THE BIOSYNTHESIS OF TERTHIENYL IN THE COMMON MARIGOLD

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ABSTRACT

THE BIOSYNTHESIS OF TERTHIENYL
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This investigation represents the first attempt to elucidate the biosynthetic pathway of the naturally occurring polythiophene, 2,2'; 5',2"-terthienyl. Marigold plants growing in nutrient solution were fed suspected precursors containing sulfur-35 or carbon-14. The radioisotopes studies were sodium sulfate-S-35, sodium hydrogen sulfide-S-35, L-methionine-S-35, DL-methionine-1-C-14, DL-methionine-2-C-14, succinic acid-2,3-C-14, DL-glutamic acid-2-C-14, and sodium acetate-1-C-14. Terthienyl was isolated from the roots at specific times from the initial feeding, and its radioactivity was determined.

Sulfur-35 was incorporated into terthienyl yielding dilution factors of specific activity ranging from 77-294 representing approximately 0.06% incorporation of the original radioactivity administered. Methionine-S-35 gave a dilution factor of 735, and no incorporation was observed from sodium hydrogen sulfide-S-35. No incorporation into terthienyl was detected from feeding DL-methionine-1-C-14, via stems, DL-glutamic acid-2-C-14, or succinic acid-2,3-C-14.

Dilution factors from other carbon-14 compounds were 1,430 from DL-methionine-2-C-14 and 10,200 to 35,100 from sodium acetate-1-C-14.

From the consideration of the dilution factors, it was ascertained that sulfur-35 was incorporated best when supplied as sodium sulfate and to a lesser extent from methionine-S-35. It was found that carbon-14 was incorporated only when supplied as methionine-2-C-14. The other carbon-14 compounds provided no incorporation to a significant extent.

Isolation of terthienyl after various time intervals of uptake of the radioisotope indicated that young plants of 8-10 weeks were most desirable for experimental work. It was suggested that terthienyl was actively metabolized and was not a "storage" product of plant metabolism. The "site of synthesis" of terthienyl was designated to be in the roots as determined from root vs stem feeding procedures. Sulfur-35 in the form of sodium sulfate and carbon-14 in the form of methionine were not incorporated into terthienyl when fed to the plant via stems, whereas incorporation was observed when fed via roots.

The lack of incorporation of carbon-14 in the form of "acetate" and of sulfur-35 in the form of hydrosulfide suggested that previous hypotheses of the biogenesis of terthienyl were not necessarily correct. From the experimental evidence obtained, a biosynthetic scheme involving homocysteine as a possible precursor was presented.

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By

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INTRODUCTION

The first naturally occurring polythiophene compound, 2,2';5',2"-terthienyl, was isolated by Zechmeister and Sease (1) from the blooms of the common marigold, Tagetes erecta L. Until that time the only other thiophene derivative known to be found in nature was a growth factor, biotin (2). The number of thiophene derivatives known to exist in nature has increased in the past eight years. In 1955 a metabolic product called "junipal" was isolated from a wood-destroying fungus, Daedalena juniperina, which infests the East African cedar, Juniperus procera Hochst (3). Sorensen and Sorensen (4) added a fourth compound to the list with the isolation of 2-phenyl-5-(alpha-propynyl)-thiophene from the essential oils of Coreopsis grandiflora, Hogg ex Sweet. Shortly after, Sorensen and Guddal (5) reported the occurrence and isolation of methyl cis-beta (5-propynyl-2-thienyl)acrylate in the roots of Chrysanthemum vulgare Bernh. Sorensen and his collaborators reported the isolation and identification of the trans isomer from the scentless mayweed, Matricaria indora (11). The nematicidal properties of the concentrate from the roots of African marigolds was investigated by Uhlenbroek and Bijloo. Two active ingredients were identified, terthienyl and a new polythiophene, 5-(3-buten-1-ynyl)-2,2'-dithienyl (6). In Sorensen's paper the

isolation of 5-methyl-5'-butadienyl-2,2'-dithienyl was reported by Mrs. Liaaen Jensen, in yet unpublished results. This supposedly is the first naturally occurring dithienyl to be discovered (11). With other workers Sorensen has identified another thiophene derivative isolated from the thistles, Berkheya macrocephala and Echinops sphaereocephalus. It was believed to have the structure of compound VIII, Table 1. Bohlmann, Bornowski, and Schonowsky isolated and identified the structures of three compound containing a single thiophene ring, two of which possessed an oxygen containing ring: XII, cis and trans isomers from Anthemus nobilis L. (also, the trans isomer from Artemisia vulgaris L.); XIII, cis and trans isomers from Santolina pinnata Viv; XIV, from Artemisia ludoviciana Nutt (15). The structures of the fourteen naturally occurring thiophene derivatives which have been reported are shown in Table 1. A recent summary of the naturally occurring thiophene compounds was published by Katritzky (12).

The present research was undertaken to obtain experimental evidence for the biosynthetic pathway of terthienyl. To understand the scope of the problem, it is best to first consider the scanty evidence from biogenetic and biosynthetic studies which have led to some speculations by a few investigators concerning the origin of the thiophene derivatives which are listed in Table 1.

