisms were more susceptible than wild-type (AB 1157) cells suggesting that DNA may be a major target of BBT. The inhibitory differences resulting from treatment with 8-MOP and BBT were quite noticeable and may indicate that the mechanism of BBT is distinct from that of 8-MOP.

TABLE III

RESPONSE OF VARIOUS MUTANTS OF *E. COLI* K12 TO BBT AND 8-MOP IRRADIATED FOR 60 MIN WITH UV-A

Dark controls were also run, but no inhibitory zones resulted. Inhibitory zones (edge to edge in mm) are followed by standard deviations.

Strain	BBT/UV-A	8-MOP/UV-A
AB 1157	20.7 (0.9)	10.0 (0.8)
JC 2926	26.3 (0.9)	19.0 (0.8)
JC 3881	25.0 (0.0)	20.0 (0.0)
JC 5519	25.0 (0.0)	16.0 (0.8)
JC 5547	24.7 (1.9)	15.3 (0.5)
JC 9239	22.7 (0.9)	15.3 (0.5)

## Discussion

To date at least thirteen thiophenes have been shown to elicit some form of photoinduced biological activity. Three of these ( $\alpha$ T, BBT and an acetylenic monothiophene) were originally isolated from members of the plant family Asteraceae and shown to cause substantial nematocidal and/or antibiotic activities in the presence of UV-A irradiation [1,10,14]. Approximately 10 other derivatives have been chemically synthesized and shown to possess varying capacities for killing the nematode *Aphelenchus avenae* in the presence of UV-A irradiation [5]. The photoactivated toxicity of BBT-OH and BBT-OAc, two more naturally derived thiophenes from members of the Asteraceae, are reported here for the first time. These studies of photobiocidal thiophenes suggest their ecological importance as protective chemicals in nature.

Comparative mechanistic studies and investigations involving the molecular targets of either natural or synthetic thiophenes are limited. Gommers et al. [5] demonstrated that several synthetic dithienyl and chlorosubstituted dithienylethenes were singlet oxygen sensitizers like  $\alpha$ T. BBT, the biosynthetic precursor of  $\alpha$ T [9], inactivated *E. coli* under aerobic conditions only which suggests that it too may kill cells via a photodynamic or  $^1\text{O}_2$  mechanism. Further study is necessary to determine whether  $^1\text{O}_2$  is in fact the excited species responsible for photosensitized killing of cells treated with BBT and UV-A.

The molecular targets of the thiophene photosensitizers have largely been unstudied. Electron microscopic studies of human erythrocytes treated with  $\alpha$ T and UV-A irradiation revealed that membranes were important targets of the  $^1\text{O}_2$  generated by the photoactivated sensitizer [8]. In another study, SDS-polyacrylamide gel electrophoresis of *E. coli* membrane-associated proteins exposed to  $\alpha$ T/UV-A showed non-specific protein crosslinking which suggested that membrane proteins were important targets of photodynamic attack [6]. Kagan et al. [7] suggested further that DNA might also be a target of photoactivated  $\alpha$ T, however, attempts to confirm this have been unsuccessful [6,8] and suggest that DNA probably does not represent an important target of the  $^1\text{O}_2$  generated by this photosensitizer.

BBT, the biosynthetic precursor of  $\alpha$ T in plants [9], also requires oxygen for expression of in vivo toxicity. The evidence indicates, however, that cellular DNA may be a molecular target of this photoactivated process. This finding stands in sharp contrast to the in vivo targets reported for  $\alpha$ T [6,8]. Two features unique to BBT may explain the apparent differing sites of action of these closely related natural products. First, the unsaturated hydrocarbon side chain associated with BBT undoubtedly imparts chemical uniqueness to the molecule. This structural difference may well affect the way in which the molecule interacts with biological membranes, possibly allowing the sensitizer access to the interior of the cell and therefore increasing the likelihood of reaction with DNA. Second, the chemical structure of BBT relates the molecule not only to the thiophenes, but also to the polyacetylenes. Polyacetylenes have been shown to represent a unique class of natural photosensitizers [11] and it is possible that this structural resemblance is reflected in a hybrid phototoxic mechanism of action. What potentially unique properties may be associ-

ated with this type of molecule are unknown, but interesting possibilities for further study pertaining to the mechanism of action of these natural photosensitizers are offered.

## References

- 1 Towers, G.H.N. (1980) *Progress in Phytochemistry, Vol. 6*, pp. 183-202, Pergamon Press, New York, NY.
- 2 Bakker, J., F.J. Gommers, I. Nieuwenhuis and H. Wynberg (1979) *J. Biol. Chem.* *254*, 1841-1844.
- 3 Wat, C.-K., W.D. MacRae, E. Yamamoto, G.H.N. Towers and J. Lam (1980) *Photochem. Photobiol.* *32*, 167-172.
- 4 Arnason, T., G.F.Q. Chan, C.-K. Wat, K. Downum and G.H.N. Towers (1981) *Photochem. Photobiol.* *33*, 821-824.
- 5 Gommers, F.J., J. Bakker and H. Wynberg (1982) *Photochem. Photobiol.* *35*, 615-619.
- 6 Downum, K.R., R.E.W. Hancock and G.H.N. Towers (1982) *Photochem. Photobiol.* *36*, 517-524.
- 7 Kagan, J., R. Gabriel and S.A. Reed (1980) *Photochem. Photobiol.* *31*, 465-469.
- 8 MacRae, W.D., G.F.Q. Chan, C.-K. Wat, G.H.N. Towers and J. Lam (1980) *Experientia* *36*, 1096-1097.
- 9 Bohlmann, F., T. Burkhardt and C. Zdero (1973) *Naturally Occurring Acetylenes*, Academic Press, London.
- 10 Chan, G.F.Q., G.H.N. Towers and J.C. Mitchell (1975) *Phytochemistry* *14*, 2295-2296.
- 11 Arnason, T., C.-K. Wat, K. Downum, E. Yamamoto, E.A. Graham and G.H.N. Towers (1980) *Can. J. Microbiol.* *26*, 698-705.
- 12 Song, P.-S. and J.R. Tapley (1979) *Photochem. Photobiol.* *29*, 1177-1197.
- 13 Gudas, L.J. and A.B. Pardee (1976) *J. Mol. Biol.* *101*, 459-477.
- 14 Gommers, F.J. (1972) *Nematologica* *18*, 458-462.