PHOTOSENSITIZING THIOPHENES FROM THE TAGETEAE

by

KELSEY DOWNUM

B.S., Colorado State University, Fort Collins, Colorado, 1975
M.S., Colorado State University, Fort Collins, Colorado, 1978

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Department of _Botany_

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date _18 August 1981_

ABSTRACT

Two separate aspects involving the thiophenes of the Tageteae (Asteraceae) were investigated. The first concerned the distribution of four thiophenes in Tagetes patula L. which were examined by high pressure liquid chromatography (HPLC). The derivatives were found to be differentially distributed throughout hydroponically grown plants. The predominant thiophenes in roots were 5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc) and 5-(buten-3-ynyl)-2,2'-bithienyl (BBT). BBT-OAc was the main derivative in shoots, whereas 2,2':5',2''-terthienyl (alpha-T) was the major compound in flower petals. BBT and one unidentified compound were found to occur in leaf glands. The levels of BBT-OAc in shoots and BBT-OAc and BBT in roots increased over the life of the plant and reached a plateau following flowering. Alpha-T in roots and both alpha-T and BBT in shoots remained at low levels over the life of the plant while 5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH) was found to be an minor component of roots or shoots.

Fourteen species from four genera of the tribe Tageteae were also screened for the presence of thiophenes by HPLC. Representatives of Dyssodia, Porophyllum, and Tagetes all contained thiophenes, but none were detected in species of Pectis.

The second part of this study concerned the photobiocidal effects of isolated thiophenes on Escherichia coli B which was used as a model biological system. Alpha-terthienyl (alpha-T), in the presence of UV-A irradiation (320nm-400nm), was

found to be a Type II photosensitizer which required oxygen for the expression of biological activity. Scavenger studies with sodium azide and BHT suggested that both singlet oxygen and superoxide were generated by the photosensitized reaction. Cellular inactivation by alpha-T was sensitive to temperature and studies with recombination deficient mutants of E. coli K-12 did not indicate that damage to cellular DNA occurred. Proteins were found to be substantially affected by the photoactivated reaction. SDS-gel electrophoresis revealed that both cytoplasmic and membrane-associated proteins might be crosslinked following treatment with alpha-T and UV-A.

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PREFACE

Plants produce many diverse chemical constituents which elicit interesting biological activities in isolated or purified form. The thiophenes are one such group of compounds synthesized by members of the plant family Asteraceae and are characteristic of the tribe Tageteae. These derivatives are toxic toward a wide variety of biological organisms when excited by longwave ultraviolet (UV-A) irradiation. Their function within the plant is still unknown and remains unstudied, but their phototoxic properties suggest that thiophenes may play a protective role in situ.

The work to be presented was a preliminary investigation into two separate areas involving thiophenes. Thiophene phytochemistry was the first area to be studied. The occurrence, distribution and levels of these biologically active compounds in one plant, Tagetes patula L. were established. The second area concerned the phototoxic mechanism of action invoked primarily by one thiophene, alpha-T. The ultimate goal of this work was to determine baseline information which might be used for future research into some of the more interesting aspects of these natural plant products. What are their in situ functions? Are they protective or do they have some other physiological or metabolic function? How do plants protect themselves from the effects of these photoactive derivatives? These are only a few of the potential research areas where this data could be useful.

SECTION I.

PHYTOCHEMICAL ASPECTS OF THIOPHENES

INTRODUCTION

Thiophenes comprise a distinct class of natural products which are characteristic of the Asteraceae, the largest flowering plant family. These sulfur containing compounds were initially isolated from petals of the "African" variety of the common marigold (Tagetes erecta L.; Tribe Tageteae) (Sease and Zechmeister, 1947). Alpha-terthienyl (alpha-T) (Figure 1) was the first natural thiophene to be identified (Zechmeister and Sease, 1947; Uhlenbroek and Bijloo, 1958). Ten years later a second thiophene, 5-(but-3-en)-2,2'-bithienyl (BBT) was isolated (Uhlenbroek and Bijloo, 1959). Additional thiophene derivatives have been reported from twelve species of Tagetes (Bohlmann and Herbst, 1962; Atkinson et al., 1965; Bohlmann et al., 1973; Bohlmann and Zdero, 1979) as well as other genera in the tribe Tageteae including two species of Dyssodia (Bohlmann and Zdero, 1976), and one species of Porophyllum (Bohlmann et al., 1973). Thiophenes have been isolated from nine tribes in addition to the Tageteae, but they have not been reported from the Astereae, Calenduleae, Cichorieae, or Mutisieae nor have they been reported from any other higher plant family (Bohlmann et al., 1973). Table I shows the occurrence of thiophenes in other tribes in the family (Sorenson, 1977).

The natural thiophenes possess one, two or three aromatic, five-membered, sulfur containing rings per molecule (Figure 2) and are biosynthetically derived from oleic acid (Bohlmann et al., 1973). Polyacetylenic intermediates, which are long chain molecules with conjugated triple bonds (Bohlmann, 1973), are

5-(4-*hydroxy-1-butenyl*)-
2,2'-*bithienyl* (*BBT*-OH)

5-(4-*acetoxy-1-butenyl*)-
2,2'-*bithienyl* (*BBT*-OAc)

5-(3-*buten-1-ynyl*)-2,2'-
bithienyl (*BBT*)

2,2':5,2'-*terthienyl*
(αT)
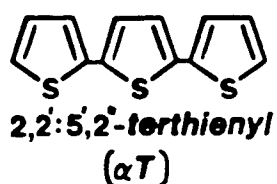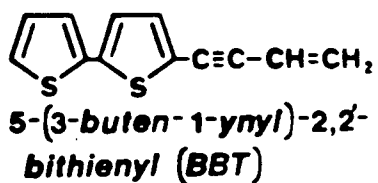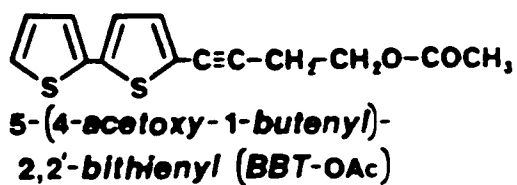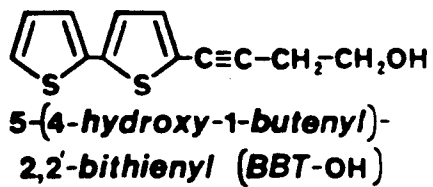
Figure 1. The thiophenes which were isolated and identified from Tagetes patula L.

Table 1. The occurrence of thiophenes within the Asteraceae (after Sorenson, 1977).

Tribe	Monothiophenes	Dithiophenes	Terthiophenes
Anthemideae	+	−	−
Arctotideae	+	+	+
Astereae	−	−	−
Calenduleae	−	−	−
Cichorieae	−	−	−
Cynareae	+	+	+
Eupatorieae	+	−	−
Helenieae	+	+	+
Heliantheae	+	+	+
Inuleae	+	+	−
Mutisieae	−	−	−
Senecioneae	+	+	−
Tageteae	+	+	+
Vernonieae	+	−	−

formed by oxidation of this eighteen carbon fatty acid. Decarboxylation of specific polyacetylenes has been proposed to lead to a twelve carbon intermediate, the "penta-yne-ene", which may be the biosynthetic precursor of thiophenes in the Asteraceae. Sulfide addition (via an unknown donor) to this intermediate, followed by cyclization(s), results in the various mono, di and terthienyl derivatives (Bohlmann et al., 1973). This aspect of metabolism, however, has not been confirmed by biosynthetic studies.

PROPOSED BIOSYNTHESIS OF THIOPHENES

$$H_3C - C \equiv C - C \equiv C - C \equiv C - C \equiv C - C \equiv C - CH = CH_2$$

$S^=$ addition

$S^=$ addition

$-CO_2$

$-CO_2$

$S^=$ addition

$-CO_2$

5-(3-buten-1-ynyl)-2,2'-bithienyl

α-terthienyl
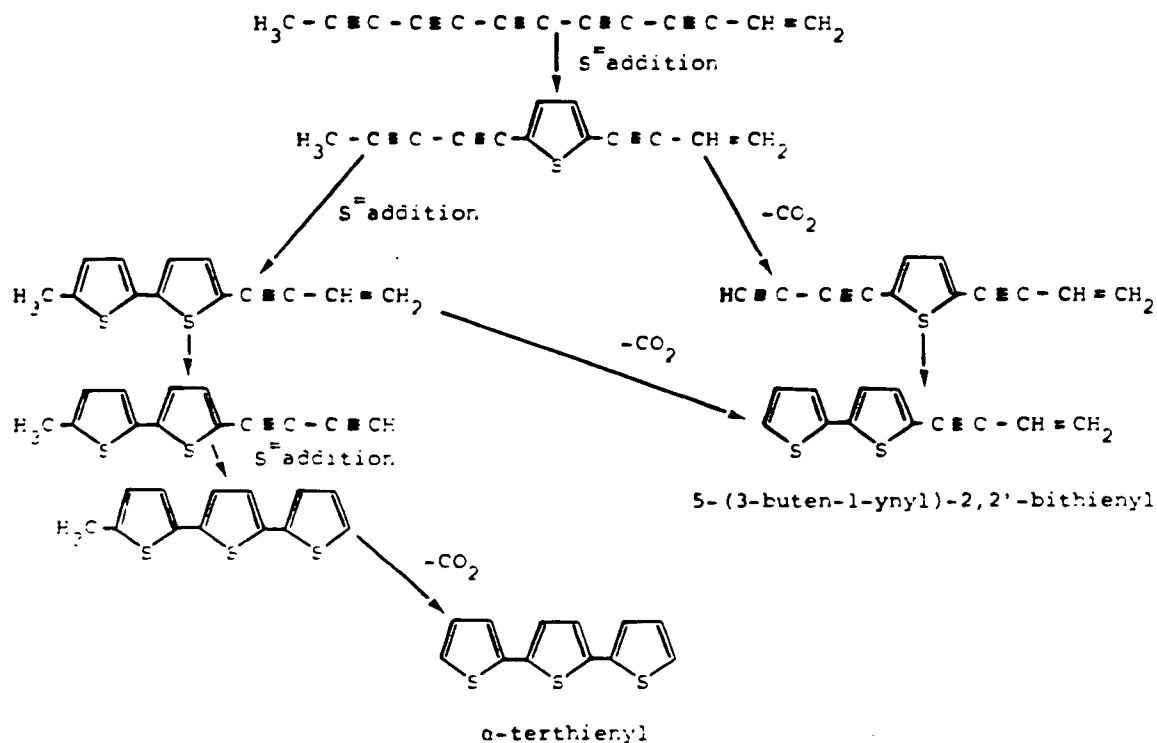
(after Bohlmann et al., 1973)

Figure 2. A proposed biosynthetic pathway leading to the formation of thiophenes from the polyacetylenic precursor, the "penta-yne-ene".

Discovery of the potent nematicidal properties of alpha-T and BBT led to the original interest in these derivatives of T. erecta (Uhlenbroek and Bijloo, 1958 and 1959). Gommers (1972) and Gommers and Geerlings (1973) subsequently showed that the nematicidal ability of alpha-T was enhanced in the presence of daylight and specifically the UV-A region of the spectrum (320 nm to 400 nm). Chan et al. (1975) reported that both alpha-T and BBT were phototoxic or bactericidial in UV-A, but caused no lethal effect in the dark. The photobiocidal or phototoxic effects of alpha-T have subsequently been shown toward a wide variety of biological organisms in addition to nematodes and include bacteria (Chan et al., 1975), fungi (Daniels, 1965), algae (Arnason et al., 1981), insects (Arnason et al., 1980; Wat et al., in press) and various other organisms (Towers, 1980). The phototoxicity of BBT has thus far only been demonstrated with bacteria (Chan et al., 1975) (a detailed account of the photoinduced toxic mechanism of action of alpha-T and BBT toward bacteria is presented in Section II. Photobiological Aspects of Thiophenes).

The toxicity of isolated thiophenes in the presence of light leads one to question their in situ significance. Do thiophenes afford plants some protective advantage as opposed to plants lacking them? Conversely, if these compounds are so toxic, how do plants protect themselves from the photoinduced effects of these toxic compounds? Answers to these questions were not sought in this study, but initial information which could perhaps give clues to possible answers was examined.

The distribution of thiophenes in members of the Tageteae has been thought to include only specific parts of the plant (Towers and Wat, 1978). Roots were found to be the primary location of alpha-T and BBT, but flower petals (Zechmeister and Sease, 1947) and the pappus of achenes (Chan et al., 1975) were also shown to possess low concentrations of these compounds. Isolation of these compounds generally involved either column or thin layer chromatography (Curtis and Phillips, 1962; Atkinson et al., 1965). The compounds were detected by fluorescence under longwave UV irradiation or by the use of a concentrated sulfuric acid solution of isatin which induced color reactions with the various thiophene derivatives. Isolation required large amounts of plant material and yields were generally low due to the low concentrations in the plant and due to instability of the compounds (Bohlmann et al., 1973). Lack of a sensitive quantitative method has hitherto prevented studies involving the distribution and accumulation of thiophenes.

Before in situ studies can be conducted, it is necessary to know the distribution and levels of the various derivatives in the plant as well as the relative toxicities of each component. This study was undertaken to determine: 1) the composition of thiophenes in T. patula; 2) their distribution throughout the plant; 3) whether specific compounds are accumulated in particular tissues; or 4) whether the levels present are sufficient to account for protection. These studies were difficult to carry out initially, because of the lack of a

suitable system for their detection and quantification. Therefore a sensitive, analytical method was developed which used high pressure liquid chromatography (HPLC). With this new technique it was possible to study thiophene derivatives in relatively crude extracts of T. patula. Qualitative distribution and quantitative accumulation of thiophenes over the life of the plant were then examined.

MATERIALS AND METHODS

Chemicals

N-bromosuccinimide, 2-bromothiophene (II), 2-methylthiophene (III), 2,5-dibromothiophene (V), and tetramethylammonium chloride (TMA) were purchased from Aldrich Chemical Company. Thiophene (I) and 2-acetylthiophene (IV) (methyl 2-thienyl ketone) were obtained from Matheson, Cole and Bell Chemicals and Eastman Kodak. Fluorescein was purchased from Sigma while isatin and vanillin were from Nutritional Biochemicals and Baker Chemical Company, respectively.

Alpha-terthienyl was synthesized by me and later by Dr. F. Garcia, Chemistry Department, National University of Mexico, using the method of Kooreman and Wynberg (1967). The standard of 5-(buten-3-ynyl)-2,2'-bithienyl was isolated from either T. patula, T. erecta, or Dyssodia papposa plant material. Reference solutions of 5-(4-hydroxy-1-butynyl)-2,2'-bithienyl (VII) and 5-(acetoxy-1-butynyl)-2,2'-bithienyl (VIII) were kindly supplied by Dr. R. Suetfeld, University of Munster,

during the later stages of this study. The compound 2,2'-dithiophene (VI) was synthesized by the method of Sease and Zechmeister (1947). All other chemicals and solvents were from laboratory stocks (A.C.S). HPLC solvents from Fisher Scientific were used for HPLC analyses. Sephadex LH-20 (25-100u, Pharmacia) was used for gel filtration chromatography.

Plant Material

Tagetes patula L. (from J.L. Hudson, Redwood City, CA) plants were used for all experiments. Plants were grown generally in the field between the months of May and September. Alternatively, plants were grown in growth chambers (Coviron, Controlled Environments LTD., Winnipeg, Mannitoba, Canada) at 25°C, 100% R.H. and a photoperiod of 16 hours light/8 hours dark. The plants required 6 to 9 weeks for flowering under either condition.

Dried Plant Specimens

Dried plants were used for the examination of thiophenes in the tribe Tageteae. Pectis filipes var. subnuda (K13230), P. imberbis (13559, 13566), P. longipes (diploid; K11375, 13500, 13508 and tetraploid; K11368, 13529, 13535), P. papposa var. papposa (K13149), and P. prostrata (K13279) were supplied by Dr. David J. Keil, California Polytechnic University, San Luis Obispo, CA. Tagetes multiflora (389a,b) and T. ellipticum (112) were collected by Timmothy Johns, University of Michigan,

Ann Arbor, MI. Dyssodia anthemidifolia , D. decipiens (58),
D. papposa , Porophyllum gracile , Tagetes coronopifolia (105),
T. filifolia (79), T. lunulata , T. lucida (111), and
T. tenuifolia (106) were supplied by Dr. G.H.N. Towers,
University of British Columbia, Vancouver, B.C. Tagetes
lemmonii (University of California Botanical Garden 74.251) was
supplied by Dr. John Strother, Herbarium, University of
California, Berkeley, CA. Tagetes lunulata, T. patula, and
T. erecta were grown in the field from seed. Duplicate voucher
specimens were identified by Dr. John Strother. Voucher
specimens were deposited in the Botany Department Herbarium,
U.B.C.

Hydroponics

Achenes of T. patula were germinated on two layers of
moistened Whatman #1 filter paper in glass petri dishes. The
dishes were covered and left on a laboratory bench top for four
to five days. Uniform seedlings approximately five centimeters
long were selected at this time and transferred to nutrient
solution following the method of Dr. A.D.M. Glass, Department
of Botany, U.B.C. (personal communication). The nutrient
solution (Table 2) was changed completely on a weekly basis.
Plants were kept in growth chambers under the previously
described conditions.

Table 2. Composition of hydroponic growth medium.
All concentrations are micromolar (uM).

Potassium nitrate	600 uM
Calcium nitrate	400
Ammonium phosphate	200
Magnesium sulfate	100
Potassium chloride	5.0
Borate	2.5
Manganese sulfate	0.2
Zinc sulfate	0.2
Copper(II) sulfate	0.05
Molybdate	0.05
Fe-EDTA	2.0

Extraction of Thiophenes

T. patula tissue was extracted by three methods. The first method was used for qualitative analyses and involved the extraction of fresh plant material. Tissue was ground in methanol in a Waring blendor (three times) and the mixture filtered on a Buchner funnel using Whatman #1 filter paper. The filtrate was diluted 1:1 with distilled water and extracted three times with equal volumes of petroleum ether (PE) (30-60°C) in a separatory funnel. The combined PE fractions were dried with anhydrous sodium sulfate and stored at -20°C. Alternatively, the PE fraction was evaporated to dryness on a rotary evaporator. Following evaporation, the residue was resuspended in HPLC grade methanol and stored at -20°C until

HPLC analysis. All procedures were conducted under dimmed room light to avoid photodegradation of the extracted compounds.

The second method was used for the isolation of reference compounds and involved the bulk extraction of freeze dried plant tissue. Fresh plants were first frozen at -80°C and then lyophilized. The dried material was separated into shoots or roots and ground to a fine powder in a Wiley mill. The powdered plant material was extracted in a Soxhlet apparatus for 24 hours with PE (30-60°C). The PE was dried over sodium sulfate and either evaporated to dryness and resuspended in methanol for HPLC analysis or left in PE for TLC or column chromatography.

The third technique was used for the extraction of thiophenes from dried herbarium specimens. One to five grams of dried material was crushed to a fine powder and placed into 20 ml of 95% ethanol. The material was allowed to extract for 3 to 4 weeks at which time the powdered plant material was removed by filtration. The ethanolic extract was then concentrated by rotary evaporation to approximately 1 to 2 ml and examined by HPLC. This method was used because it was simple and allowed the simultaneous extraction of all samples. Thiophenes were extracted successfully from seven year old dried tissue.

Identification of Thiophenes

The thiophenes isolated from T. patula were analyzed by various spectroscopic methods. Ultraviolet absorption spectra and mass spectra were used for comparison with published data

for structural verification. UV spectra were recorded in spectral grade methanol using either a Pye-Unicam SP8-100 UV/Vis or a Unicam SP-800A UV/Vis spectrophotometer. Emission and excitation spectra were determined in either methanol or ethanol on a Perkin-Elmer 650-10s fluorescence spectrophotometer with a Perkin-Elmer 150 Xenon power supply. Infra-red spectroscopy was conducted on a Unicam SP-200G grating spectrophotometer using either chloroform or carbon disulfide. Mass spectral analyses were recorded with a Hewlett Packard Model 5985B GLC-MS by the Chemistry Department at Simon Fraser University. Chromatographic separation of compounds was carried out with a capillary SE-30 column and a temperature program from 80°C to 220°C at 15°C/min.

Chromatography

TLC

Thin layer chromatography was used for qualitative analysis and small scale isolation of compounds. Separations were carried out on unactivated silica gel with and without fluorescent indicator. Analytical sheets (purchased from Eastman Kodak) and Silica Gel G (type 60) (Brinkmann Instruments, Canada) spread to a wet thickness of 0.5 mm on glass plates were used for analytical and preparative TLC, respectively.

The chromatograms were developed in one dimension in rectangular chambers without equilibration using one of three

solvent systems:

> A.   PE (30-60°C):Acetone (99:1)
>
> B.   PE
>
> C.   PE:Diethyl Ether (9:1)

Solvent system A was used mainly.

Thiophenes were detected by their characteristic fluorescence under longwave UV or by reaction with three spray reagents. The spray reagents used included:

> 1) <u>Vanillin Spray</u> (Picman <u>et al</u>., 1980)
>> 0.5g vanillin
>>
>> 9 ml 95% ethanol
>>
>> 0.5 ml conc. sulfuric acid
>>
>> 3 drops glacial acetic acid

> 2) <u>Isatin Spray</u> (Curtis and Phillips, 1962)
>> 0.4% isatin in conc. sulfuric acid

> 3) <u>Succinimide-Fluorescein Spray</u> (specific for sulfur compounds)
>> spray solution I: 0.35 g N-bromosuccinimide
>>
>>   100 ml methyl chloroform
>>
>> spray solution II: a. 0.33 g fluorescein
>>
>>   100 ml 0.1 N NaOH

b. 3 ml of a.

100 ml of 95% ethanol

Column Chromatography

Column chromatography on silica gel was initially used to isolate standard compounds from crude plant extracts and to purify products from organic syntheses. Columns were developed with PE (30-60°C) followed by increasing amounts of acetone to elute the more polar thiophenes. Isolated compounds were checked for purity by HPLC.

High Pressure Liquid Chromatography

Qualitative and quantitative analyses of thiophenes were carried out on a Varian Series 5000 LC. A Variscan 634 S spectrophotometer set to 350 nm was used to detect eluted compounds. Varian MicroPak MCH-10 (4 mm x 30 cm) and MCH-10' (8 mm x 50 cm) octadecylsilane reverse-phase columns were used to separate derivatives in analytical and preparative modes, respectively. An isocratic solvent system consisting of acetonitrile and water (72:28) containing 10 mM each of potassium phosphate buffer (pH 3.2 to 3.5) and TMA was used to elute thiophenes following the method of Phillips and Towers (1981). All liquid chromatography was conducted at room temperature using flow rates of 1 ml/min for analytical work

and 3 to 5 ml/min for preparative work.

Qualitative HPLC Studies

The distribution of thiophenes in hydroponically grown T. patula was examined by HPLC. The roots, shoots and flowers from mature flowering plants were extracted by the first method described (see Extraction of Thiophenes). Leaf glands were ruptured with capillary pipettes. The oil was collected by capillary action and then transferred into HPLC grade methanol by forcing air through the pipettes. Approximately 800 to 1000 glands were sampled from several plants. This oil, the shoot, root and flower extracts were examined immediately.

Quantitative HPLC Studies

Quantitation of thiophene derivatives in crude plant extracts was carried out by reverse-phase HPLC with UV detector set to 350 nm. Stock solutions of each thiophene standard were prepared by dilution into 95% ethanol. Concentration curves were determined by filling the 20 ul injection loop with the various diluted standards and measuring resulting peak areas from the chart recorder. At least five injections of each dilution were made. Peak areas were determined by multiplying the peak height (H) in cm by the width at half height (W1/2) in cm. All values were then multiplied by the recorder expansion factor (S) to correct for recorder sensitivity.

$$\text{Peak Area} = H \times W1/2 \times S$$

The data was analyzed by linear regression and the line of best fit used for concentration determinations from crude extracts. All concentration curves had coefficient of determination values (r^2) of 0.99 or better.

Two studies involved quantitative HPLC. The first was the determination of thiophene levels in mature, flowering T. patula plants grown hydroponically. Concentrations of the known thiophenes were determined for roots, shoots (stems and leaves), immature leaves, mature leaves, and flowers. Tissue was extracted by the first method described (see Extraction of Thiophenes). Thiophene levels were determined immediately following extraction.

In the second study, determination of thiophene concentrations from early seedling stage through flowering was investigated. For this, approximately 45 T. patula seedlings were grown under hydroponic conditions. Five plants, selected randomly, were harvested at 2 to 3 week intervals. The plants were pooled and frozen at $-80°C$, lyophilized, and extracted by Soxhlet extraction. The extract was immediately analyzed by HPLC and thiophene concentrations determined by comparison of peak areas with the concentration curves of the appropriate standard. Standard deviations associated with HPLC injection generally varied by less than 5%.

Phototoxicity of Thiophene Standards

The biological activities of BBT-OH (VII), BBT-OAc (VIII), BBT (IX) and alpha-T (X) were examined by the method of Chan et al. (1975). E. coli, Pseudomonas aeruginosa, and Saccharomyces cerevisiae were tested. E. coli was grown in nutrient broth (Difco). P. aeruginosa was grown in PP2 (Difco) medium while S. cerevisiae was grown in Sabouraud dextrose broth (Difco). Cultures were grown overnight at either 30°C (yeast) or 37°C (bacteria) with shaking at 250 rpm. The overnight cultures were diluted (1:100) in fresh media and 0.1 ml spread onto agar plates with appropriate nutrients. Whatman #1 filter paper discs (7 mm in diameter) loaded with 5 ug to 10 ug of each standard were placed on the lawns and the plates were incubated for 30 min. Following incubation, the plates were irradiated for one hour by four Sylvania F20T12/BLB lamps (see Irradiation Sources in Section II), incubated overnight and then scored for inhibitory zones. Unirradiated control plates were also prepared for comparison.

RESULTS AND DISCUSSION

Figure 3 lists the structures of thiophenes used in this study. I to VI do not occur naturally in members of the Tageteae, but were used for comparative purposes and in synthetic work. VII, VIII, IX and X were isolated from Tagetes patula L.

I) R=H ; R'=H

II) R=Br; R'=H

III) R=CH₃; R'=H

IV) R=COCH₃; R'=H

V) R=Br; R'=Br

VI) R=H; R'=H

VII) R=H; R'= C≡C−CH₂−CH₂OH

VIII) R=H; R'= C≡C−CH₂−CH₂O−COCH₃

IX) R=H; R'= C≡C−CH=CH₂

X) R=H   R'=H
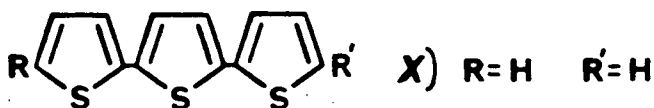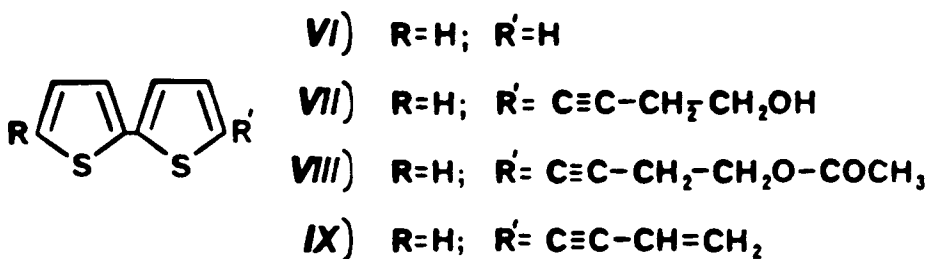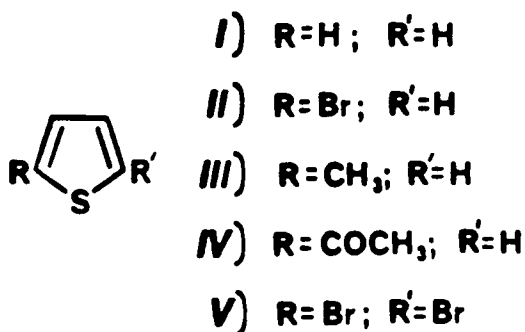
Figure 3. Chemical structures of the thiophenes used in these studies. Compounds I to VI are not found naturally whereas compounds VII to X were isolated from <u>Tagetes patula</u> L.

Structural Verification of Standards

Structural verification of the naturally occurring thiophenes involved spectroscopic methods. Appendix I lists the data obtained from reference compounds isolated from T. patula or synthesized chemically. UV, IR, fluorescence, and MS spectra for most compounds are given. UV absorption maxima are followed by published extinction coefficients in parentheses. Mass spectra showing relative intensities of m/e peaks ($\geq 10\%$) are also given. Fluorescence data showing excitation maxima are followed by emission maxima (fluorescence) in parentheses. Verification of the structures of VII, VIII, IX and X were based on comparison of spectral data with previously published results. The structure of alpha-T (X) was also confirmed by chemical synthesis.

Chromatography

Separation of thiophene derivatives from T. patula was accomplished by four chromatographic methods. Thin layer (TLC) and column chromatography were used for qualitative analysis and preparative isolation of thiophenes from crude plant extracts. Table 3 lists TLC data for qualitative separation of various thiophenes. Compounds VII, VIII, IX, and X were found to fluoresce under longwave UV irradiation (320 nm to 400 nm) while the thiophenes (I thru VI) did not. Of the two reagents used for visualization, vanillin yielded the most vivid and characteristic color reactions. Vanillin also seemed to be more

sensitive than isatin for detection of BBT-OH (VII) and BBT-OAc (VIII). Neither compound gave color reactions with isatin but did react with vanillin at the same concentrations. Presumably the compounds were below the level of detectability of isatin although Picman et al. (1980) found the two reagents to be equally sensitive for detection of alpha-T (0.05 ug). BBT (IX) and alpha-T (X) were easily distinguished by color reaction with either vanillin or isatin. All derivatives reacted with the succinimide-fluorescein spray which is specific for sulfur containing compounds.

Reverse-phase high pressure liquid chromatography (HPLC) was used for qualitative and quantitative analyses of the thiophenes in crude plants extracts as well as for preparative work. Coupled with a UV detector, HPLC was the most useful and versatile chromatographic method for thiophenes. Table 4 lists uncorrected retention times (R(t)), corrected retention times (R(t')= R(t) - to), relative retention (a), and resolution values (R) of the four naturally derived thiophene reference compounds. Elution of standards was in the order of decreasing polarity. BBT-OH (VII), the most polar compound, was retained least (R(t')= 3.5 min) and was eluted before the more non-polar thiophenes BBT-OAc (VIII) (R(t')= 5.0 min), BBT (IX) (R(t')= 7.2 min) and alpha-T (X) (R(t')= 8.9 min), respectively.

Thiophenes isolated from crude extracts of T. patula by preparative TLC, column chromatography or preparative HPLC were submitted to combined GLC-MS analysis for structural elucidation.

Table 3. Color reactions of thiophenes with vanillin and
isatin spray reagents, UV fluorescence and Rf
values (in Solvent System A).

| Compound | Reagent | | UV | |
	Vanillin	Isatin	Fluorescence	Rf
thiophene (I)	violet	gray	-	0.11
2-bromothiophene (II)	turquoise	-	-	0.93
2-methylthiophene (III)	green	gray	-	0.11
2-acetothiophene (IV)	red to orange	brown	-	0.74
2,5-dibromo-thiophene (V)	purple	purple	-	0.90
2,2'-dithiophene (VI)	blue	NT	-	0.75
BBT-OH (VII)	violet	-	blue	0.00
BBT-OAc (VIII)	blue	-	blue	0.09
BBT (IX)	greenish blue	green	blue	0.67
Alpha-T (X)	olive green	blue	blue	0.61

- no response   NT - Not tested

Thin layer and column chromatography have been used
extensively for the separation of thiophenes from crude plant
extracts (Zechmeister and Sease, 1947; Uhlenbroek and Bijloo,
1958, 1959; Chan et al., 1975; Selva et al., 1978) and for the
purification of synthetic thiophenes (Sease and Zechmeister,

Table 4. Retention times (R(t)), corrected retention
times (R(t')), relative retention (a),
and resolution (R) of thiophene standards
separated by HPLC (to = 1.9 min).

Thiophene Standard	R($\underline{t}$)	R($\underline{t}$')	$\underline{a}$	R
BBT-OH (VII)	5.4 min	3.5 min		
			1.43	3.53
BBT-OAc (VIII)	6.9	5.0		
			1.44	4.00
BBT (IX)	9.1	7.2		
			1.24	3.09
Alpha-T (X)	10.8	8.9		

HPLC parameters are listed in Materials and Methods.

1947; Uhlenbroek and Bijloo, 1960; Atkinson et al., 1965;
Patrick and Honegger, 1974). These techniques were useful for
the initial location and isolation of thiophenes from plants
when coupled with UV fluorescence and color reaction with
vanillin (Picman et al., 1980), but were not acceptable for
quantitative analyses of compounds. Reverse-phase HPLC proved
to be the best chromatographic technique for quantitation.

Qualitative Analysis of Thiophenes

The distribution of thiophenes in different parts of hydroponically grown T. patula plants was investigated by HPLC and fluorescence microscopy. The UV absorbing compounds extracted from the roots of eight week old plants are shown in Figure 4. By comparison with the corrected retention times (R(t')) of standards, BBT-OH (VII), BBT-OAc (VIII), BBT (IX), and alpha-T (X) were all found to be present along with several other unidentified compounds. BBT-OAc (VIII) and BBT were the predominant derivatives in roots. Shoot material (leaves and stems) contained the same thiophene derivatives as roots (Figure 5) but in quite different proportions. BBT-OAc (VIII) was still prevalent in shoots, but BBT was a much less important derivative. An unidentified compound with a R(t') of 6.5 minutes was found to be a major shoot component. Flowers (Figure 6) also contained VII, VIII, IX and X. In addition, two unidentified components with R(t')'s of 6.5 and 10.1 minutes were present in substantial amounts. Of the known thiophenes, alpha-T (X) was the major derivative in flowers, although BBT-OAc (VIII) was well represented. It should be mentioned that the unidentified compounds (R(t') 6.5 and 10.1 minutes) displayed similar UV absorption spectra to other thiophenic derivatives (see Appendix I) and are probably also thiophenes.

The leaf glands of marigolds have been studied for their volatile, insect repelling components (de Mucciarelli and Montes, 1970; de Villiers et al., 1971; Okoth, 1973). They have been shown to contain a number of monoterpenes (e.g. tagetone
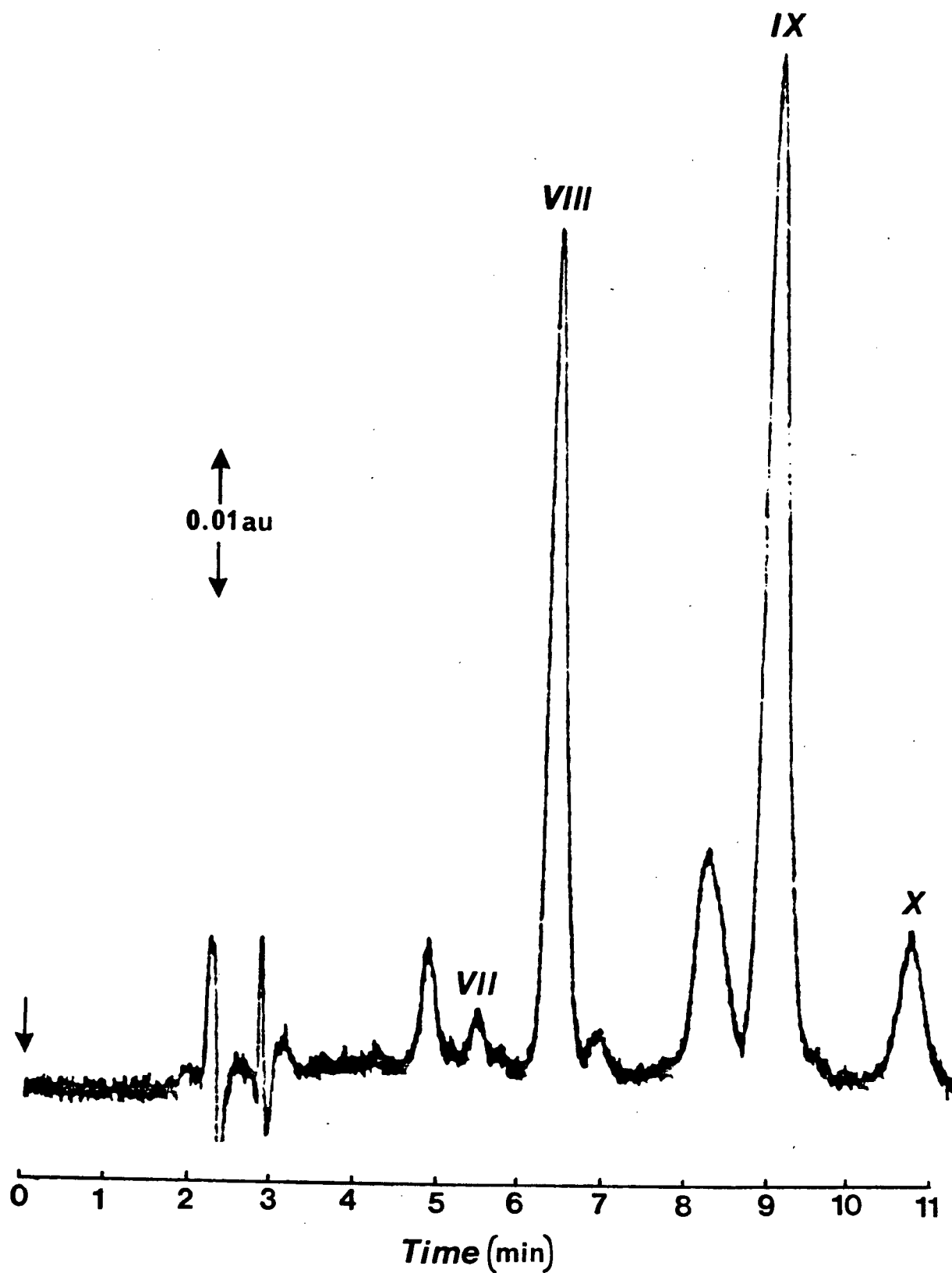
Figure 4. HPLC trace of the UV absorbing compounds which were extracted from the roots of 8 week old hydroponically grown T. patula.

Figure 5. HPLC trace of the UV absorbing compounds which were extracted from the shoots of 8 week old hydroponically grown *T. patula*.

Figure 7. HPLC trace of the UV absorbing compounds in the oil of leaf glands from 8 week old hydroponically grown *T. patula*.

Figure 6. HPLC trace of the UV absorbing compounds which were extracted from flower petals of hydroponically grown I. patula.

and tagetenone), but thiophenes were not found. BBT and one unidentified component (R(t') 6.5 minutes), however, were detectable by HPLC (Figure 7). Anatomically, leaf glands are associated with resin canals. The presence of thiophenes in leaf glands might also indicate their presence in the resin canals as well. The resin canal system could offer a convenient transport system in the plant for these non-polar compounds.

Fluorescence microscopy of fresh T. patula tissue was conducted to determine whether concentrations of thiophenes could be detected visually. Their intense blue to violet fluorescence under longwave UV was suggestive that this technique might be useful for detection of thiophenes at the cellular or tissue level. Root, stem, leaf and flower sections were examined, however no fluorescence was found which would specifically indicate their location in cells or tissues. The absence of fluorescence in tissue excited by longwave UV could indicate that either thiophenic derivatives are not concentrated in specific regions of the plant or that their fluorescence is quenched in situ, preventing their detection by this method.

Quantification of Thiophenes

Thiophene levels in plants were measured by HPLC. Quantification by this new technique involved the use of standard concentration curves which were determined separately for each reference compound. Figures 8, 9, 10 and 11 give the relationships between detector response (recorded as peak area)

and amount of standard injected for each reference compound. It was necessary to determine standard curves for each compound individually since the extinction coefficients differed at the monitoring wavelength (350 nm). This technique was sensitive enough to quantify thiophenes in the nanomolar (10^{-9} M) range which allowed analyses of as little as 1 g of fresh tissue.

Crude T. patula extracts from different regions of plants (75 days old) were determined quantitatively (Table 5; page 36). Roots showed the highest concentrations of VIII and IX. Alpha-T (X) was prevalent in flowers and BBT-OH was a minor component throughout the plant. Analysis of immature and mature leaf tissue revealed that growing leaves contained considerably higher levels of thiophenes than mature tissue. Concentration differences between older and younger leaf tissue may be a result of the loss of compounds by: 1) degradation in older tissue resulting from either biochemical or photo-induced mechanisms; 2) transport from older tissue to younger tissue; 3) volatilization from glands; or 4) dilution in older tissue. Conversely, younger tissue may actively synthesize thiophenes which perhaps could account for higher concentrations in younger tissue.

The fate of thiophenes over the life of the plant was also investigated quantitatively. Figure 12 shows the levels of VIII, IX and X from root extracts of hydroponically grown plants. BBT-OAc (VIII) and BBT (IX) were the main thiophene components in crude root extracts during early seedling growth. Both derivatives increased in concentration for the first 80
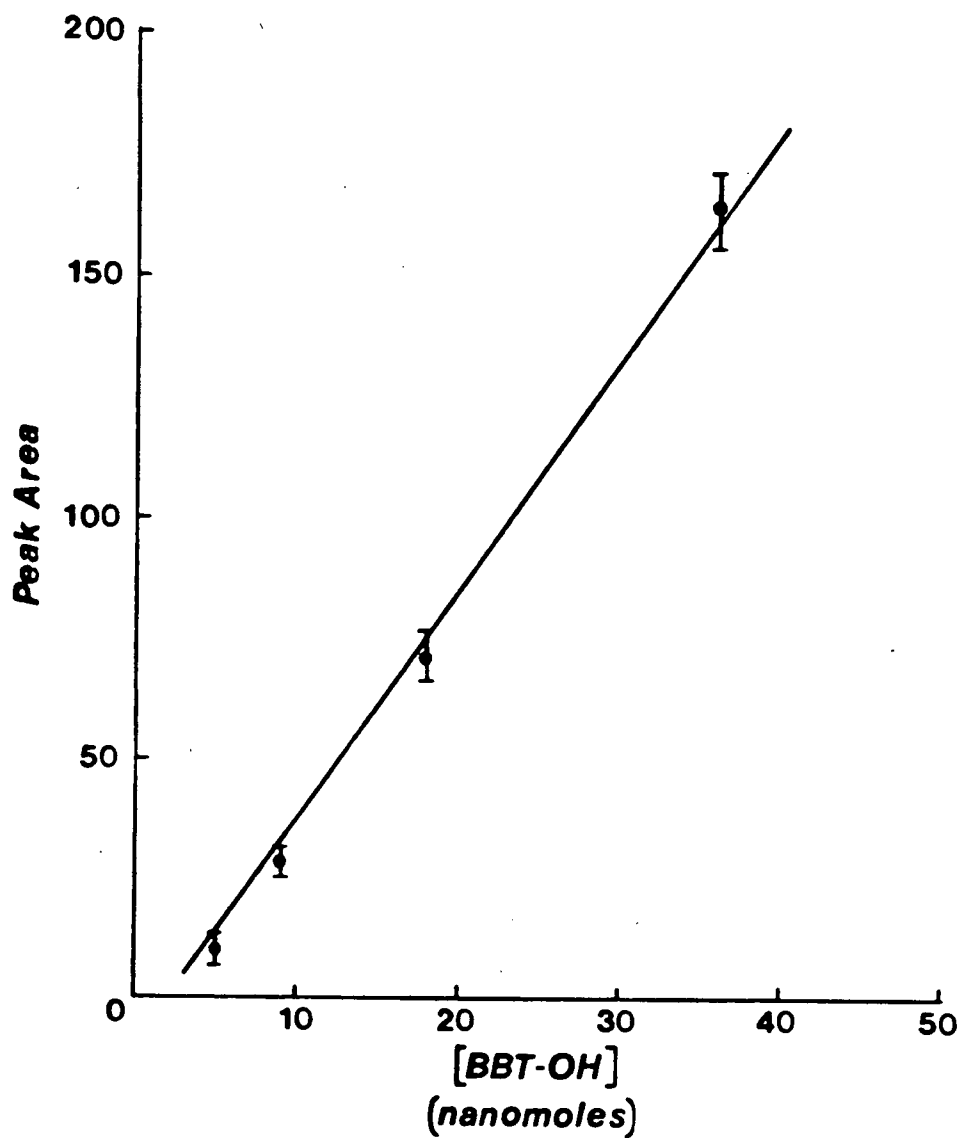
Figure 8. HPLC concentration curve for BBT-OH (VII) which relates peak area (detector response) and amount of standard injected (nanomoles).
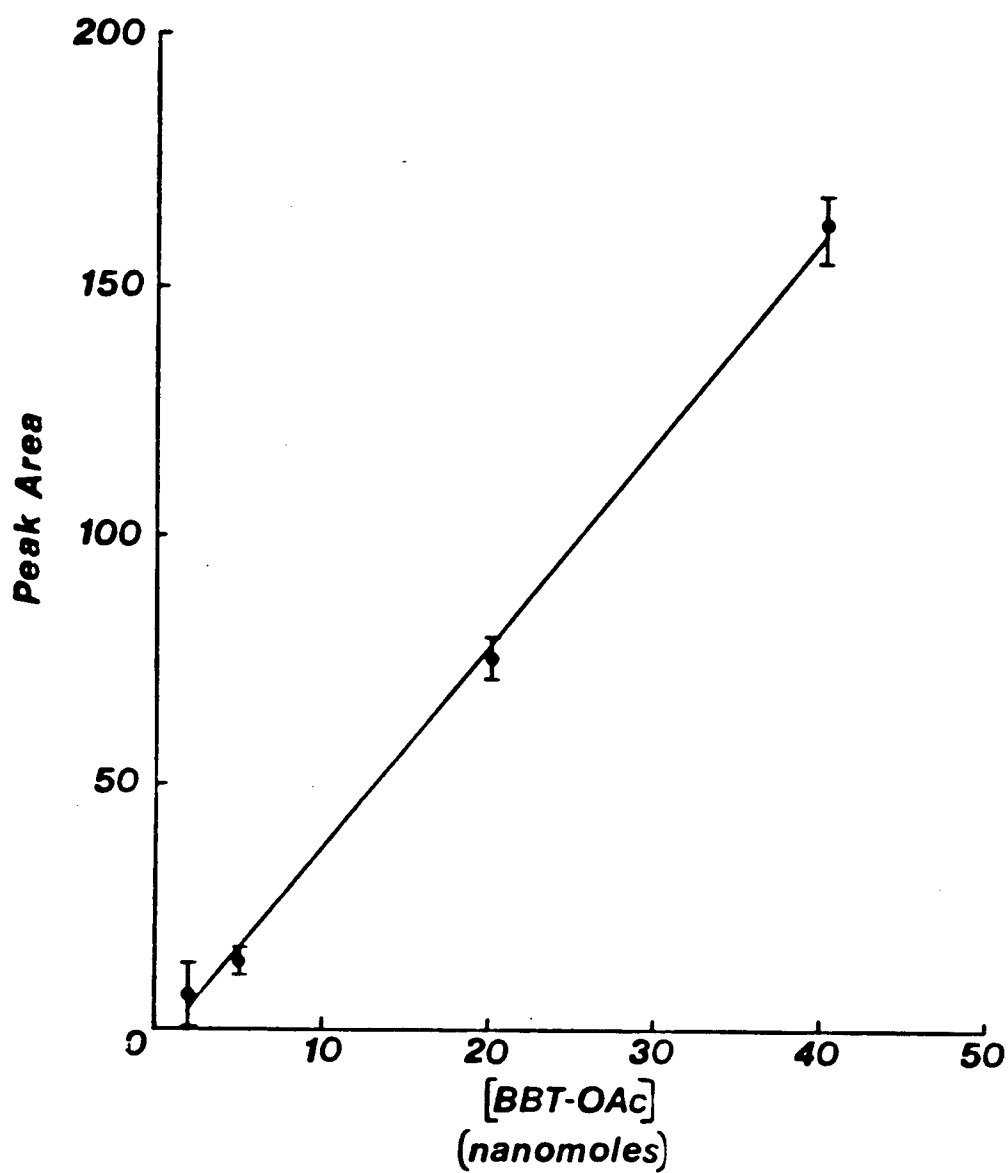
Figure 9. HPLC concentration curve for BBT-OAc (VIII) which relates peak area (detector response) and amount of standard injected (nanomoles).
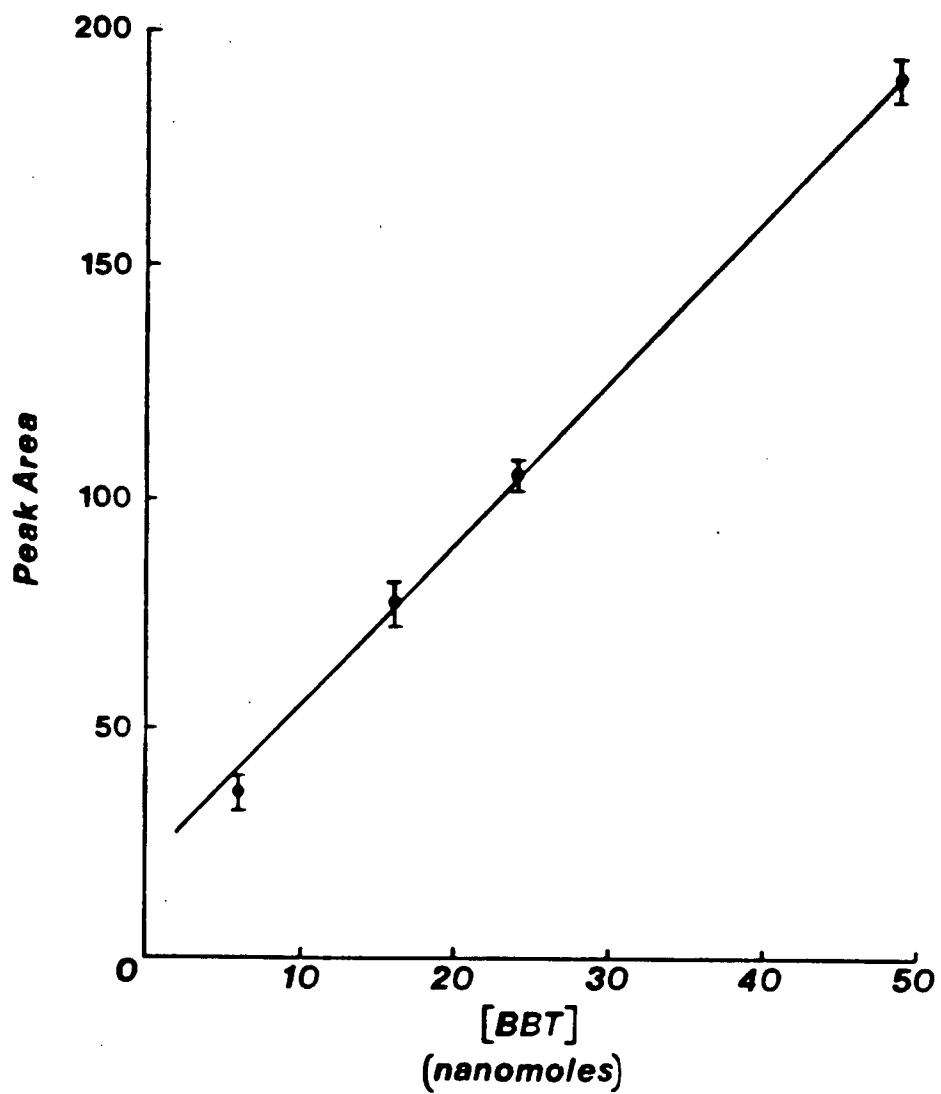
Figure 10. HPLC concentration curve for BBT (IX) which relates peak area (detector response) and amount of standard injected (nanomoles).
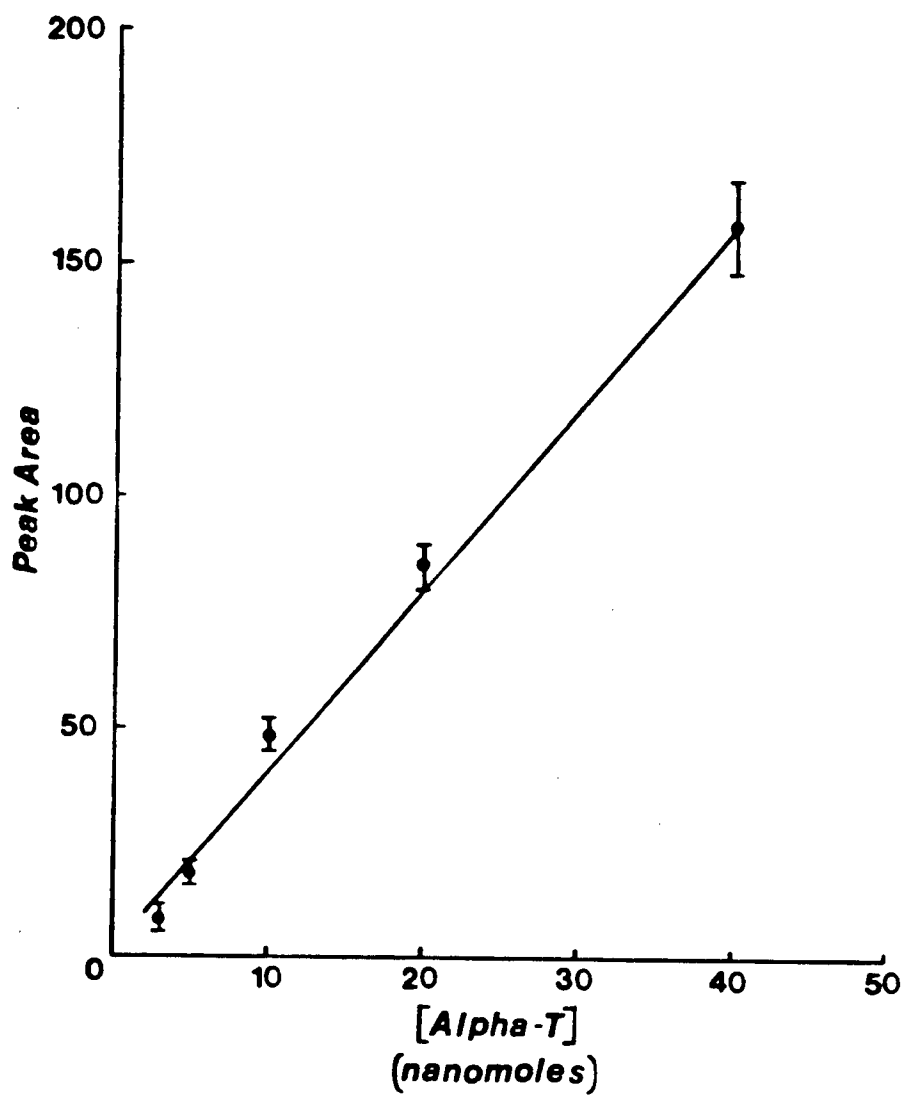
Figure 11. HPLC concentration curve for alpha-T (X) which relates peak area (detector response) and amount of standard injected (nanomoles).
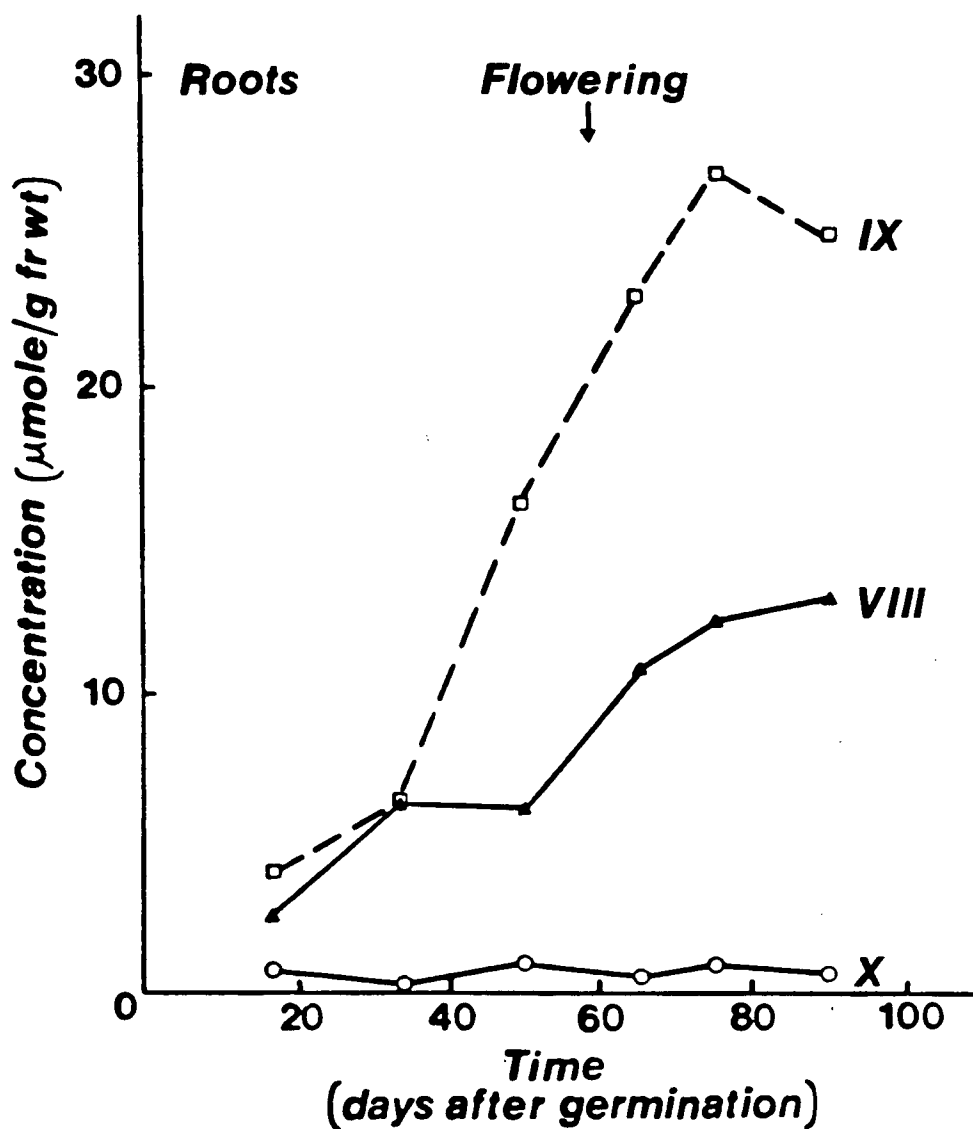
Figure 12. The concentration (umole/g fr wt) of BBT-OAc (VIII) (▲———▲), BBT (IX) (□――□) and alpha-T (X) (O———O) in the roots of hydroponically grown T. patula over time.

Table 5. Thiophene levels in crude extracts of hydropon-
ically grown plants. Standard deviation of three
injections are listed in parentheses. All
concentrations are in micromoles per gram fresh
weight (uM/g fresh wt).

Compound	Root	Shoot	Immature Leaf	Mature Leaf	Flower
BBT-OH (VII)	0.2(0.0)	0.2(0.0)	0.3(0.1)	-	-
BBT-OAc (VIII)	12.3(0.3)	6.4(0.2)	1.5(0.3)	0.6(0.1)	0.5(0.1)
BBT (IX)	26.7(1.1)	0.6(0.1)	0.4(0.0)	-	0.4(0.1)
Alpha-T (X)	0.5(0.2)	0.5(0.1)	0.5(0.1)	-	1.3(0.2)

- below quantifiable levels

days after germination but reached a plateau after that time.
The concentration of alpha-T (X) changed very little over the
experimental period.

BBT-OAc (VIII) was the dominant thiophene in shoots
(Figure 13). BBT (IX) and alpha-T (X) were equally concentrated
and did not increase appreciably over time. BBT-OAc (VIII), on
the other hand, increased until around 75-80 days when an
equilibrium concentration was reached. The leveling of BBT-OAc
(VIII) in the shoot and BBT-OAc (VIII) and BBT (IX) in the
roots followed flowering quite closely. The same time dependent
trends were also observed during replications of these
experiments.
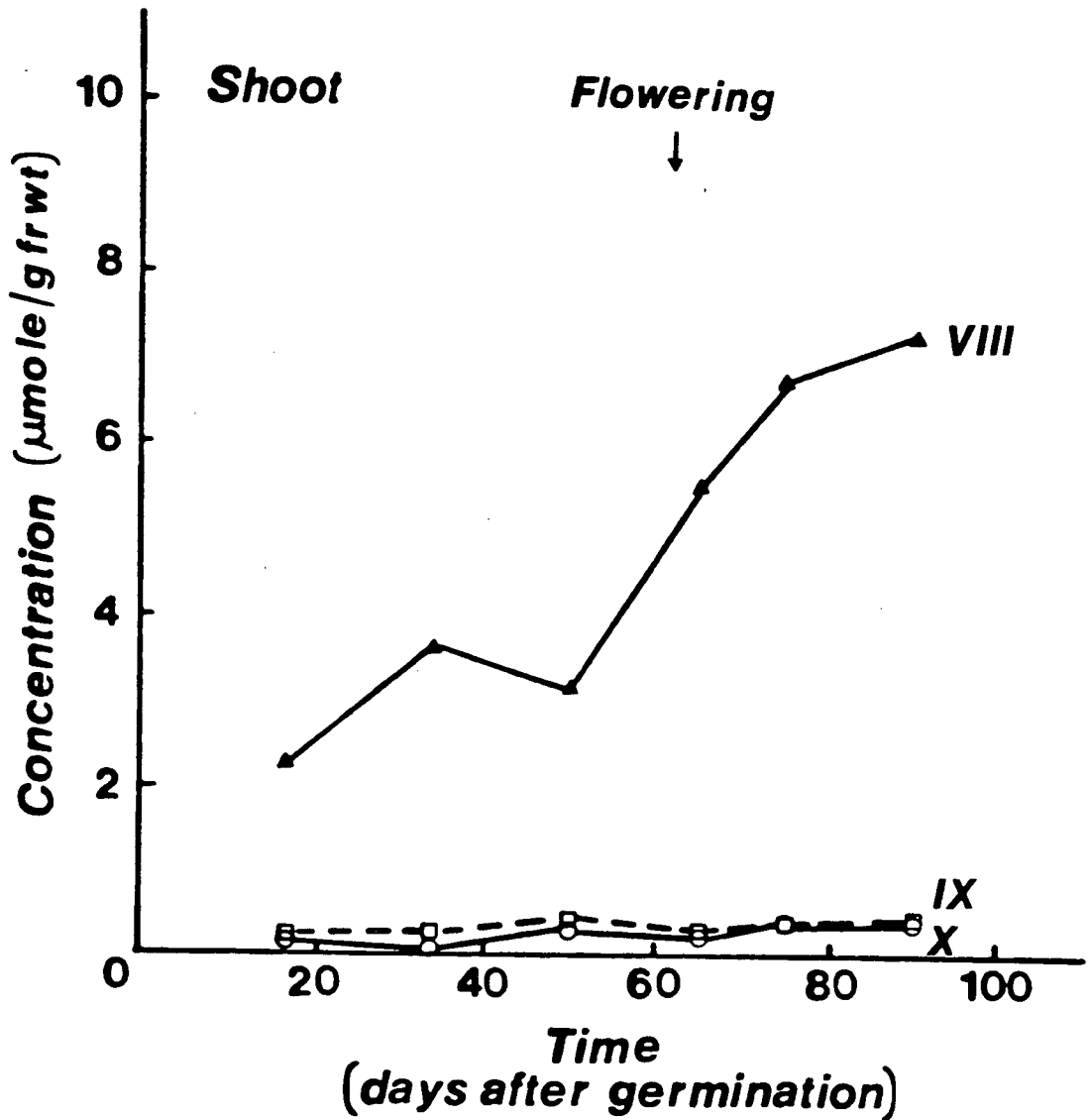
Figure 13. The concentration (umole/g fr wt) of BBT-OAc (VIII) (▲——▲), BBT (IX) (□— —□) and alpha-T (X) (O———O) in shoots of hydroponically grown T. patula over time.

Distribution of Thiophenes in the Tageteae

The Tageteae comprises 16 to 18 genera distributed generally in the warmer areas of North and South America (Keil, 1975; Strother, 1977). Chemically, the tribe is characterized by the lack of sesquiterpene lactones or pyrrolizidine alkaloids, characteristic of many other tribes of the Asteraceae, and by the presence of thiophenic and specific monoterpenoid derivatives (Rodriguez and Mabry, 1977). Fifteen species which represent the four largest genera of the tribe (Dyssodia, Pectis, Porophyllum, and Tagetes) were examined for the presence of BBT-OH (VII), BBT-OAc (VIII), BBT (IX) and alpha-T (X). Table 6 lists the results of this survey. Bithienyl and terthienyl derivatives were common in the genera Dyssodia, Porophyllum and Tagetes . Thiophenes , however, were totally lacking in species of Pectis.

Based on morphological characters, Pectis has no close relatives among other genera of the tribe (Keil, 1975; Strother, 1977). The chemical uniqueness of the genus is reflected by the fact that 20 out of approximately 80 species thus far examined display the Kranz syndrome or C4 metabolism (Smith and Turner, 1975). The lack of thiophenes found in this study may be further evidence that Pectis, the largest genus in the Tageteae, occupies a more distant relationship with other members of the tribe.

Table 6. Distribution of thiophenes within the tribe Tageteae.

Plant Species	VII	VIII	THIOPHENES IX	X
Dyssodia anthemidifolia Benth.			+	+
D. decipiens Bartl.	+	+	+	+
D. papposa (Vent.) Hitchc.	+	+	+	+
Pectis filipes Harv. & Gray				
P. imberbis A. Gray				
P. longipes A. Gray				
diploid				
tetraploid				
P. papposa Harv. & Gray				
P. prostrata Cav.				
Porophyllum gracile Benth.	+	+	+	+
P. ruderale	(+)	(+)	(+)	(+)e
P. lanceolatum DC.			(+)	(+)c
Tagetes coronopifolia Willd.	+	+	+	+
T. elliptica Sm.			(+)	(+)b,c
T. erecta L.	+	+	+	+

Table 6. (continued)

Plant Species	VII	VIII	IX	X
Tagetes filifolia Lag.		+	(+)	(+)b
T. glandulifera Schrank.	(+)	(+)	(+)	b
T. gracilis DC.	(+)	(+)	(+)	(+)d
T. indica	(+)	(+)	(+)	(+)b
T. lemmonii A. Gray	+	+	+	+
T. lunulata Ort.	+	+	+	+
T. lucida Cav.	(+)	(+)	(+)	(+)b
T. minuta L.	(+)	+	+	+ a
T. multiflora HBK.			+	+
T. patula L.	+	+	+	+
T. signata Bartl.		(+)	(+)	(+)b
T. tenuifolia Cav.	+	(+)	+	+ b
T. terniflora HBK.	(+)	(+)	(+)	(+)d
T. zypaquirensis H.and B.			(+)	d

a. Atkinson et al., 1965

b. Bohlmann et al., 1973

c. Chan et al., 1979

d. Bohlmann and Zdero,1979

e. Bohlmann et al., 1980

Biological Activity of Thiophene Standards

Compounds VII, VIII, IX and X were all found to give positive phototoxic bioassays (Table 7) using E. coli B, P. aeruginosa and S. cerevisiae. Alpha-T and BBT have previously been shown to kill bacteria in the presence of UV-A irradiation (Chan et al., (1975); also see Section II). BBT-OH (VII) and BBT-OAc (VIII), however, had never been examined before. The unidentified shoot and flower component with R(t') of 6.5 minutes was also found to be phototoxic to E. coli and S. cerevisiae. The mechanism of thiophene phototoxicity was explored in more detail in Section II (see Photobiological Aspects of Thiophenes).

Table 7. Phototoxicity of thiophenes to microorganisms.

Test Organisms	BBT-OH	BBT-OAc	BBT	Alpha-T
Bacteria				
Escherichia coli B	+	+	+	+
Pseudomonas aeruginosa	+	+	+	+
Yeast				
Saccharomyces cerevisiae	+	+	+	+

CONCLUSION

This study was a initial investigation into the distribution of thiophenes in members of the tribe Tageteae. A new method of separating and detecting these derivatives was developed using high pressure liquid chromatography with UV detection system. This technique was used to examine the thiophenic profile of one species, Tagetes patula L. Standard reference compounds of BBT-OH, BBT-OAc, BBT and alpha-T were first isolated and identified, and then their distribution was examined. The constituents in roots were found to be different from those of shoots. BBT-OAc and BBT were the major components of roots while BBT-OAc was the dominant compound in shoots. Alpha-T was the main derivative in flower petals and BBT in leaf oil glands. The concentration of BBT-OAc and BBT in roots increased over the life of the plant until flowering when the levels stabilized. BBT-OAc also increased in concentration in shoots over time. Alpha-T in roots and both alpha-T and BBT in shoots remained at fairly low levels over the lifetime of the plant.

HPLC was also used to screen 14 species from the tribe Tageteae for thiophene derivatives. Members from four genera were examined for the presence of the reference thiophenes common to T. patula. All of the species of Dyssodia, Porophyllum, and Tagetes contained some of the reference thiophenes, but species of Pectis showed no thiophenes in detectable concentrations.

The phototoxic effects of isolated alpha-T and BBT have

been demonstrated toward a wide spectrum of biological organisms. BBT-OH and BBT-OAc were therefore also examined for photobiocidal properties. The phototoxicities of BBT-OH and BBT-OAc were shown using E. coli, P. aeruginosa and S. cerevisiae as assay organisms. If the phototoxic properties of these compounds can be extrapolated to other biological systems, as have been with alpha-T, then it seems possible that these compounds could play an important protective role in situ.

SECTION II.

PHOTOBIOLOGICAL ASPECTS OF THIOPHENES

INTRODUCTION

Thiophenes were first isolated and characterized from petal extracts of "African marigold", Tagetes erecta L. (Zechmeister and Sease, 1947). The major thiophenes identified, alpha-terthienyl (alpha-T) and 5-(buten-1- ynyl)-2,2'-bithienyl (BBT), were screened for antibiotic activity against several bacteria including Escherichia coli and Pseudomonas ovali, but no biological response was found. Eighteen years later, Daniels (1965) observed that marigold seeds (achenes) caused a "phototoxic" response when placed on lawns of Candida albicans and irradiated with longwave (non-germicidal) UV light, but that no halo of growth inhibition occurred in the dark. The compounds later found responsible for this photoactivated antibiotic effect were identified as alpha-T and BBT (Chan et al., 1975). Since these initial findings thiophenes in the presence of UV-A irradiation (320-400 nm) have been shown to be toxic to bacteria and yeasts (Chan et al., 1975) as well as algae (Arnason et al., 1981), trematode cercaria larvae (Graham et al., 1980), nematodes (Gommers and Geerlings, 1973; Gommers et al., 1980), mosquito and blackfly larvae (Arnason et al., 1980; Wat et al., in press) and fish (Towers, 1980). Alpha-T has also been shown to elicit severe erythema and prolonged hyperpigmentation in human and guinea pig skin irradiated with UV-A (Chan et al., 1977; Towers et al., 1979).

.The photobiocidal effects of the thiophenes have stimulated interest in their in vivo mechanism of action. Photosensitizers have been defined as chemicals which cause

other components in a system to react when excited by radiation (Turro and Lamola, 1977). Photosensitized reactions occur primarily as one of two types as discussed by Krinsky (1977) and Spikes (1977). Initially, a ground state sensitizer molecule ($^{\circ}S$) is excited to a singlet excited state (^{1}S) by the absorption of a photon (hv) (1). The ^{1}S state is short lived (10^{-9} to $10^{-6}s$) and can undergo intersystem crossing over to a longer lived (10^{-3} to 10s) triplet-excited state (^{3}S).

$$\overset{hv}{^{\circ}S ----> {}^{1}S ----> {}^{3}S} \qquad (1)$$
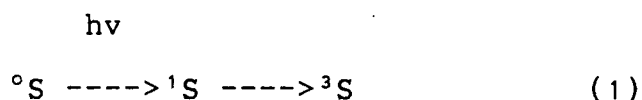
The ^{3}S state, being longer lived, has the capacity to initiate Type I or Type II reactions. Type I reactions (2) proceed via radical or redox mechanisms generally in the absence of molecular oxygen.

$$Type\ I \qquad {}^{3}S + A ----> S^{\overline{\cdot}} + A^{\underline{\cdot}} \qquad (2)$$
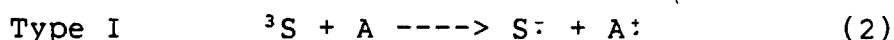
Type II or photodynamic reactions (3) involve energy transfer between molecular oxygen and ^{3}S in a spin conserved reaction.

$$Type\ II \qquad {}^{3}S ----> {}^{\circ}S + {}^{1}O_2 \qquad (3)$$
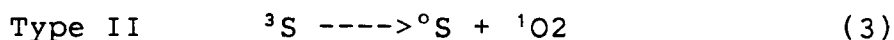
This mechanism accounts for nearly all of the quenching of ^{3}S by molecular oxygen and generates $^{\circ}S$ and singlet oxygen ($^{1}O_2$) which is responsible for the photooxidation of many biological molecules. A less efficient reaction between ^{3}S and molecular

oxygen involves electron transfer and is a special case of a Type I mechanism (4).

$$^3S ----> S^{\dot{+}} + O_2^{\dot{-}} \qquad (4)$$

This pathway leads to the formation of superoxide radical ($O_2^{\dot{-}}$) and a semi-oxidized form of the sensitizer ($S^{\dot{+}}$). Both of these radicals may react with various biological molecules.

Furocoumarins or psoralens are undoubtedly the most extensively studied group of non-photodynamic or Type I photosensitizers. In vivo, 8-methoxypsoralen (8-MOP) kills microorganisms by photoinduced monoadduct formation and interstrand crosslinkages with DNA (Song and Tapley, 1979). The photobinding is temperature independent and does not require oxygen (Oginsky et al., 1959); Musajo and Rodighiero, 1972). On the other hand, the in vivo photosensitization by the polyacetylene, phenylheptatriyne (PHT), recently investigated by Arnason et al. (1980) was found to inactivate E. coli more efficiently in the absence of oxygen. Unlike 8-MOP, PHT does not crosslink DNA (Wat et al., 1977) and thus represents a new class of Type I photosensitizer.

More than 400 photodynamic compounds are known which sensitize biological molecules by a Type II or singlet oxygen mechanism (Santamaria and Prino, 1972). The in vivo targets of these sensitizers are quite variable and are believed to depend on the cellular location of the sensitizer, the singlet oxygen generating efficiency of the sensitizer during irradiation and

the susceptibility of the target molecule and/or structure to photooxidation by singlet oxygen.

The location of the sensitizer is dictated by its penetrability into the cell. Ito (1978) has suggested that photodynamic compounds may inactivate cells by reacting either outside the cell if the sensitizer is excluded or from within if the compound can pass the cytoplasmic membrane. He also points out that a unique feature of photodynamic action is that the generation site of singlet oxygen and the reaction site may be different. In fact, in an aqueous environment, singlet oxygen may diffuse and react up to 100 nm from the generation site (Wagner et al., 1980). This is quite distinct from Type I sensitizers which react through radical mechanisms in which the sensitizer and substrate must be in close proximity at the time of reaction.

In vitro and in vivo studies with various Type II photosensitizers have shown that many biologically important molecules are susceptible to photooxidation by singlet oxygen. Free amino acids, e.g. tryptophan, and amino acid residues in proteins (Dubbelman et al., 1980), unsaturated fatty acid residues in lipids (Rawls and Van Santen, 1970), cholesterol (Teng and Smith, 1973; Suwa et al., 1978), and nucleic acids (Webb et al., 1979) have all been shown to react with singlet oxygen. This damage to biological molecules in vivo is expressed by loss of membrane integrity resulting from lipid peroxidation and crosslinking or destruction of membrane proteins (Dubbelman et al., 1980; Lamola and Doleiden, 1980),

inactivation of cytoplasmic enzymes (Yamamoto et al., 1979) or genetic damage (Webb et al., 1979),respectively.

The in vivo mechanisms of action of different thiophenes are interesting and may have significant in situ ramifications. Alpha-T is the only naturally derived thiophene to be examined thus far, but differences of opinion regarding its mechanism of action already exist. A Type I or radical mechanism was proposed by Kagan et al. (1980) who reported: 1) that photosensitization of E. coli by alpha-T was not oxygen dependent; 2) in the presence of UV-A, labelled alpha-T covalently bonded to calf thymus DNA and Candida utilis DNA; and 3) that covalent bonding did not involve cross linking of double stranded DNA as with 8-MOP. However, the Type II or photodynamic nature of alpha-T has been demonstrated in several studies using various techniques. Bakker et al. (1979) showed that the in vitro inactivation of glucose-6-phosphate dehydrogenase was oxygen dependent. Acetylcholinesterase inactivation and photohemolysis of human erythrocytes as well as cellular inactivation of E. coli were all enhanced under aerobic conditions (Wat et al., 1980; Arnason et al., 1981). A singlet oxygen mechanism has been indicated by the work of both Bakker's group and Towers' group (Arnason et al., 1981). Damage to cytoplasmic membranes of human erythrocytes has been postulated as a possible site of alpha-T photosensitized attack (Yamamoto et al., 1979; Wat et al., 1980).

The in vivo mechanism of action of two thiophenes were examined in this study. Alpha-T was the primary sensitizer

studied, although several important aspects of BBT photosensitization were also investigated. Data will be presented which show that the phototoxicity of alpha-T toward E. coli : 1) is due to alpha-T and not to a photoproduct; 2) appears to follow multiple-hit inactivation kinetics; 3) is oxygen dependent; 4) may involve singlet oxygen and possibly other excited species of oxygen; 5) is not affected by the inability of various mutants to perform genetic recombination or post-replication repair; 6) is sensitive to irradiation temperature; and 7) probably involves photodynamic attack of the cytoplasmic membrane which is reflected in crosslinking of membrane proteins. BBT photosensitization, on the other hand, appears to follow single-hit inactivation kinetics of E. coli and is affected by the ability of mutants to perform genetic recombination and post-replication repair, which suggests the involvement of cellular DNA.

MATERIALS AND METHODS

Irradiation Sources

Three sources of UV-A irradiation (320 nm to 400 nm) were commonly used during these studies (the particular source is specified in each experimental section). Fluorescent sources consisted of either four horizontal Sylvania F20T12/BLB lamps or two vertical Sylvania F15T8/BL lamps. A 1000 watt xenon arc lamp (Orion Corporation) was used to establish the action spectrum of alpha-T. Wavelength bands were selected at 10 nm

intervals between 320 nm and 400 nm by interference filters (Corion Corporation, half-bandwidth 10 nm).

Irradiance of the fluorescent UV-A sources as well as the wavelength specificity and irradiance of the interference filters used in conjunction with the xenon lamp were measured with a Research Radiometer (International Light Inc.). Irradiance versus wavelength for the fluorescent lamps are shown graphically in Figure 14. The irradiance determined at the maximum wavelength of each interference filter along with several pertinent dosimetric parameters used in preparing the action spectrum are listed in Table 8.

Survival Curves

The methods used to determine the survival of E. coli in response to varying concentrations of alpha-T and BBT irradiated with UV-A were essentially those of Arnason et al. (1980). Overnight cultures of E. coli B-23(+) (Microbiology Department, UBC) were grown in nutrient broth at 37°C with shaking. The cells were centrifuged twice in sterile physiological saline (PSS, 0.87% NaCl) at 1000 x g for 10 minutes and resuspended in sterile PSS to an optical density of 0.1 at 520 nm using a Spectronic 20 photometer. Three ml aliquots of the resulting bacterial suspension were aseptically pipetted into quartz cuvettes and appropriate amounts of either alpha-T or BBT were added in 20 ul of 95% ethanol. The cuvettes were incubated with shaking for 30 minutes at 37°C followed by irradiation with Sylvania F20T12/BLB lamps at a distance of 15
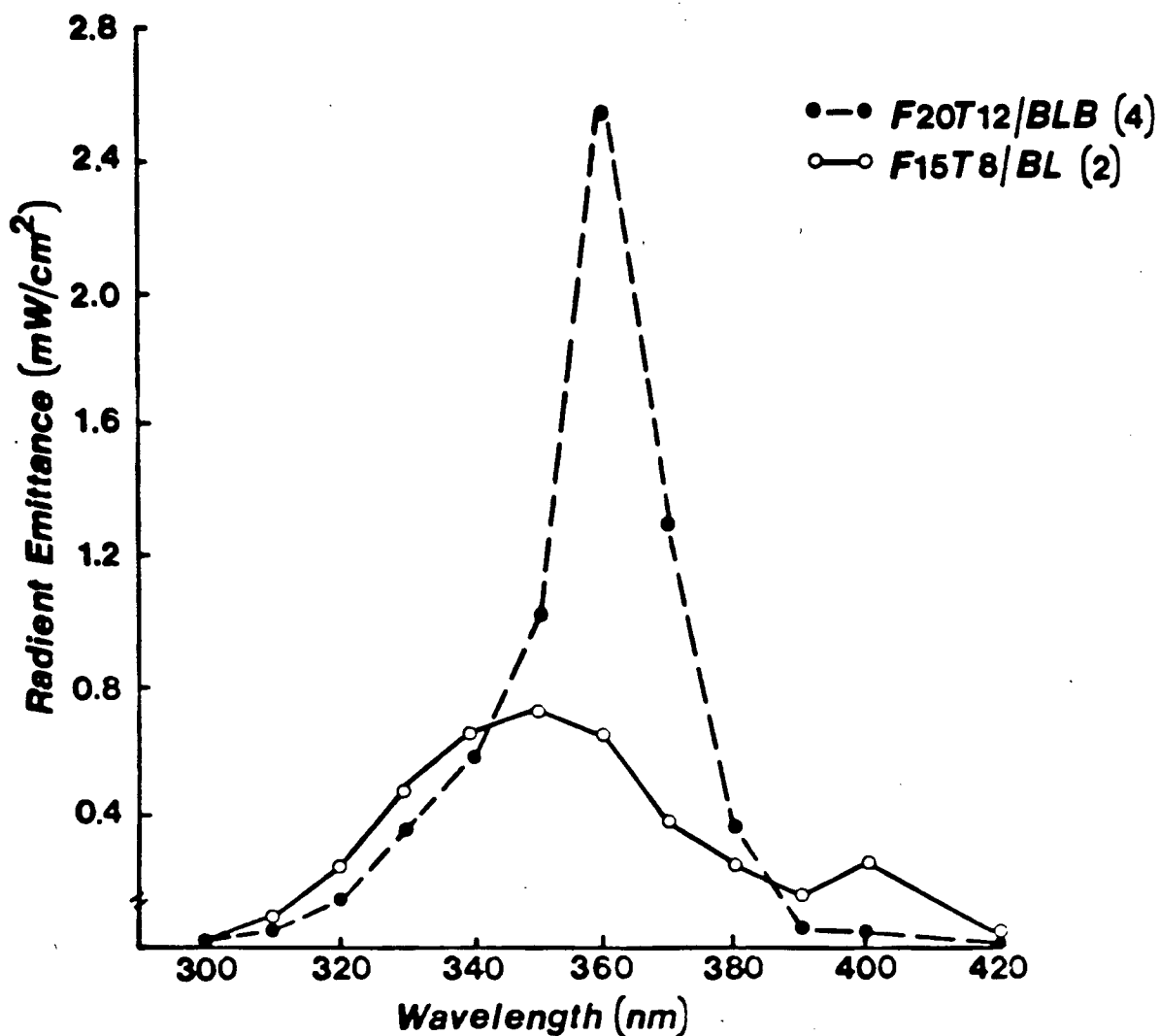
Figure 14. Radient emittance (mW/cm2) versus wavelength of the fluorescent sources used throughout these studies. Measurements were taken 10 cm from the F20T12/BLB lamps and 20 cm from the F15T8/BL lamps.

Table 8. Irradiance at the wavelength maximum of each interference filter with several pertinent dosimetric parameters used to determine the action spectrum of alpha-T

WAVELENGTH (nm)	IRRADIANCE (mW $\times 10^4$ cm^{-1})	ENERGY FLUENCE (J $\times 10^2$ cm^{-1})	ENERGY/hv (J $\times 10^{17}$ hv^{-1})	hv FLUENCE (# hv cm^{-2})
310	6.9	8.2	6.4	1.3
320	7.1	8.5	6.2	1.4
330	7.0	8.4	6.0	1.4
340	7.0	8.4	5.9	1.4
350	7.0	8.4	5.7	1.5
360	7.0	8.4	5.5	1.5
370	7.0	8.4	5.4	1.6
380	7.3	8.8	5.2	1.7
390	7.0	8.4	5.1	1.7
400	7.0	8.4	5.0	1.7

cm (see Irradiation Sources). Samples of 0.1 ml were removed at appropriate times during irradiation; this necessitated successive removal and subsequent replacement of cuvettes under the UV-A lamps. Controls containing 1 ug/ml of alpha-T and BBT were assayed simultaneously in the dark as well as cells which were irradiated without photosensitizer. Samples were diluted in PSS and plated on nutrient agar. All experiments were repeated at least three times and the data presented are representative.

Bacterial Preparation

Liquid cultures of E. coli B and Pseudomonas aeruginosa were grown to stationary phase at 37°C. Aerobic cultures were shaken at 250 rpm while anaerobic cultures were grown in a BBL GasPak Anaerobic System with GasPak O_2 combuster and CO_2 generator packs to create and maintain anaerobic conditions. An inorganic salt medium (Hindennach and Henning, 1975) was used to culture cells and either 0.4% glucose or succinate was added as the carbon source for E. coli and P. aeruginosa, respectively. Cultures of P. aeruginosa also received potassium nitrate as a terminal electron acceptor for anaerobic respiration. Subcultures in fresh medium with a final dilution of 1 in 100 were used for experiments unless indicated otherwise.

Assay Procedure

A 3-necked, borosilicate reaction flask (Multi-purpose jacketed reactor from Pierce Chemical Co.) with a 10ml capacity, equipped with stir bar, gas inlet, and leur-lok sampling port was used for all irradiation experiments unless otherwise indicated. Initially, 9 ml of BM2 medium with appropriate supplements was added to the sterilized reaction flask. Either medical air (Linde, Type I), He (Linde, 99.995%), or He/O_2 (4:1) was passed through a millipore filter (Type HA, 0.45 um) then bubbled through the growth medium for 15 minutes prior to addition of cells. After flushing with the appropriate

gas, 1 ml of 1 in 10 diluted cells was added to the reaction flask. The cells were incubated for one hour in air, 3 hours in He and 30 minutes in He/O2. Alpha-T was then added in 95% ethanol and the cultures were incubated for 30 minutes in the dark and then irradiated with continuous gas bubbling through the medium. Samples of 0.5 ml were withdrawn using a sterile 1 ml syringe at various times during incubation and irradiation. Viable cell numbers were determined by diluting and plating followed by colony counting after 24 hours incubation at 37°C.

Alpha-Terthienyl Action Spectrum

E. coli B was used to determine the action spectrum of alpha-T between 320 nm and 400 nm. After cell survival was determined for alpha-T (see Survival Curves) a concentration was chosen which would give effective cellular inactivation. The concentration of alpha-T chosen was 1ug/ml.

These studies were carried out in sterile microtitration plates (Gibco Scientific) having 96 wells per plate. Basal mineral salts medium containing 0.4% glucose (150 ul) was added to each of the wells. This was followed by the addition of approximately 1000 cells suspended in BM2 (100 ul). The bacterial suspension was prepared by diluting an overnight culture into fresh BM2 (1 in 10^5). The sensitizer was then added in 5 ul of 95% ethanol to give a final concentration per well of 1 ug/ml. The plates were incubated at 37°C for 30 minutes and then irradiated.

The irradiation source was a 1000 watt xenon arc lamp (see

Irradiation Sources). The distance from the source was adjusted to give 0.7 W/m² at each of the wave bands. Following calibration, plates were irradiated at each wavelength for two minutes at room temperature followed by incubation at 37°C for 18 hours. A plate containing the sensitizer and bacteria, but unirradiated, was also prepared as a dark control.

Bacterial growth (turbidity) was measured spectrophotometrically at 620 nm using a Titertek Multiscan Photometer (Flow Laboratories). The optical density of the wells on each irradiated and control plate was determined immediately following irradiation and after 18 hours incubation in the dark. Changes in turbidity were averaged to give a single representative change in OD. The resulting OD was a direct reflection of the initial cellular inactivation and/or indication of cellular damage incurred during irradiation. The ratio between OD's of irradiated and non-irradiated plates yielded a measure of the relative effectiveness of the dose (sensitizer + irradiation). Dividing this value by the number of incident photons gives the relative photon efficiency at each wavelength. This value was plotted against wavelength for the action spectrum. The data presented are averages of at least three experiments conducted on different days and are plotted with standard deviation.

Rec Mutant Assay

Isogenic strains of E. coli K-12 recombination deficient (rec) mutants were obtained from Dr. A. J. Clarke, University of California, Berkeley. The strains and their relevant characteristics are listed in Table 9. All strains were grown in nutrient broth at 37°C with shaking at 250 rpm. Overnight cultures were diluted 1 in 100 and 0.1 ml was spread onto nutrient agar plates. Six plates were prepared for each strain. Sterile Whatman #1 filter paper discs which had been previously loaded with alpha-T (10 ug/disc), BBT (10 ug/disc), bleomycin (15 ug/disc) and 8-MOP (20 ug/disc) were placed onto the agar plates which were then incubated for 1 hour at 37°C. Three replicate plates were kept dark for controls while the other three replicate plates were irradiated by four Sylvania F20T12/BLB lamps for 1 hour. All plates were then incubated in the dark for 48 hours at 37°C. Inhibitory zones (edge to edge) were measured in millimeters. The experiment was repeated three times on separate days. Representative data from one experiment is presented with standard deviations.

Scavenger Studies

All procedures regarding preparation of E. coli B cultures, irradiation and plating for viable cell counts were described previously (see Assay Procedure). Stock solutions of 1M sodium azide and 50 mM butylated hydroxytoluene (BHT) were prepared fresh for all experiments by dissolving the

Table 9. Strains of E. coli used and their relevant characteristics (Bachmann and Low, 1980).

Strain	Parent	Phenotypic Trait
AB 1157	AB 1133(K12)	wild type
JC 2926	AB 1157	rec A⁻
JC 3881	AB 1157	rec A,B,C⁻
JC 5519	AB 1157	rec B,C⁻
JC 5547	AB 1157	rec B,C,F⁻
JC 9239	AB 1157	rec F⁻

appropriate chemical in distilled water and 95% ethanol, respectively. The azide solution was filter sterilized (Millipore, Type HA) while the BHT solution was used without further sterilization. Aliquots of each stock solution were added aseptically to cultures yielding final concentrations of 20 mM sodium azide and 50 uM BHT. The scavengers were added to bacterial suspensions 15 minutes after alpha-T addition and followed by 15 minutes incubation in the dark before irradiation. All experiments were repeated at least three times. The data presented are representative.

Temperature Studies

All procedures regarding preparation of E. coli B cultures, irradiation and survival assay were described previously (see Assay Procedure). Cells were inoculated into the reaction flask containing air saturated BM2 growth medium at 37°C and incubated for 30 minutes. Alpha-T (1 ug/ml) in 95%

ethanol was then added and the cells incubated in the dark for a further 30 minutes. The temperature of the culture was then adjusted to the desired irradiation temperature and equilibrated for 15 minutes prior to irradiation for 30 minutes.

The effect of incubation temperature on the photosensitized inactivation of E. coli by alpha-T was investigated by lowering the culture temperature to 5°C for 15 minutes before the addition of alpha-T. Following this prechill and 30 minutes incubation with alpha-T at 5°C in the dark, the culture was irradiated at 5°C and cell survival determined. All graphs represent at least three separate experiments.

Crosslinking of E. coli Proteins

Cultures of E. coli B were grown overnight to stationary phase at 37°C with shaking in BM2 containing 0.4% glucose. Two, 10 ml portions were subcultured in 990 ml of fresh BM2 separately and grown to an optical density of 0.6 at 520 nm. The cells were harvested by centrifugation at 8000 rpm for 10 minutes and resuspended in 100 ml of 10 mM phosphate buffer pH 7.0. The resulting suspension (approximately 3×10^9 cells/ml) was incubated with slow shaking for 15 minutes at 37°C prior to the addition of 1 ug/ml or of alpha-T in 95% ethanol. The suspension was then incubated for 30 minutes and subsequently irradiated by two horizontal Sylvania F15T8/BL lamps suspended 20 cm above the suspensions. Ten ml aliquots were removed prior to alpha-T addition, after 30 minutes incubation with alpha-T

and following 15 and 30 minutes UV-A irradiation. Viable cell counts were determined from each of the samples which were kept dark and on ice during the course of the experiment.

Cells were broken in a French press at 14,000 psi after the addition of pancreatic DNase I (20 ug/ml; Sigma). The resulting broken cell suspensions were centrifuged at 10,000 x g for 15 minutes to remove the remaining intact cells. The suspension was then centrifuged at 180,000 x g for 1 hour in a Beckman 50 Ti rotor. The pellet containing membranes and ribosomes was resuspended in 0.5 ml distilled water. The supernatant containing soluble or cytoplasmic proteins was ethanol precipitated with 0.5% NaCl and twice the volume of ethanol (18 ml). The soluble proteins were collected after overnight precipitation at -20°C by centrifugation in an Eppendorf Model 54-12 centrifuge.

SDS-Polyacrylamide Gel Electrophoresis

Slab gel electrophoresis was used to separate the soluble and pelletable proteins of E. coli following irradiation in the presence of alpha-T. The basic method was previously described by Lugtenberg et al. (1975). Separating or lower gels of 7% (vol/vol) acrylamide were prepared from a stock acrylamide solution containing 30% (wt/vol) acrylamide and 0.8% (wt/vol) N,N'-methylenebisacrylamide. The gels were polymerized by the addition (per 12.5 ml of gel mix) of 20 ul of TEMED (N,N,N',N'-tetramethylethylenediamine) and 0.165 ml of 1% (wt/vol) ammonium persulfate. The stacking or upper gels were also

prepared from the 30:0.8 acrylamide stock and was polymerized by the addition (per 5 ml of gel mix) of 10 ul TEMED and 0.12 ml of 1% ammonium persulfate.

Protein samples were assayed by the method of Sandermann and Strominger (1972). Solubilization of proteins was accomplished by dilution of the sample in an equal volume of solubilization reduction mix containing 4% (wt/vol) SDS, 10% (vol/vol) 2-mercaptoethanol, twofold-concentrated upper gel buffer and 20% (vol/vol) glycerol followed by heating at 88°C for 10 minutes in a Temp-Block module heater (Lab Line Instruments Inc., Melrose Park, Ill.) filled with glyerol. Approximately 20 ug of protein was added per well. The gels were run for 3 to 3-1/2 hours at a constant voltage of 150 volts. The separated proteins were stained using Coomassie Brilliant Blue (Hancock and Carey, 1979).

RESULTS AND DISCUSSION

Survival Curves

The synergistic action of alpha-T and UV-A irradiation or BBT and UV-A on the survival of E. coli is demonstrated in Figures 15 and 16. The differences in cellular inactivation caused by these biosynthetically related thiophenes suggest that the compounds may have different mechanisms of action. Figure 15 shows the survival of cells exposed to varying doses of alpha-T and UV-A. All of the curves had a pronounced lag or shoulder after the start of irradiation which was followed by
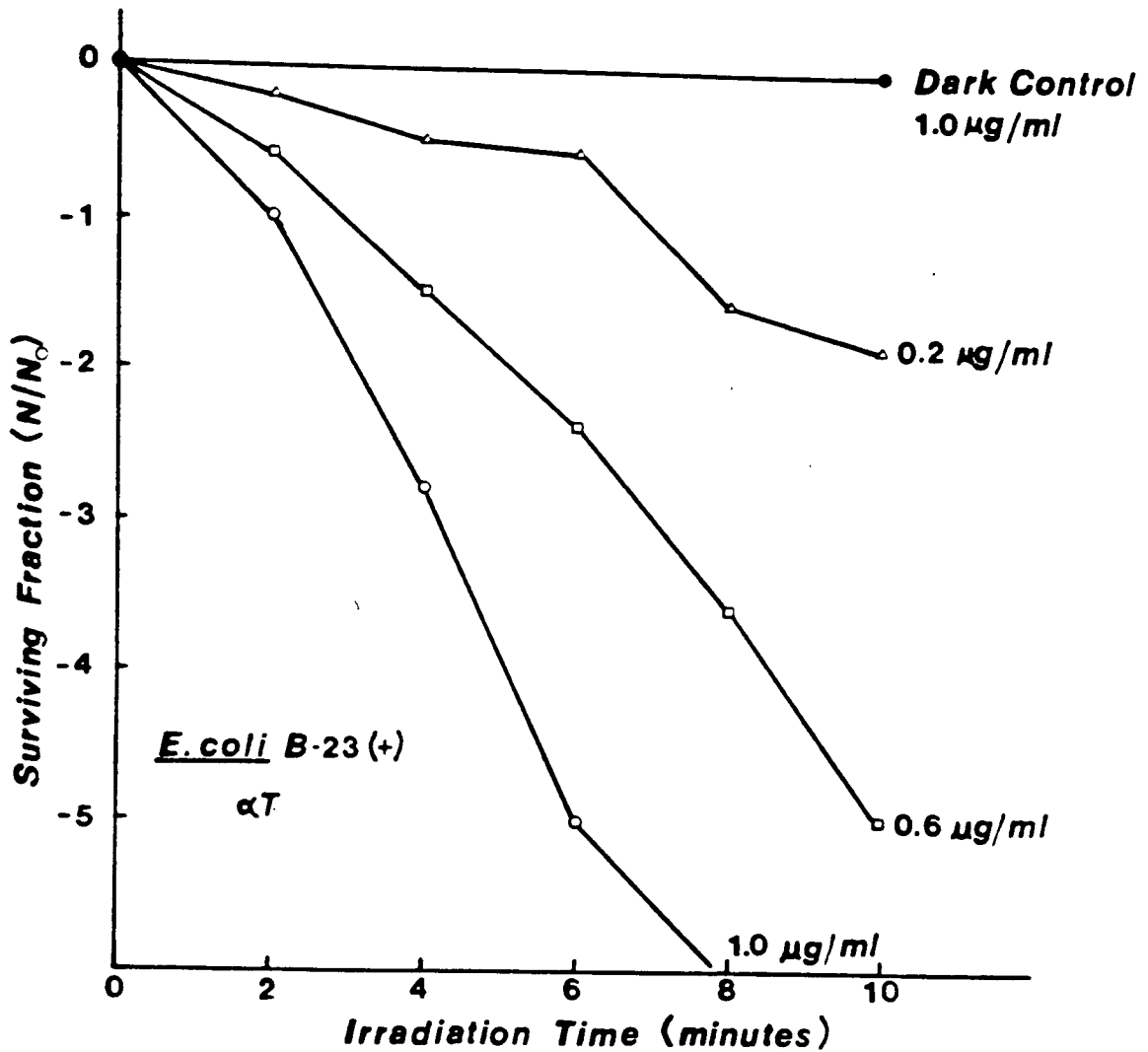
Figure 15. Survival curves of E. coli B-23(+) in response to various doses of alpha-T and UV-A irradiation.

an exponential decline in cell viability. This type of survival is similar to the action of many photosensitizers on microorganisms (Oginsky et al., 1959; Smith and Hanawalt, 1969; Grossweiner and Smith, 1981; Grover et al., 1981) and has been interpreted to mean that cells were either capable of accumulating considerable damage before death or that they could repair incurred cellular damage before a critical point when exponential killing was allowed to proceed (Zimmer, 1961; Elkind and Whitmore, 1967; Clayton, 1971). It was observed during these and other experiments with alpha-T and E. coli that colonies resulting from treated cells displayed considerable size variation. This variation may be a reflection of the extent of damage incurred by cells during irradiation.

Inactivation of cells by BBT and UV-A (Figure 16) was found to be quite different from that caused by alpha-T plus irradiation. Survival curves showed immediate exponential decline in cell viability which may indicate that the cellular target and/or mechanism of photosensitized killing is distinct from that of alpha-T.

BBT, unlike alpha-T, contains an unsaturated, four carbon side chain which resembles the structure of a polyacetylene from which the thiophenes in nature are ultimately derived (Bohlmann et al., 1973). The polyacetylenes are believed to represent a new class of natural photosensitizers (Towers, 1980). Phenylheptatriyne (PHT), which has been studied in the most detail, does not show enhanced photoactivity to E. coli in oxygen (Arnason et al., 1980) and does not form interstrand
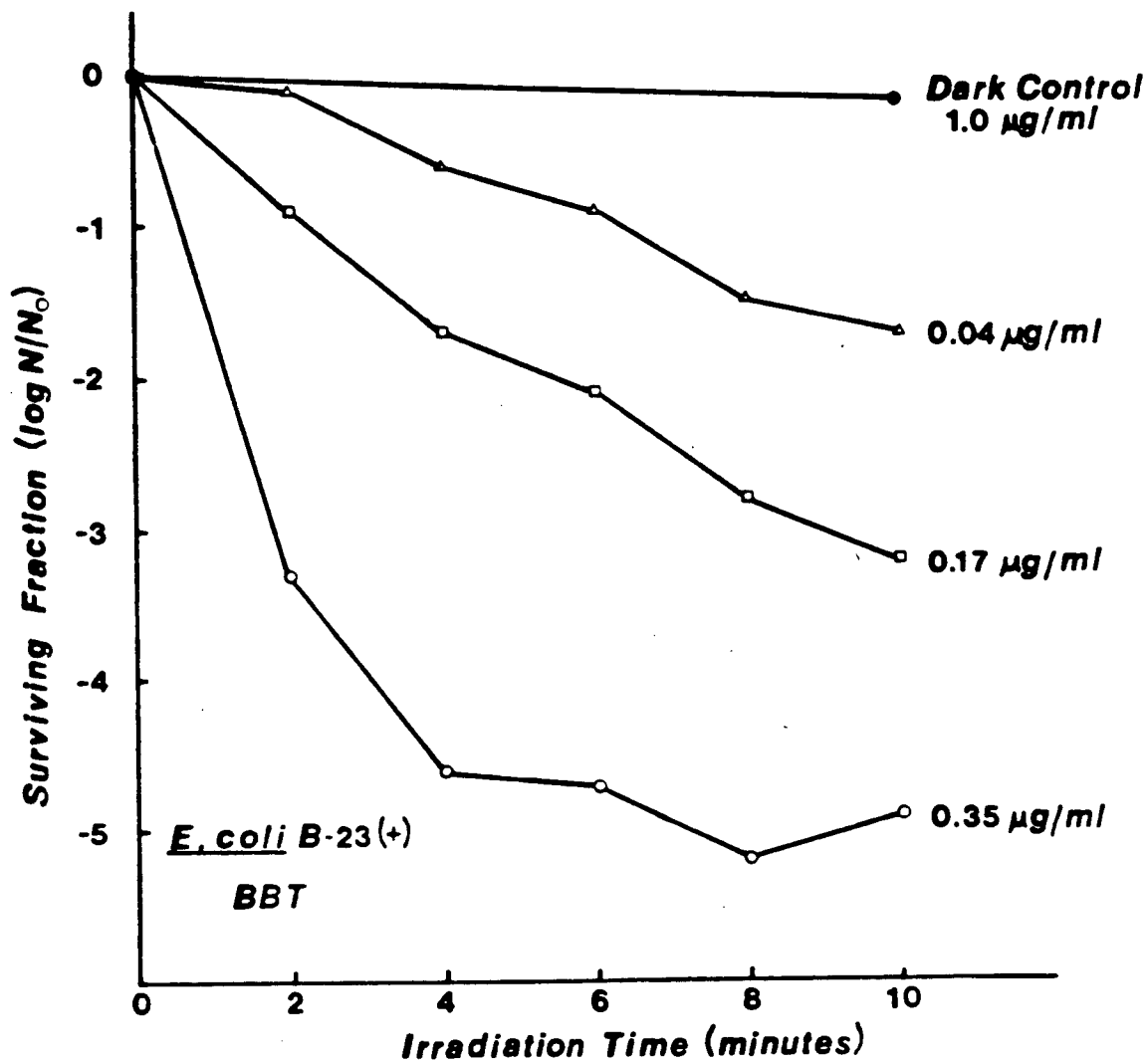
Figure 16. Survival curves of E. coli B-23(+) in response to various doses of BBT and UV-A irradiation.

linkages with DNA (Wat et al., 1977) like the furocoumarins. Based on these and other data to be presented, BBT appears to display an intermediate reaction mechanism between that of alpha-T and that of PHT.

Action Spectrum

The action spectrum for E. coli survival following treatment with alpha-T and UV-A versus the absorption spectrum for alpha-T is shown in Figure 17. A close agreement between activity and light absorption was observed. In both curves, the maximum was at 350 nm with declining effectiveness at shorter and longer wavelengths. These curves provide good evidence that alpha-T, rather than a photoproduct formed during irradiation, was the primary absorbing molecule responsible for the photosensitized reaction. Had a photoproduct been formed a different action spectrum would have resulted which would have resembled the absorption spectrum of the photoproduct.

This result agrees with the action spectrum of alpha-T on mosquito larvae (Arnason et al., 1980). The maximum wavelength found in that study was 360 nm. The 10 nm difference could be due to light scattering amongst larvae. This would be greatly reduced by using very dilute suspensions of E. coli cells.
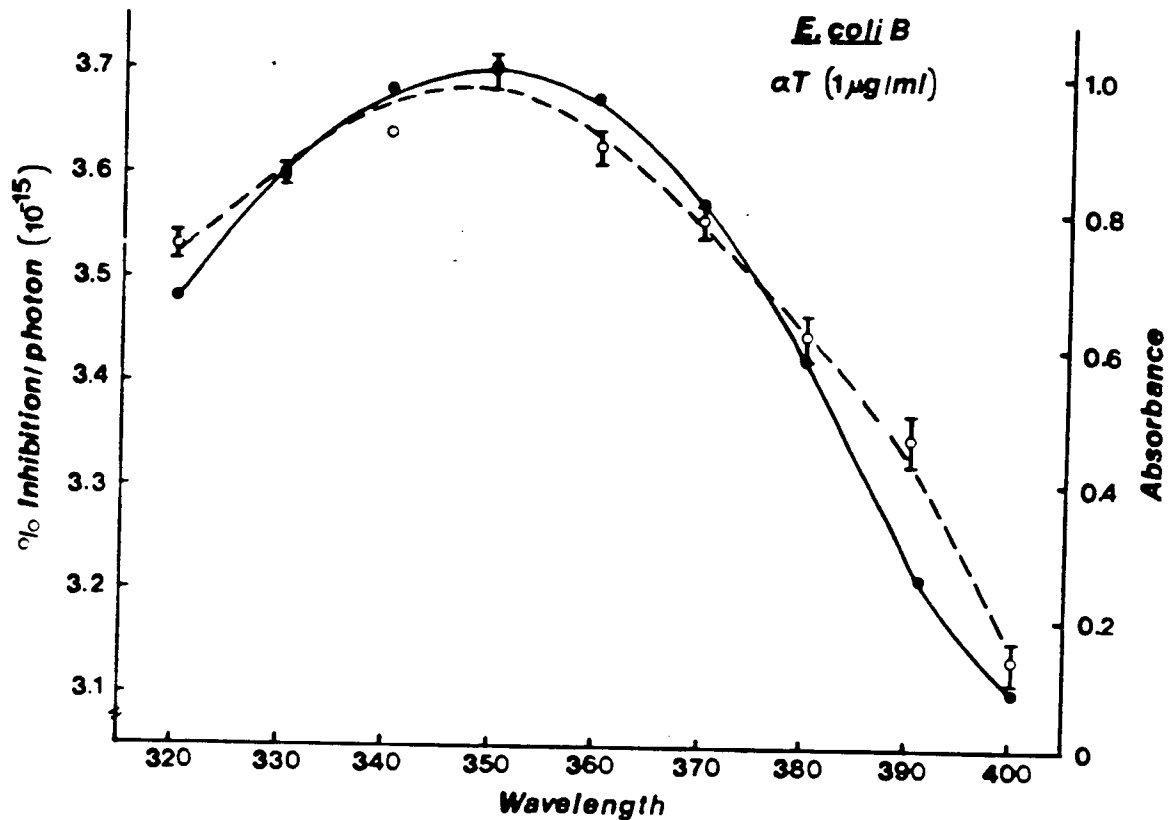
Figure 17. The photoinduced bactericidal action (O---O) on E. coli B compared with the absorption (●————●) of alpha-T at different wavelengths between 320nm and 400nm.

Studies on Repair Deficient Mutants of E. coli

The inability of various mutants of E. coli to perform genetic recombination or post-replication repair was used to indicate whether DNA damage could be caused by the action of alpha-T or BBT. Recombination deficient (rec) mutants are unable to incorporate extrachromosomal DNA into their genome or to repair damaged sites on the DNA (Smith, 1977). These damaged sites could include pyrimidine dimers, monoadducts or crosslinkages in the DNA strand or nicks in the DNA. It was thought that recombination mutants would show greater sensitivity to compounds which damage DNA than wild type cells which were capable of repair. This was shown to be the case in the experiments reported here with bleomycin and 8-MOP (Table 10), two compounds which have been shown to react with DNA. Bleomycin, which causes the degradation of intracellular DNA (Onishi et al., 1973), inhibits DNA synthesis (Suzuki et al., 1968), and induces the synthesis of rec A protein in E. coli (Gudas and Pardee, 1976), was used as a non-photoactivated control. Greater zones of inhibition were exhibited by all of the rec⁻ strains as compared with wild type (AB 1157). This result agrees with a recent study which showed that growing cultures of repair deficient strains of E. coli were more sensitive to the effect of bleomycin (Yamamoto and Hutchinson, 1979) than wild type. 8-MOP, on the other hand, which kills microorganisms via photoinduced monoadduct formation and interstrand crosslinkages with DNA (Song and Tapley, 1979), was selected as a photoactivatible control. This DNA intercalator

Table 10. Response of various E. coli rec mutants to alpha-T, BBT, bleomycin, and 8-MOP in UV-A and dark. Inhibitory zones (mm) are followed by standard deviations in parentheses.

Strain	Alpha-T UV-A	BBT UV-A	Bleomycin		8-MOP UV-A
			UV-A	Dark	
AB 1157	15.5(0.9)	20.7(0.9)	20.7(1.2)	21.0(0.8)	10.0(0.8)
JC 2926	17.3(1.2)	26.3(0.9)	31.7(0.5)	28.7(0.9)	19.0(0.8)
JC 3881	16.3(0.5)	25.0(0.0)	32.7(0.5)	27.3(2.5)	20.0(0.0)
JC 5519	16.0(0.8)	25.0(0.0)	28.7(1.2)	26.7(1.2)	16.0(0.8)
JC 5547	16.3(0.9)	24.7(1.9)	32.7(2.1)	24.7(2.6)	15.3(0.5)
JC 9239	14.7(0.5)	22.7(0.9)	24.2(0.5)	21.7(0.5)	15.3(0.5)

also inhibited mutant strains more than strain AB 1157, the wild type. This finding agrees with the results of Grossweiner and Smith (1981) who recently showed that repair deficient strains of E. coli were more sensitive to 8-MOP and UV-A than wild type cells.

The data further show that the rec⁻ strains and the parental wild type (AB 1157) were equally sensitive to alpha-T. Had the mutants been more sensitive than AB 1157 then this would have been good evidence for alpha-T damage to DNA. This agrees with one other published study by MacRae et al. (1980a) who showed that alpha-T and UV-A do not increase the frequency of chromosomal aberations in cultured Syrian hamster cells. In another study, however, Kagan's group (Kagan et al., 1980) found the opposite result, that alpha-T did react with calf thymus DNA in vitro and Candida utilis DNA in vivo. This

finding may be explained by the high concentrations of alpha-T which were used (40 ug/ml as compared with 1 ug/ml used in these studies) along with the long irradiation times (2 to 4 hours). It can be argued that alpha-T may react with DNA given a sufficient concentration and irradiation time, however evidence for reaction of alpha-T with DNA was not found in this in vivo study using bactericidal concentrations.

BBT, although biosynthetically related to alpha-T, elicited a very different response from the E. coli mutants. All rec⁻ strains except JC 9239 (rec F) were more sensitive to BBT and UV-A than the wild type (AB 1157). This may indicate that the mechanism of action of BBT could involve DNA damage in cellular killing.

No inhibitory zones were formed in response to any of the photoactivated compounds in the dark. Intercalation of 8-MOP into DNA in the dark may cause the inhibition of DNA synthesis (Song and Tapley, 1979), but inhibitory zones were not found with the concentration of 8-MOP used in this study.

Aerobic-Anaerobic Studies

The photoinduced antibiotic activity of alpha-T was found to occur only in the presence of air or oxygen and was independent of the aerobic or anaerobic status of cells just prior to irradiation. Cultures of E. coli grown and irradiated aerobically in either air or He/O2 (4:1) in the presence of alpha-T showed a marked decrease in cell viability (Figure 18).

The treatment of anaerobically grown cells irradiated under He with UV-A and alpha-T caused no decrease in cell survival (Figure 19). Cells grown in He and transferred to an air atmosphere during irradiation were killed in the presence of alpha-T. There was a brief delay in the start of killing, however, which was probably related to the diffusion rate of oxygen in the suspension. In contrast, cells respiring aerobically were protected from the effects of alpha-T by the introduction of He prior to irradiation.

Similar results were obtained with Pseudomonas aeruginosa (Figure 20), although higher doses of alpha-T (2.5 ug/ml) were necessary to yield equivalent decreases in cell viability. Control conditions with alpha-T in the dark or UV-A irradiation of cells in the absence of alpha-T may have had a small effect on the survival of E. coli and P. aeruginosa. Jagger (1976) has demonstrated that exposure of E. coli to UV-A induces growth delay which may have occurred in these studies, however this response was not investigated.

The requirement of oxygen for phototoxicity of alpha-T is consistent with a Type II reaction mechanism (Krinsky, 1977; Smith, 1977). The sensitivity of E. coli and P. aeruginosa to treatment with alpha-T and UV-A was not found to be a function of the type of cellular respiration in different cultures as recently suggested (Arnason et al., 1981). Instead, cellular inactivation was only dependent on the availibility of oxygen during irradiation.

Wat et al. (1980) studied the effect of alpha-T and UV-A
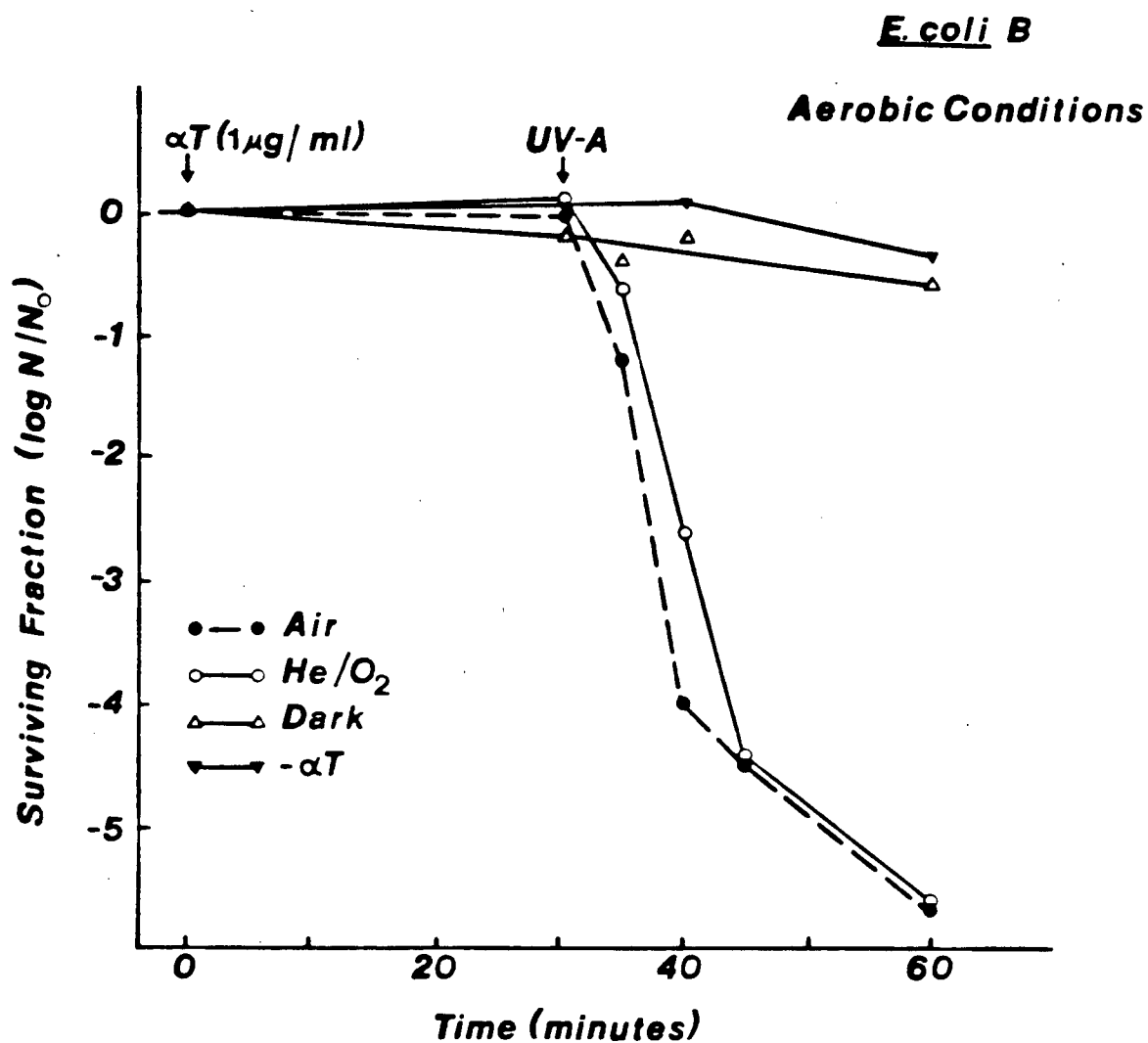
Figure 18. The survival of aerobically grown E. coli B irradiated with UV-A in the presence of alpha-T under aerobic conditions. Alpha-T was added to cultures at 0 minutes and irradiation was started at 30 minutes.
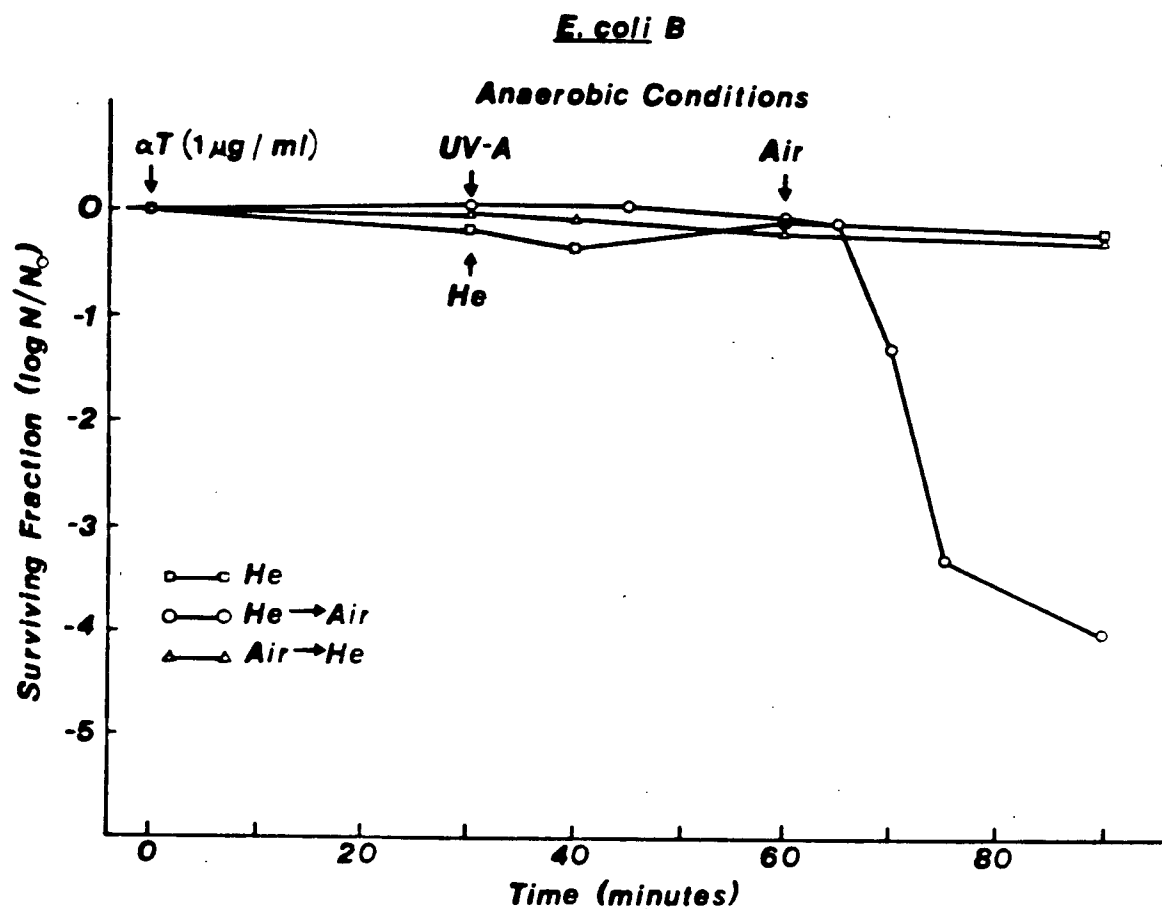
Figure 19. The survival of anaerobically grown E. coli B irradiated with UV-A in the presence of alpha-T under aerobic and anaerobic conditions. Alpha-T was added to cultures at 0 minutes and irradiation was started at 30 minutes. Cells initially in He (o————o) were transferred to air at 60 minutes. Cells initially in air (△————△) were switched to He at 30 minutes.
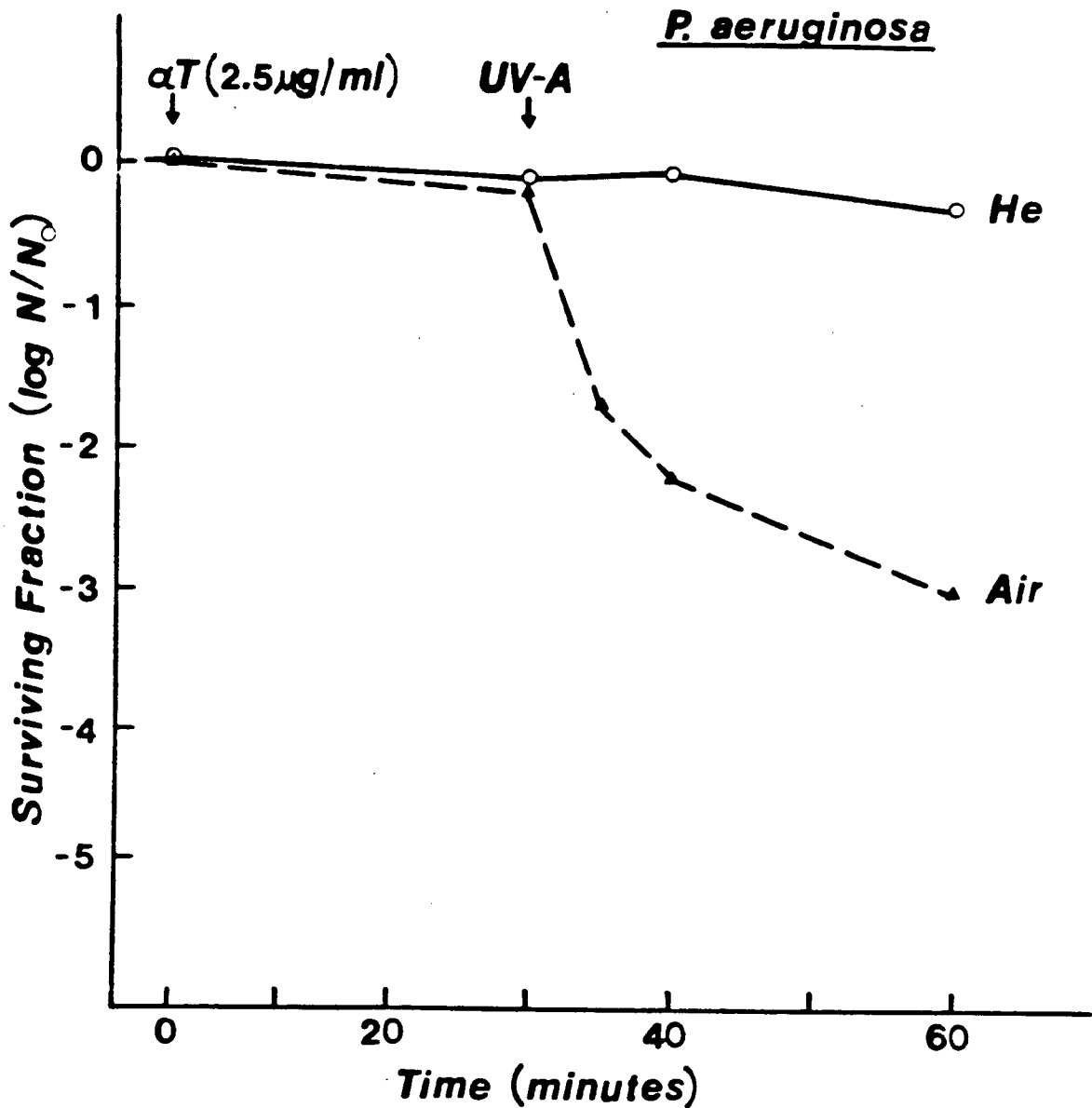
Figure 20. The survival of <u>Pseudomonas aeruginosa</u> in the presence of alpha-T and UV-A irradiation under aerobic and anaerobic conditions. Alpha-T was added to cultures at 0 minutes and irradiation was started at 30 minutes.

irradiation on human erythrocytes. Their results clearly showed that both hemolysis and RBC acetylcholinesterase inactivation were oxygen dependent. Arnason et al. (1981) also demonstrated that the survival of starved, stationary phase cells irradiated in the presence of alpha-T was oxygen dependent. Kagan et al. (1980), on the other hand, concluded that alpha-T was a non-photodynamic phototoxic compound or a Type I photosensitizer. This result cannot be explained in view of the findings presented here. The action of alpha-T was unequivocally dependent on oxygen under the conditions of the experiments reported.

Effect of Scavengers on Alpha-T Phototoxicity

The inactivation rate of E. coli cells exposed to alpha-T (1 ug/ml) and UV-A irradiation in the presence of 20 mM azide (Figure 21) was less than the killing rate of cells in the absence of azide (control). The most obvious difference involved the survival of azide treated cells. Control cultures showed more than two orders of magnitude greater inactivation following 10 minutes irradiation than cells in the presence of azide. Azide, an effective quencher of singlet oxygen in vitro (Hasty et al., 1972) and in vivo (Ito, 1978), was not found to affect cell viability during the 15 minutes dark period prior to UV-A exposure. Cellular protection indicates that singlet oxygen may be involved in the phototoxic mechanism of alpha-T (Ito, 1978). Arnason et al. (1981) also demonstrated protection of starved, stationary phase E. coli cells from alpha-T and UV-

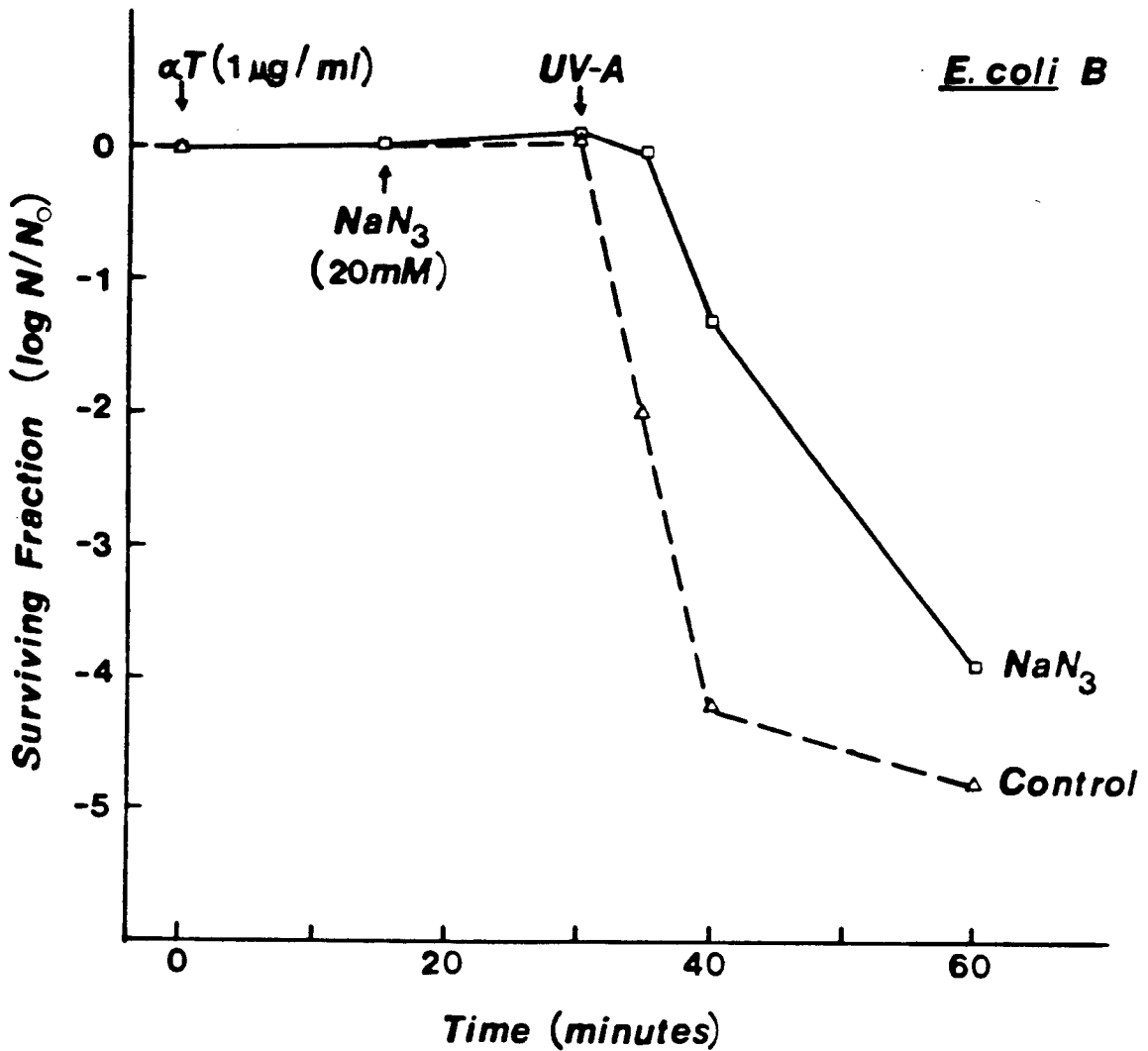Figure 21. The effect of 20 mM sodium azide on the survival of E. coli B in the presence of alpha-T and UV-A irradiation. Alpha-T was added to cultures at 0 minutes, azide at 15 minutes, and irradiation was started at 30 minutes.

A by 23 mM azide. Kagan et al. (1980), on the other hand, found no protection of Candida utilis by 0.153 mM azide and concluded that cellular killing by alpha-T did not involve singlet oxygen. They studied cellular growth rates rather than inactivation rates, which made it necessary for them to use a sub-inhibitory concentration of azide (0.153 mM). This concentration was approximately 100 times lower than that recommended by Ito (1978) to demonstrate in vivo protection from singlet oxygen. Kagan's group was unable to use higher concentrations of this cytotoxic compound because of its inhibitory effect on respiration (i.e. growth). Inactivation rates rather than growth rates were measured in the experiments reported here because of this effect.

BHT, a free radical scavenger (Foote, 1976), was also added to E. coli suspensions to determine its effect on the photosensitized reaction (Figure 22). The effect of BHT on cell survival was less dramatic than with azide, although a significant reduction in the rate of killing over the irradiation period was observed. This indicates the involvement of a Type I or radical component in the phototoxic mechanism of alpha-T and UV-A. The existence of a radical mechanism was originally indicated by the work of Arnason et al. (1981), who showed that E. coli, in the presence of alpha-T and UV-A was protected by the exogenous addition of superoxide dismutase. This alternate pathway was further indicated by the protection of BHT presented here. As with azide, no bactericidal effects were observed with 50 uM BHT and alpha-T in the dark period

Figure 22. The survival of E. coli B in the presence of alpha-T, UV-A irradiation and 50 uM BHT. Alpha-T was added to cultures at 0 minutes, BHT at 15 minutes, and irradiation was started at 30 minutes.

preceding irradiation.

The possibility of enhanced cellular protection by the simultaneous addition of 20 mM azide and 50 uM BHT was also investigated (Figure 23). Slight protection was observed, but differences in killing rates occurred only during the first five minutes of irradiation. Protection above that observed with either scavenger alone was not found. The simultaneous presence of the three compounds (azide, BHT and alpha-T) may be toxic to the cells, which could explain the lack of enhanced protection. This aspect, however, was not studied further.

The participation of singlet oxygen in the reaction mechanism of alpha-T has also been demonstrated in vitro. Bakker et al. (1979) showed that glucose-6-phosphate dehydrogenase inactivation by alpha-T was protected by various quenchers of singlet oxygen including azide, histidine, tryptophan, and methionine. They also reported enhanced enzymatic inactivation in D2O where the lifetime of singlet oxygen is increased ten fold (Merkel et al., 1972).

Temperature Studies

The effect of irradiation temperature on the photodynamic inactivation of E. coli by alpha-T was investigated (Figure 24). Cells grown and incubated with the sensitizer at 37°C were irradiated at various temperatures between 5°C and 42°C. In general, cellular inactivation showed a temperature effect. Survival during irradiation at 42°C showed single-hit kinetics and high rates of killing. As temperatures were lowered the lag
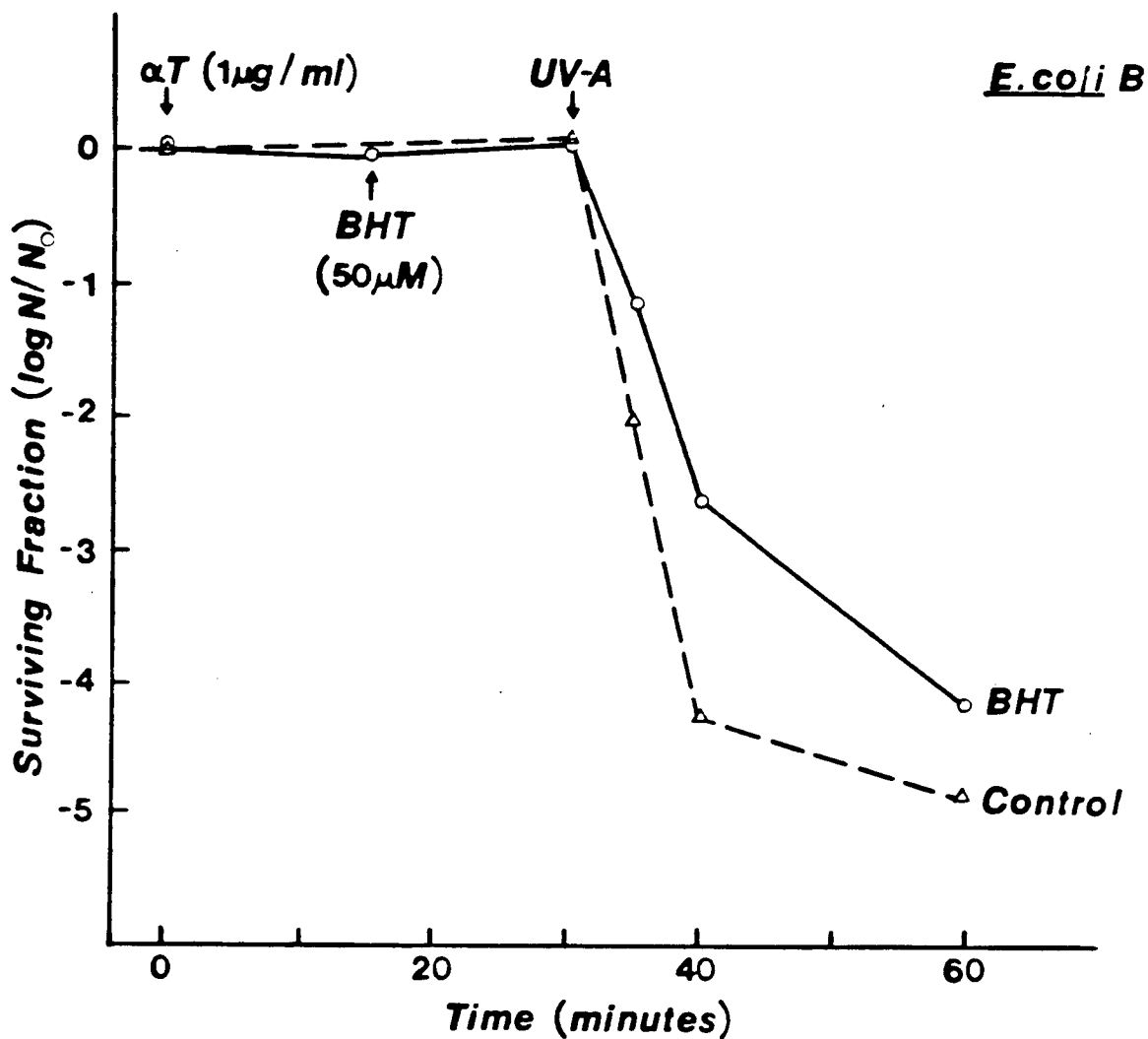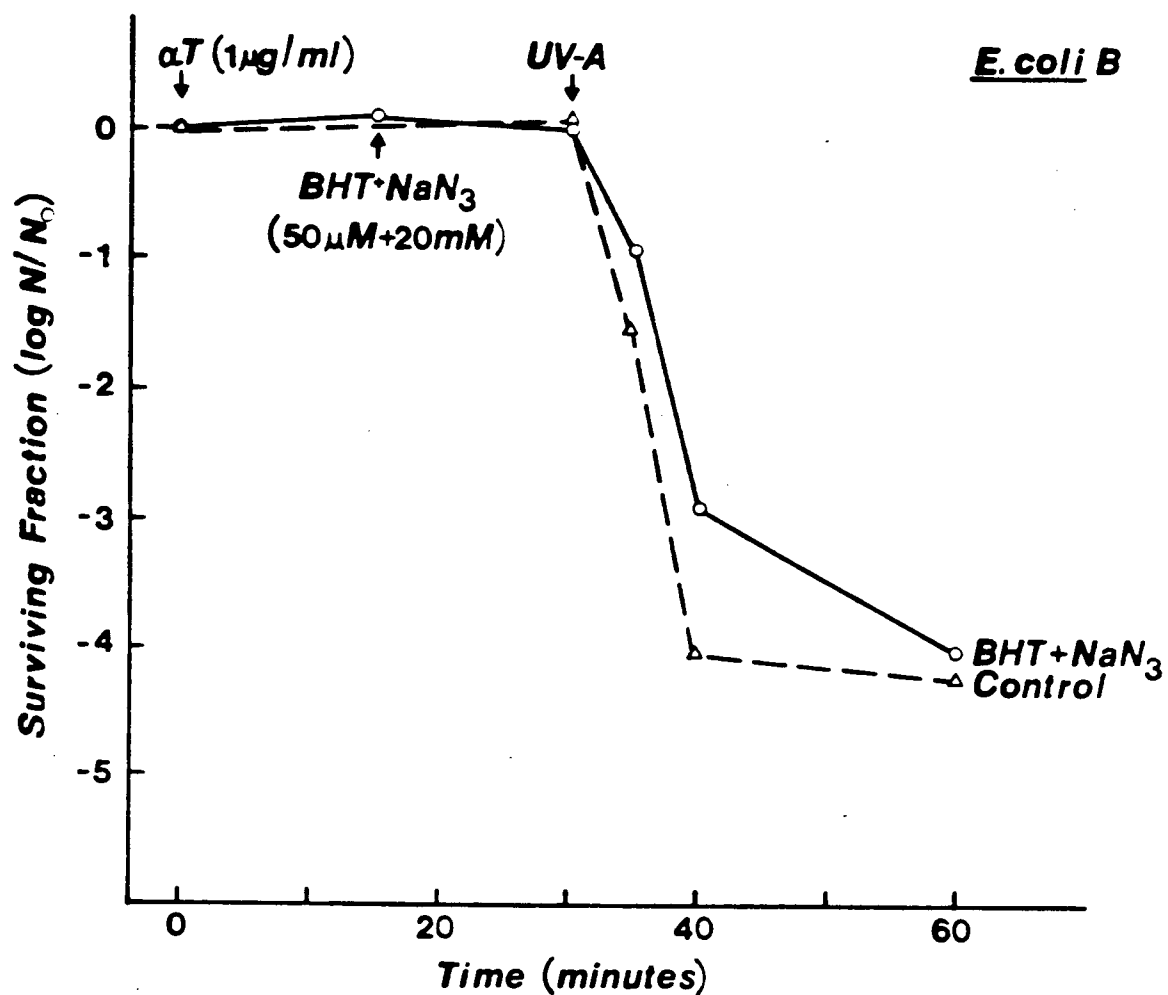
Figure 23. The survival of E. coli B in the presence of alpha-T, UV-A irradiation, 20 mM azide and 50 uM BHT. Alpha-T was added to cultures at 0 minutes, azide and BHT at 15 minutes and irradiation was started at 30 minutes.
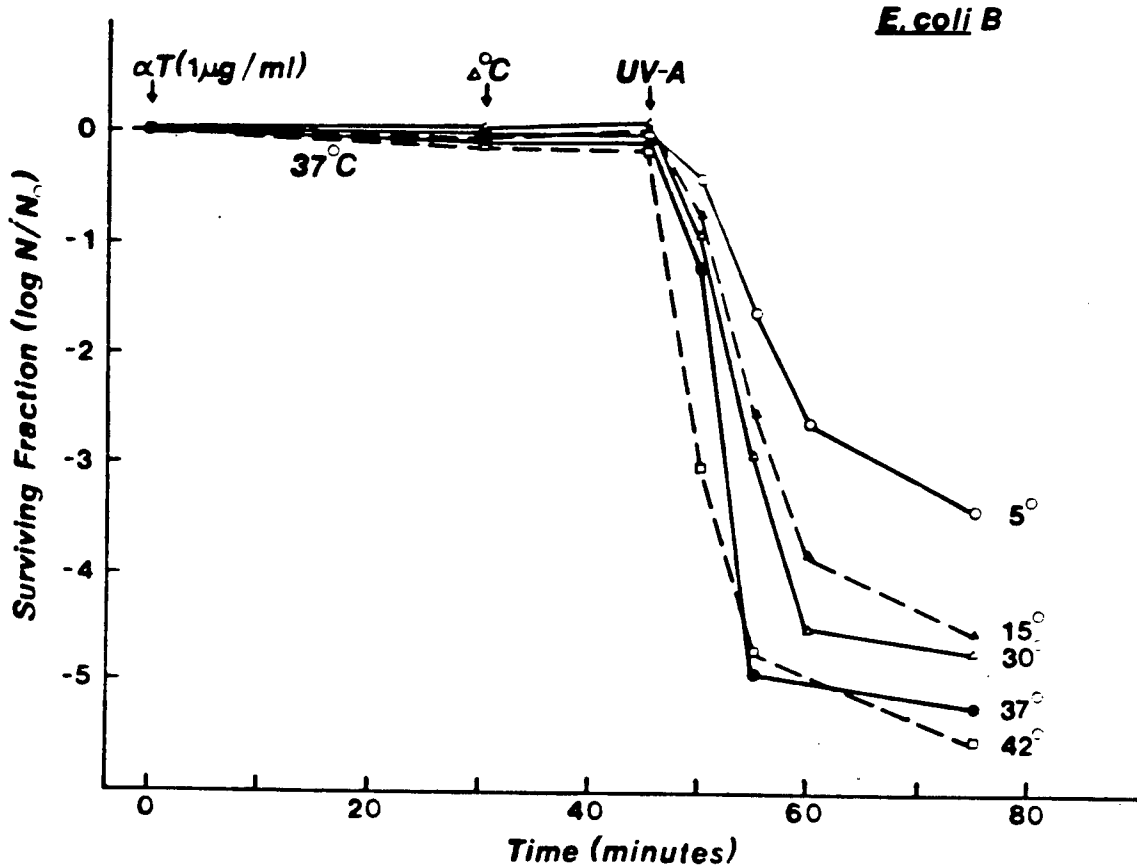
Figure 24. The survival of E. coli B in the presence of alpha-T and UV-A irradiation at different irradiation temperatures between 5 and 42 C. All cultures were incubated with alpha-T at 37 C for 30 minutes. The culture temperatures were then adjusted to the irradiation temperatures and allowed to equilibrate for 15 minutes prior to irradiation (45 minutes).

or shoulder regions of the graphs became more pronounced and were followed by slower killing rates. The most protection, or greatest resistance to inactivation, was at 5°C.

Recent studies by various workers have shown in vivo phototoxicity responses which are affected by temperature. Reduced inactivation rates of Saccharomyces cerevisiae were demonstrated at lower temperatures in response to photosensitization by both hematoporphyrin (HP) and toluidine blue (TB) (Stenstrom et al., 1980; Ito, 1981). The same trend was also seen in an E. coli-TB system (Wakayama et al., 1980) and in a human erythrocyte-protoporphyrin (PP) system (Dubbelman et al., 1980).

Figure 25 gives the Arrhenius relationship between rate of inactivation during the logarithmic phase (log of inactivation rate, k) and temperature (1/°K) from the data of Figure 24. A linear response was obtained for inactivation between 5°C and 37°C with an apparent activation energy of 6.63 Kcal/mole. The rate of cellular killing at 42°C did not show the linear nature of the other rates, which suggests the possibility of the involvement of additional factors participating in cellular inactivation. These factors may be related to effects of temperature on DNA, proteins, or membrane components.

It has been suggested that temperature effects on photodynamic killing result from changes in the fluidity of cytoplasmic membranes. Ito (1981) studied the effects of temperature on photodynamic killing of S. cerevisiae by TB. An Arrhenius plot of the data showed a slight discontinuity at 21-
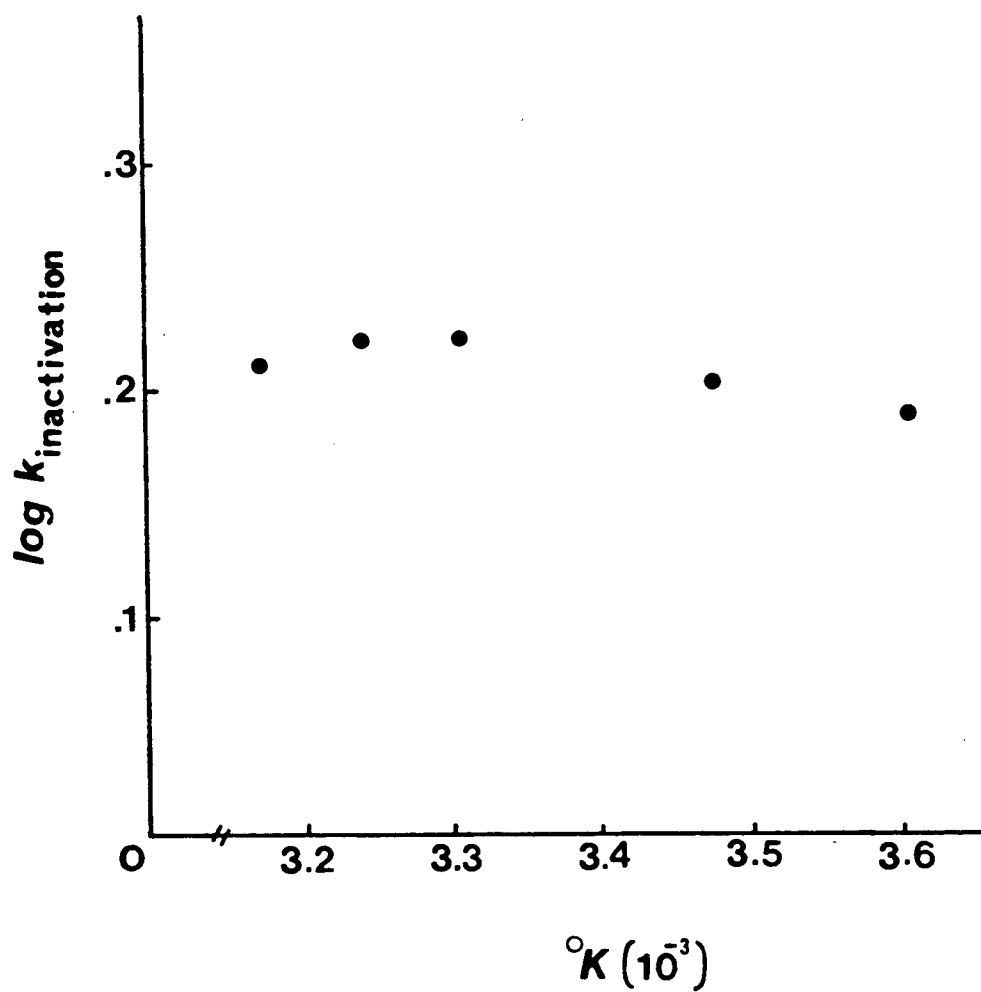
Figure 25. The Arrhenius relationship of the data presented in Figure 24 (Ea = 6.6 kcal/mole).

22°C; Ito (1981) suggested that this was a reflection of the cytoplasmic membrane phase transition. This is the temperature where the lipid matrix changes from a liquid crystalline state to a gel or ordered state. Cohn and Tseng (1977) reported a substantial increase in the photodynamic killing efficiency of S. cerevisiae by eosin Y at 30°C which was not seen at 27°C. They concluded that the marked dependence of inactivation rate on temperature might have been caused by a temperature dependent increase in membrane fluidity which either allowed singlet oxygen to migrate more readily or caused a temperature induced change in lipid-protein interactions or in membrane protein conformation which resulted in increased sensitivity. E. coli cytoplasmic membranes show a very broad phase transition beginning at approximately 10°C below the growth temperature (Nakayama et al., 1980). No critical point was found near this temperature as can be seen in Figure 25 and therefore no direct relationship between photodynamic killing of E. coli by alpha-T and membrane phase transition could be drawn in the present study.

A recent study also indicates the participation of membranes in the photodynamic action of alpha-T. MacRae et al. (1980b) demonstrated lesions in the cytoplasmic membranes of human erythrocytes irradiated in the presence of alpha-T. This indication of membrane damage might reflect involvement of either the lipid or protein components of membranes or an interaction between the two.

The effect of incubation temperature on cellular

inactivation by alpha-T was also studied. Cultures incubated with alpha-T at 5°C and 37°C for 30 minutes and subsequently irradiated at 5°C gave nearly identical survival curves (Figure 26). This result shows that incubation temperature is apparently not a critical factor for the photodynamic action of alpha-T on E. coli. Although E. coli cytoplasmic membranes at 5°C retain some fluid lipid domains (Jackson and Cronan, 1978), the significant fluidity differences between 5°C and 37°C preincubated membranes was not found to influence the subsequent activity of alpha-T. This means that the temperature effects observed in Figure 24 do not result from an inhibition of alpha-T transport into the cell at the lower temperature (otherwise the 37°C preincubated cells in Figure 26 would more closely resemble the 37°C treated cells in Figure 24). This is suggestive evidence that alpha-T need not necessarily enter the cell to cause cell death subsequent to irradiation.

Effects of Alpha-T on Cellular Proteins

Figure 27 shows an electropherogram of the soluble (A-C) and membrane associated (E-H) proteins from E. coli B before and after treatment with 1 ug/ml alpha-T and UV-A. The soluble proteins before addition (A) and after 30 minutes dark incubation with alpha-T (B) showed no obvious differences. Proteins from irradiated cells (C), however, showed reduced definition which was accompanied with general blurring of protein bands. Membrane-associated proteins showed the results of photosensitization even more dramatically. No visible
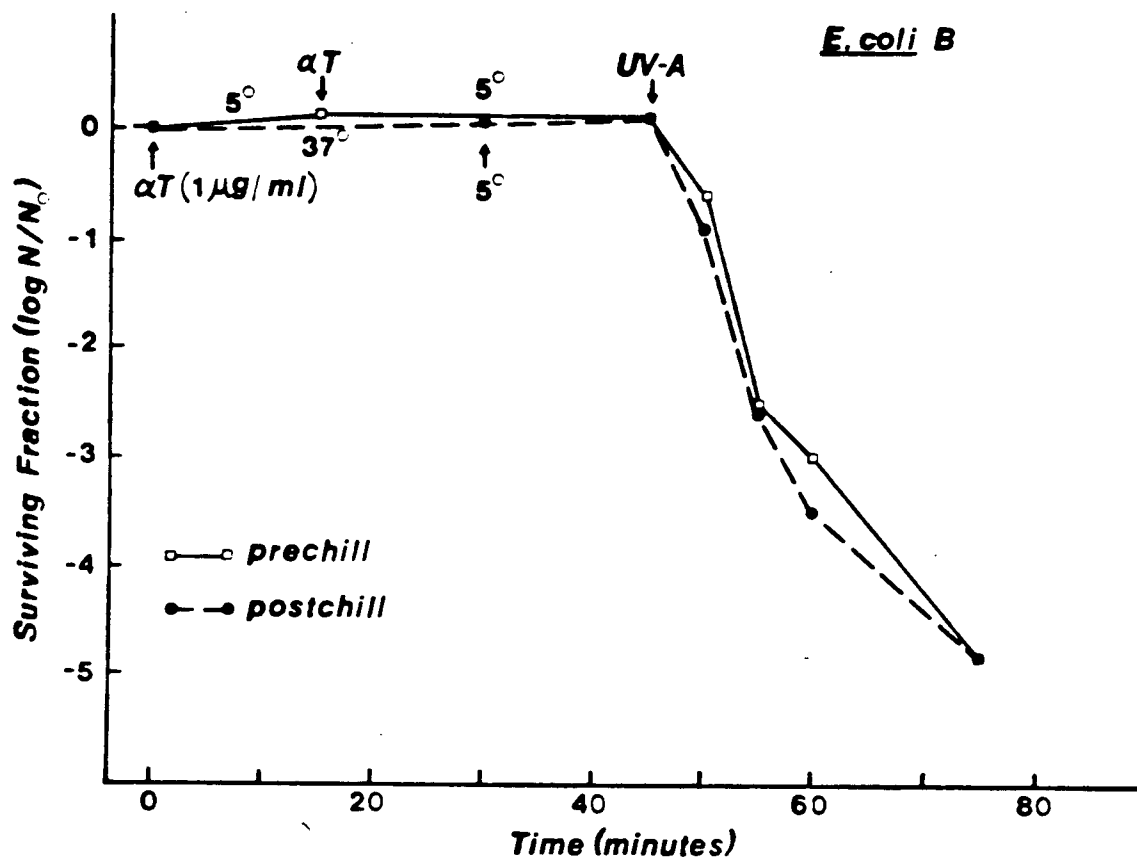
Figure 26. The effect of incubation temperature on the survival of E. coli B irradiated in the presence of alpha-T at 5 C. Prechill treated cultures (□———□) were first cooled to 5 C prior to adding alpha-T and then incubated at 5 C for 30 minutes while postchill treated cultures (●———●) were incubated with alpha-T for 30 minutes and then cooled to 5 C for 15 minutes. Irradiation of all cultures was started at 45 minutes.
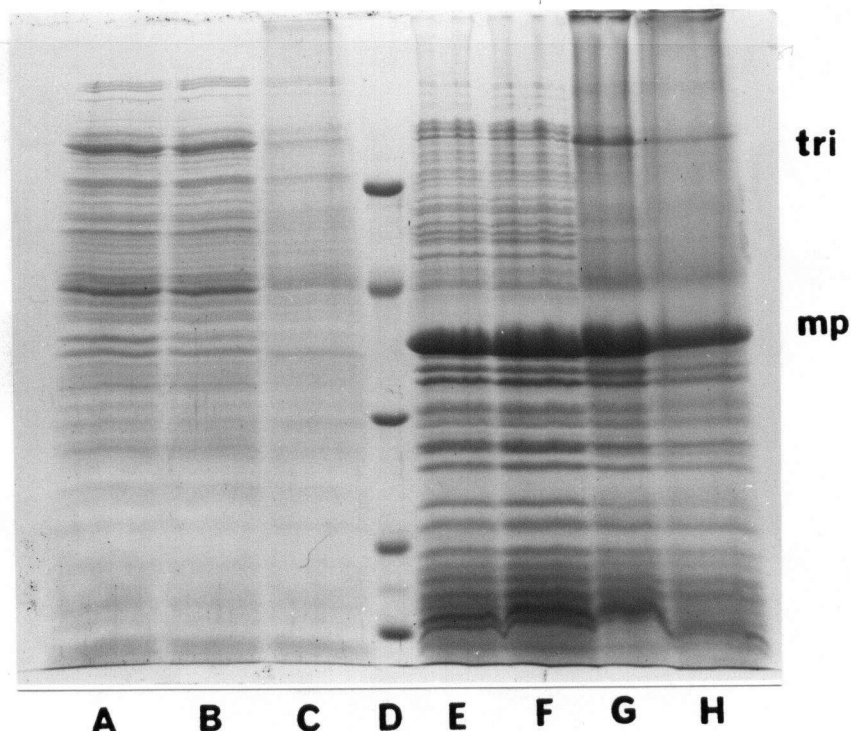
Figure 27. SDS-gel electropherogram of the soluble or cytoplasmic proteins (A-C) and the membrane-associated proteins (E-H) of E. coli B. Standards (D) included BSA (67,000), ovalbumin (45,000), pepsin (34,700), carbonic anhydrase (30,000), trypsinogen (25,000), trypsin inhibitor (20,000) and lysozyme (14,300). A) soluble proteins before treatment; B) after 30 minutes dark incubation with 1ug/ml alpha-T; C) after 30 minutes UV-A; E) membrane-associated proteins before treatment; F) after 30 minutes dark incubation with 1ug/ml; G) after 15 minutes UV-A and H) after 30 minutes UV-A. mp- 'matrix protein'; tri- trimer of 'matrix protein'. Note the aggregation of protein at the tops of columns G and H and the non-specific blurring of proteins in C, G and H above 'matrix protein'.

differences in proteins before addition and following 30 minutes dark incubation with 1 ug/ml alpha-T were evident in columns E and F. The proteins in columns G and H, however, show the effects following 15 and 30 minutes irradiation, respectively. The higher molecular weight proteins were the most affected. General blurring of bands was found in addition to protein aggregation at the top of gels.

Photodynamic modification of membrane protein from human erythrocytes sensitized by protoporphyrin has also been reported (DeGoeij et al., 1975 and 1976; Girotti, 1976; Lamola and Doleiden, 1980). SDS gel electrophoresis of protein from treated cells revealed the blurring of protein bands and the appearance of protein aggregates at the top of gels (DeGoeij et al., 1976). This effect was postulated to result from crosslinking of membrane proteins following photooxidation of amino acid residues by singlet oxygen (Dubbelman et al., 1980), but might also result from protein degradation. The effect on erythrocytes closely matches the photodynamic effect of alpha-T on E. coli proteins. The crosslinking or degradation of membrane proteins appears nearly identical. The photosensitized effect on soluble protein ,however, is a noteworthy difference. The cytoplasmic or soluble proteins of E. coli also were found to be susceptible to photooxidation and subsequent crosslinking into aggregates. The soluble proteins (cytoplasmic enzymes) of red blood cells have been shown to respond variably to different photodynamic sensitizers. Alpha-T caused only slight effects on enzymatic activity prior to cell lysis while

methylene blue was responsible for considerable inactivation of cytoplasmic enzymes (Yamamoto et al., 1979). These differences probably reflect the differing abilities of these sensitizers to penetrate the cell membrane. Lysis of E. coli was not found to result from photosensitization by alpha-T despite substantial crosslinking of the proteins. Intact cells could still be observed microscopically following 30 minutes irradiation in the presence of alpha-T, although most cell movement had ceased. This suggests that the peptidoglycan layer or cell wall remained intact. Observations at the electron microscopic level were not made to determine whether alpha-T induced membrane lesions in outer membranes as observed in red blood cells.

CONCLUSION

The in vivo mechanism of action of alpha-T and BBT was studied. Alpha-T was found to require oxygen for the expression of photoinduced bactericidal action. Cellular killing of E. coli resembled multiple-hit kinetics and was not dependent on the prior respiratory status of cells before irradiation in the presence of alpha-T. This Type II sensitizer was shown to elicit bactericidal activity at different wavelengths between 320 nm and 400 nm which resembled the absorption of alpha-T in the same wavelength range. This suggested that alpha-T, rather than a photoproduct, was the sensitizer molecule. Protection of E. coli cells by sodium azide and BHT indicated the participation of both singlet oxygen and free radicals in the

inactivation of cells. Studies with recombination deficient mutants of E. coli did not suggest that damage to cellular DNA resulted from attack by the photosensitizer. Cellular proteins, however, were dramatically affected by UV-A irradiation in the presence of alpha-T. SDS gel electrophoresis revealed that both cytoplasmic and membrane proteins of E. coli might be crosslinked by the action of alpha-T in the presence of UV-A irradiation.

BBT was also studied but to a lesser extent. A different mechanism of action from alpha-T was indicated. Cellular killing of E. coli followed different inactivation kinetics than killing caused by alpha-T and studies with recombination deficient mutants suggested the possibility of damage to DNA in the mechanism of cellular inactivation.

APPENDIX I

The following is a listing of the spectral data obtained from the reference compounds isolated from T. patula or synthesized chemically. UV, IR, MS and fluorescence spectra for most compounds are given. UV absorption maxima are followed by published extinction coefficients in parentheses. Mass spectra showing relative intensities of m/e peaks are also given. Fluorescence data showing excitation maxima are followed by emission maxima (fluorescence) in parentheses (nm). Verification of the structures of VII, VIII, IX and X were based on comparison of spectral data with previously published results. The structure of alpha-T was also confirmed by chemical synthesis.

2,2':5',2"-terthienyl (alpha-T) MW 248; UV(nm) 350(22400), 252(9100); IR(1/cm) 830,800,690; MS (m/e≥10%) 69(18), 124(10), 127(19), 171(14), 203(18), 248(100), 249(18), 250(15); fluorescence(nm) 350(425).

5-(buten-1-ynyl)-2,2'-bithienyl (BBT) MW 216; UV(nm) 346(27800) 254(9750); IR(1/cm) 3070, 2200, 1600, 975, 920, 850; MS(m/e≥10%) 95(13), 171(18), 215(11), 216(100), 217(18); fluorescence(nm) 346(410).

5-(4-acetoxy-1-butenyl)-2,2'-bithienyl (BBT-OAc) MW 279; UV(nm) 333(21800), (325)(21500), 246(5540); MS (m/e≥10%) 43(47), 95(16), 108(14), 149(11), 171(14), 203(18), 216(100), 217(18), 218(22), 276(19); fluorescence(nm) 333(390).

5-(4-hydroxy-1-butenyl)-2,2'-bithienyl (BBT-OH) MW 234; UV(nm) 333(22700), (327)(22500), 240(6600); IR(1/cm) 3650,

3600, 3400, 3060, 1050, 845; fluorescence(nm) 333(387).

LITERATURE CITED

Arnason, T.; Swain, T.; Wat, C.-K.; Graham, E.A.; Partington, S.; Towers, G.H.N. and Lam, J. 1980. Mosquito larvicidal activity of polyacetylenes from species in the Asteraceae. Biochem. System. Ecol. In press.

Arnason, T.; Wat, C.-K.; Downum, K.; Yamamoto, E.; Graham, E.A. and Towers, G.H.N. 1980. Photosensitization of Escherichia coli and Saccharomyces cerevisiae by phenylheptatriyne from Bidens pilosa . Can. J. Microbiol. 26:698-705.

Arnason, T.; Stein, J.R.; Graham, E.A.; Wat, C.-K.; Towers, G.H.N. and Lam, J. 1981. Phototoxicity to selected marine and freshwater algae of polyacetylenes from species in the Asteraceae. Can. J. Bot. 59:54-58.

Arnason, T.; Chan, G.F.Q.; Graham, E.A.; Wat, C.-K.; Downum, K. and Towers, G.H.N. 1981. Oxygen requirement for UV mediated cytotoxicity of alpha-terthienyl to E. coli and S. cerevisiae. Photochem. Photobiol. 33:821-824.

Atkinson, R.E.; Curtis, R.F. and Phillips, G.T. 1965. Naturally-occurring thiophens. Bithienyl derivatives from Tagetes minuta L. J. Chem. Soc. 7109-7115.

Bachmann, B.J. and Low, K.B. 1980. Linkage map of Escherichia coli K-12. Edition 6. Microbiol. Rev. 44:1-56.

Bakker, J.; Gommers, F.J.; Nieuwenhuis, I. and Wynberg, H. 1979. Photoactivation of the nematicidal compound alpha-terthienyl from roots of marigolds (Tagetes species). A possible singlet oxygen role. J. Biol. Chem. 254:1841-1844.

Bohlmann, F. and Herbst, P. 1962. Uber die Inhaltsstoffe von Tagetes- Arten. Polyacetylenverbindungen, XLIII. Chem. Ber. 95:2945-2955.

Bohlmann, F.; Burkhardt, T. and Zdero, C. 1973. Naturally
    Occurring Acetylenes. London. Academic Press.

Bohlmann, F. 1973. Naturally occurring acetylenes. In:
    Phytochem. Vol. III. Pergamon Press. pp. 112-131.

Bohlmann, F. and Zdero, C. 1976. Uber inhaltsstoffe der gattung
    Dyssodia. Chem. Ber. 109:901.

Bohlmann, F. and Zdero, C. 1979. Uber dimere terpenketone aus
    Tagetes gracilis. Phytochem. 18:341-343.

Bohlmann, F.; Jakupovic, J.; Robinson, H. and King, R.M. 1980.
    A dithienylacetylene from Porophyllum ruderale. 19:2760.

Chan, G.F.Q.; Towers, G.H.N. and Mitchell, J.C. 1975.
    Ultraviolet- mediated antibiotic activity of thiophene
    compounds of the Tagetes. Phytochem. 14:2295-2296.

Chan, G.F.Q.; Prihoda, M.; Towers, G.H.N. and Mitchell, J.C.
    1977. Phototoxicity evoked by alpha-terthienyl. Contact
    Dermititis. 3:215-216.

Chan, G.F.Q.; Lee, M.M.; Glushka, J. and Towers, G.H.N. 1979.
    Photosensitizing thiophenes in Porophyllum, Tessaria and
    Tagetes. Phytochem. 18:1566.

Clayton, R. 1971. Light and Living Matter. Vol 2. McGraw-Hill,
    New York.

Cohn, G.E. and Tseng, H.Y. 1977. Photodynamic inactivation of
    yeast sensitized by Eosin Y. Photochem. Photobiol. 26:
    465-474.

Curtis, R.F. and Phillips, G.T. 1962. Thin-layer chromatography
    of thiophene derivatives. J. Chromatog. 9:366-368.

Daniels, F. 1965. A simple microbiological method for
    demonstrating phototoxic compounds. J. Inves. Der. 44:
    259-263.

DeGoeif, A.F.P.M.; ver Vergaert, P.H.J.T.H. and van Steveninck,
     J. 1975. Photodynamic effects of protoporphyrin on the
     architecture of erythrocyte membranes in protoporphyrin
     and in normal red blood cells. Clin. Chim. Acta. 62:287-
     292.

DeGoeif, A.F.P.M.; van Straalen, R.J.C. and van Steveninck, J.
     1976. Photodynamic modification of proteins in human red
     blood cell membranes, induced by protoporphyrin. Clin.
     Chim. Acta. 71:485-494.

Dubbelman, T.M.A.R.; Haasnoot, C. and van Steveninck, J. 1980.
     Temperature dependence of photodynamic red cell membrane
     damage. Biochem. Biophys. Acta. 601:220-227.

Elkind, M. and Whitmore, G. 1967. In: The Radiobiology of
     Cultured Mammalian Cells. pp. 237-308. Gordon and Beach.
     New York.

Foote, C.S. 1976. Photosensitized oxidation and singlet oxygen:
     Consequences in biological systems. In: Free Radicals in
     Biology. Vol. II. Ed. W.A. Pryor. Academic Press, New
     York, pp. 85-133.

Girotti, A.W. 1976. Photodynamic action of protoporphyrin IX on
     human erythrocytes: cross-linking of membrane proteins.
     Biochem. Biophys. Res. Commun. 72:1367-1374.

Gommers, F.J. 1972. Increase of the nematicidal activity of
     alpha-terthienyl and related compounds by light.
     Nematologica 18:458-462.

Gommers, F.J. and Geerlings, J.W.G. 1973. Lethal effect of near
     ultraviolet light on Pratylenchus penetrans from roots of
     Tagetes. Nematologica 19:389-393.

Gommers, F.J.; Bakker, J. and Smits, L. 1980. Effects of
     singlet oxygen generated by the nematicidal compound
     alpha-terthienyl from Tagetes on the nematode Aphelenchus
     avenae. Nematologica 26:369-375.

Graham, K.; Graham, E.A. and Towers, G.H.N. 1980. Cercaricidal activity of phenylheptatriyne and alpha-terthienyl, naturally occurring compounds in species of Asteraceae (Compositae). Can. J. Zoo. 58:1955-1958.

Grossweiner, L.I. and Smith, K.C. 1981. Sensitivity of DNA repair-deficient strains of Escherichia coli K-12 to various furocoumarins and near-ultraviolet radiation. Photochem. Photobiol. 33:317-323.

Grover, N.B.; Margalit, A.; Zaritsky, A.; Ben-Hur, E. and Hansen, M.T. 1981. Sensitivity of exponentially growing populations of Escherichia coli to photo-induced psoralen-DNA interstrand crosslinks. Biophys. J. 33:93-106.

Gudas, L.J. and Pardee, A.B. 1976. DNA-synthesis inhibition and induction of protein X in Escherichia coli. J. Mol. Biol. 101:459-477.

Hancock, R.E.W. and Carey, A.M. 1979. Outer membrane of Pseudomonas aeruginosa: heat- and 2-mercaptoethanol-modifiable proteins. J. Bacteriol. 140:902-910.

Hasty, N.; Merkel, P.B.; Radlick, P.; and Kearns, D.R. 1972. Role of azide in singlet oxygen reactions - Reaction of azide with singlet oxygen. Tetra. Lett. 1:49-52.

Hindennach, I. and Henning, U. 1975. The major proteins of Escherichia coli outer cell envelope membrane. Preparative isolation of all major membrane proteins. Eur. J. Biochem. 59:207-213.

Ito, T. 1978. Cellular and subcellular mechanisms of photodynamic action: The singlet oxygen hypothesis as a driving force in recent research. Photochem. Photobiol. 28:493-508.

Ito, T. 1981. Does membrane attaching photodynamic action reflect the so-call phase transition? Photochem. Photobiol. 33:117-120.

Jackson, M.B. and Cronan, J.E. 1978. An estimate of the minimum amount of fluid lipid required for the growth of Escherichia coli Biochem. Biophys. Acta 512:472-479.

Jagger, J. 1976. Effects of near-ultraviolet radiation on microorganisms. Photochem. Photobiol. 23:451-454.

Kagan, J.; Gabriel, R. and Reed, S.A. 1980. Alpha-terthienyl, a non-photodynamic phototoxic compound. Photochem. Photobiol. 31:465-469.

Keil, D.J. 1975. Flora of Panama (Family 184. Compositae). VI. Tageteae. Annals Missouri Bot. Gar. 62:1220-1241.

Kooreman, H.J. and Wynberg, H. 1967. Chemistry of polythienyls. 3. Synthesis of terthienyls. Rec. Tr. Chim. 86:37-43.

Krinsky, N.I. 1977. Singlet oxygen in biological systems. TIBS Feb. 35-38.

Lamola, A.A. and Doleiden, F.H. 1980. Cross-linking of membrane proteins and protoporphyrin-sensitized photohemolysis. Photochem. Photobiol. 31:597-601.

Lugtenberg, J.; Meijers, J.; Peters, R.; van der Hoek, P. and van Alphen, L. 1975. Electrophoretic resolution of the 'major outer membrane protein' of Escherichia coli K-12 into four bands. FEBS Lett. 58:254-258.

MacRae, W.D.; Chan, G.F.Q.; Wat, C.-K.; Towers, G.H.N. and Lam, J. 1980a. Examination of naturally occurring polyacetylenes and alpha- terthienyl for their ability to induce cytogentic damage. Experientia. 36:1096-1097.

MacRae, W.D.; Irwin, D.A.J.; Bisalputra, T. and Towers, G.H.N. 1980. Membrane lesions in human erythrocytes induced by the naturally occurring compounds alpha-terthienyl and phenylheptatriyne. Photobiochem. Photophysiol. 1:309-318.

Merkel, P.B.; Nilsson, R. and Kearns, D.R. 1972. Deuterium effects on singlet oxygen lifetimes in solutions- New test of singlet oxygen reactions. J. Am. Chem. Soc. 94: 1030-1031.

de Mucciarelli, P.S.H.L. and Montes, A.L. 1970. La esencia de
    Tagetes minuta L. Annales de la Sociedan Cientifica
    Argentina. 190:145-166.

Musajo, L. and Rodighiero, G. 1972. Mode of photosensitizing
    action of furocoumarins. Photophysiol. 7:115-147.

Nakayama, H.; Mitsui, T.; Nishihara, M. and Kito, M. 1980.
    Relation between growth temperature of E. coli and phase
    transition temperature of its cytoplasmic and outer
    membranes. Biochem. Biophys. Acta. 601:1-10.

Oginsky, E.L.; Green, G.S.; Griffith, D.G. and Fowlks, W.L.
    1959. Lethal photosensitization of bacteria with 8-
    methoxypsoralen to long wave length ultraviolet
    radiation. J. Bacteriol. 78:821-833.

Okoth, J. 1973. Tagetes minuta L., as a repellent and
    insecticide against adult mosquitoes. East African Med.
    J. 50:317-322.

Onishi, T.; Shimada, K. and Takagi, Y. 1973. Effects of
    bleomycin on E. coli strains with various sensitivities
    to radiations. Biochem. Biophys. Acta. 312:248-258.

Patrick, T.B. and Honegger, J.L. 1974. Synthesis of 5-ethynyl-
    2,2'- bithienyl and related compounds. J. Org. Chem. 39:
    3791-3793.

Phillips, D.W. and Towers, G.H.N. 1981. Reversed-phase high-
    performance liquid chromatography of red algal
    bromophenols. 206:573-580.

Picman, A.K.; Ranieri, R.L.; Towers, G.H.N. and Lam, J. 1980.
    Visualization reagents for sesquiterpene lactones and
    polyacetylenes on thin-layer chromatograms. 189:187-198.

Rawls, H.R. and Van Santen, P.J. 1970. A possible role for
    singlet oxygen in initiation of fatty-acid autooxidation.
    J. Am. Oil. Chem. Soc. 47:121-125.

Rodriguez, E. and Mabry, T.J. 1977. Tageteae-chemical review. In: The Biology and Chemistry of the Compositae. Academic Press. New York. pp. 785-797.

Sandermann, H. and Strominger, J.L. 1972. Biosynthesis of peptidoglycan of bacterial cell wall. 27. Purification and properties of C-55-isoprenoid alcohol phosphokinase from Staphylococcus aureus. J. Biol. Chem. 247:5123-5131.

Santamaria, L. and Prino, G. 1972. List of the photodynamic substances. In: Research Progress in Organic, Biological and Medicinal Chemistry. Vol. III. Part I. Eds. Gallo, U. and Santamaria, L. North Holland Publishing Co., Amsterdam. pp.

Sease, J.W. and Zechmeister, L. 1947. Chromatographic and spectral characteristics of some polythienyls. J. Amer. Chem. Soc. 69:270-273.

Selva, A.; Arnone, A.; Mondelli, R.; Sprio, V.; Ceraulo, L.; Petruso, S.; Plescia, S. and Lamartina, L. 1978. Cardopatine and isocardopatine, two novel cyclobutane substances from Cardopatium corymbosum. Phytochem. 17: 2097-2100.

Smith, K.C. and Hanawalt, P.C. 1969. Molecular Photobiology. Academic Press, New York.

Smith, K.C. 1977. Ultraviolet effect on molecules and cells. In: The Science of Photobiology. Ed. Smith, K.C. Plenum Press. pp. 113-142.

Smith, B.N. and Turner, B.L. 1975. Distribution of Kranz syndrome among Asteraceae. Amer. J. Bot. 62:541-545.

Song, P.-S. and Tapley, J.R. 1979. Photochemistry and photobiology of psoralens. Photochem. Photobiol. 29:1177-1197.

Sorenson, N.A. 1977. Polyacetylenes and conservatism of chemical characters in the Compositae. In: The Biology and Chemistry of the Compositae. Vol.I. Academic Press. New York. pp. 385-409.

Spikes, J.D. 1977. Photosensitization. In: The Science of Photobiology. Ed. Smith, K.C. Plenum Press, New York, pp. 87-112.

Stenstrom, A.G.K.; Moan, J.; Brunborg and Eklund, T. 1980. Photodynamic inactivation of yeast cells sensitized by hematoporphyrin. Photochem. Photobiol. 32:349-352.

Strother, J.L. 1977. Tageteae-systematic review. In: The Biology and Chemistry of the Compositae. Vol. II. Academic Press. New York. pp. 769-782.

Suwa, K.; Kimura, T. and Schaap. A.P. 1978. Reaction of singlet oxygen with cholesterol in liposomal membranes. Effect of membrane fluidity on the photooxidation of cholesterol. Photochem. Photobiol. 28:469-473.

Suzuki, H.; Nagai, K.; Yamaki, H.; Tanaka, N. and Umezawa, H. 1968. Mechanism of action of bleomycin. Studies with growing cultures of bacterial and tumor cells. J. Antibiotics 21:379-386.

Teng, J.I. and Smith, L.L. 1973. Sterol metabolism. 24. Unlikely participation of singlet molecular-oxygen in several enzyme oxygenations. J. Am. Chem. Soc. 95:4060-4061.

Towers, G.H.N. and Wat, C.-K. 1978. Biological activities of polyacetylenes. Rev. Latinamer. Quim. 9:162-170.

Towers, G.H.N.; Arnason, T.; Wat, C.-K.; Graham, E.A.; Lam, J. and Mitchell, J.C. 1979. Phototoxic polyacetylenes and their thiophene derivatives (Effects on human skin). Contact Dermititis. 5:140-144.

Towers, G.H.N. 1980. Photosensitizers from plants and their photodynamic action. In: Progress in Phytochemistry. Vol. 6. Pergamon Press, New York, pp. 183-202.

Turro, N.J. and Lamola, A.A. 1977. Photochemistry. In: The Science of Photobiology. Ed. Smith, K.C. pp. 63-86.

Uhlenbroek, J.H. and Bijloo, J.D. 1958. Investigations on nematicides. I. Isolation and structure of a nematicidal principle occurring in Tagetes roots. Recueil. 77:1004-1009.

Uhlenbroek, J.H. and Bijloo, J.D. 1959. Investigations on nematicides. II. Structure of a second nematicidal principle isolated from Tagetes roots. Recueil. 78:382-390.

Uhlenbroek, J.H. and Bijloo, J.D. 1960. Investigations on nematicides. III. Polythienyls and related compounds. Recueil. 79:1181-1196.

de Villiers, D.J.J.; Garbers, C.F. and Laurie, R.N. 1971. Synthesis of tagetenones and their occurrence in oil of Tagetes minuta. Phytochem. 10:1359-1361.

Wagner, S.; Taylor, W.D.; Keith, A. and Snipes, W. 1980. Effects of acridine plus near ultraviolet light on Escherichia coli membranes and DNA in vivo. Photochem. Photobiol. 32:771-779.

Wakayama, Y.; Takagi, M. and Yano, K. 1980. Photosensitized inactivation of E. coli cells in toluidine blue-light system. Photochem. Photobiol. 32:601-605.

Wat, C.-K.; Biswas, R.K.; Graham, E.A.; Bohm, L.; Towers, G.H.N. and Waygood, E.R. 1977. Ultraviolet-mediated cytotoxic activity of phenylheptatriyne from Bidens pilosa L. J. Nat. Prod. 42:103-111.

Wat, C.-K.; MacRae, W.D.; Yamamoto, E.; Towers, G.H.N. and Lam, J. 1980. Phototoxic effects of naturally occurring polyacetylenes and alpha-terthienyl on human erythrocytes. Photochem. Photobiol. 32:167-172.

Wat, C.-K.; Prasad, S.K.; Graham, E.A.; Partington, S.; Arnason, T.; Towers, G.H.N. and Lam, J. Photosensitization of invertebrates by natural polyacetylenes. System. and Ecol. In press.

Webb, R.B.; Hass, B.S.; and Kubitschek, H.E. 1979. Photodynamic effects dyes on bacteria. 2. Genetic effects of broad-spectrum visible-light in the presence of acridine-dyes and methylene-blue in chemostat cultures of _Escherichia coli_. Mutat. Res. _59_:1-13.

Yamamoto, E.; Wat, C.-K.; MacRae, W.D. and Towers, G.H.N. 1979. Photoinactivation of human erythrocyte enzymes by alpha-terthienyl and phenylheptatriyne, naturally occurring compounds in the Asteraceae. FEBS Lett. _107_:134-136.

Yamamoto, K. and Hutchinson, F. 1979. Response to bleomycin of _Escherichia coli_ mutants deficient in DNA repair. J. Antibiotics. _32_:1181-1185.

Zechmeister, L. and Sease, J.W. 1947. A blue-fluorescing compound, terthienyl, isolated from marigolds. J. Amer. Chem. Soc. _69_:273-275.

Zimmer, K.G. 1961. Studies on Quantitative Radiation Biology. Hafner Publishing Co., New York, pp. 21-27.