TABLE 1.--Structures of Naturally Occurring Thiophene Compounds

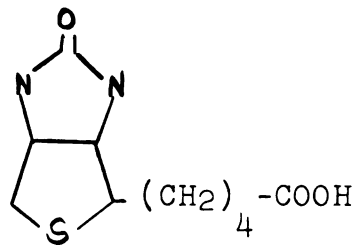
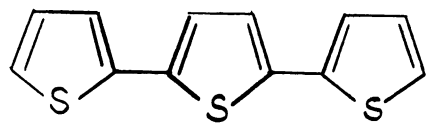
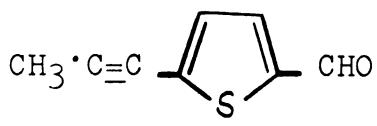
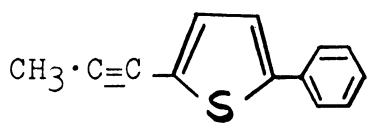
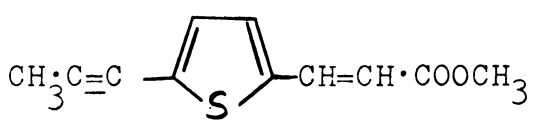
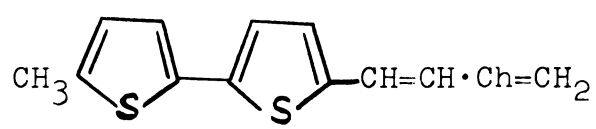
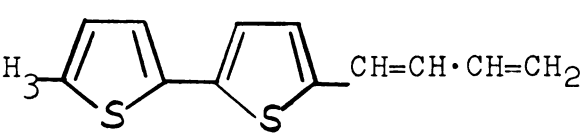
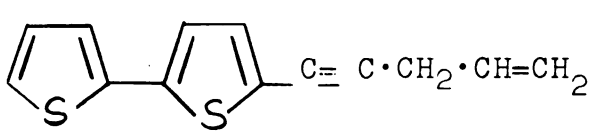
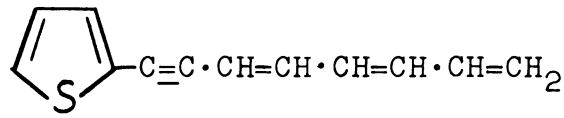
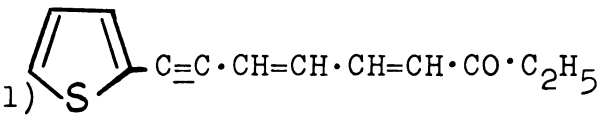
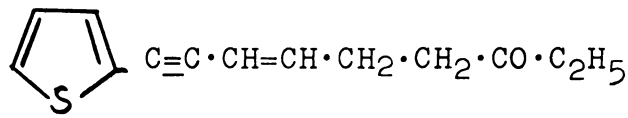
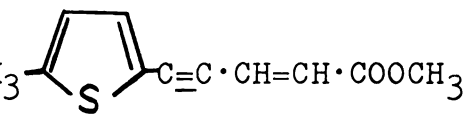
Compound	Structure
I Biotin	
II 2,2';5',2"-Terthienyl	
III "Junipal"	
IV 2-Phenyl-5-(alphapropynyl)-thiophene	
V Methyl-cis-beta (5-propynyl-2-thienyl) acrylate	
VI 5-Methyl-5'-butadienyl-2,2'-dithienyl	
VII 5-Methyl-5'-butadienyl-2,2'-dithienyl	
VIII 5-(4-Penten-1-ynyl)-2,2'-dithienyl	

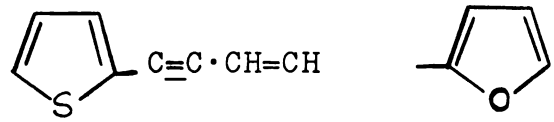
Table 1. continued:

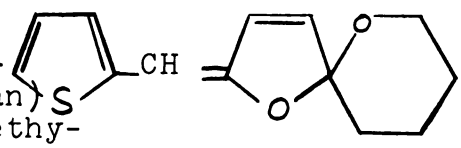
IX 2-(3,5,7-octatrien-1-ynyl)-thiophene 

X 2-(7-keto-3,5-nonadien-1-ynyl) thiophene 

XI 2-(7-keto-3-nonen-1-ynyl)-thiophene 

XII Methyl-5 (5-methyl-2-thienyl)-pent 4-yne-2-enylate 

XIII 1-Thienyl-4-furanyl-but-1-yne-3-ene 

XIV 1-Spiro(2'-oxo-tetra-hydropyran) 4-(2"-thienylmethylene)furan-2-ene 

Compounds II and IV-XIV have in common not only the thiophene nucleus and unsaturated linkages in the latter eleven, but all are found in plants of the Natural Order Compositae. This order includes such plants as the daisy, dandelion, aster, ragweed, and wormwood. These plants have small flowers or florets borne in dense involucrate beads which resemble single flowers (7). Of the living seed plants the Compositae comprise one tenth of the total number including herbs, shrubs, and trees embracing the most highly developed family in the vegetable kingdom. Depending upon a personal choice of definition, there exist today somewhere between 15,000 and 100,000 species. A simplified summary of the family of the Compositae is given in Table 2. This was taken from Sorensen (11) who based the data on the most recent botanical treatment of the family performed by Hoffman in 1889.

Of significance is the fact that all of the plants of the Compositae which produce the thiophene derivatives also have been the sources of another class of naturally occurring compounds, the polyacetylenes. Because of the proximity of polyacetylenes and unsaturated thiophene derivatives in the same order of plants, an intimate interrelationship of these two classes of compounds was suggested. Challenger speculated that the occurrence of polyacetylenes with low hydrogen content lends support to them as the origin of terthienyl (2). His suggested

TABLE 2.--A Simplified Survey of the Family of the Compositae

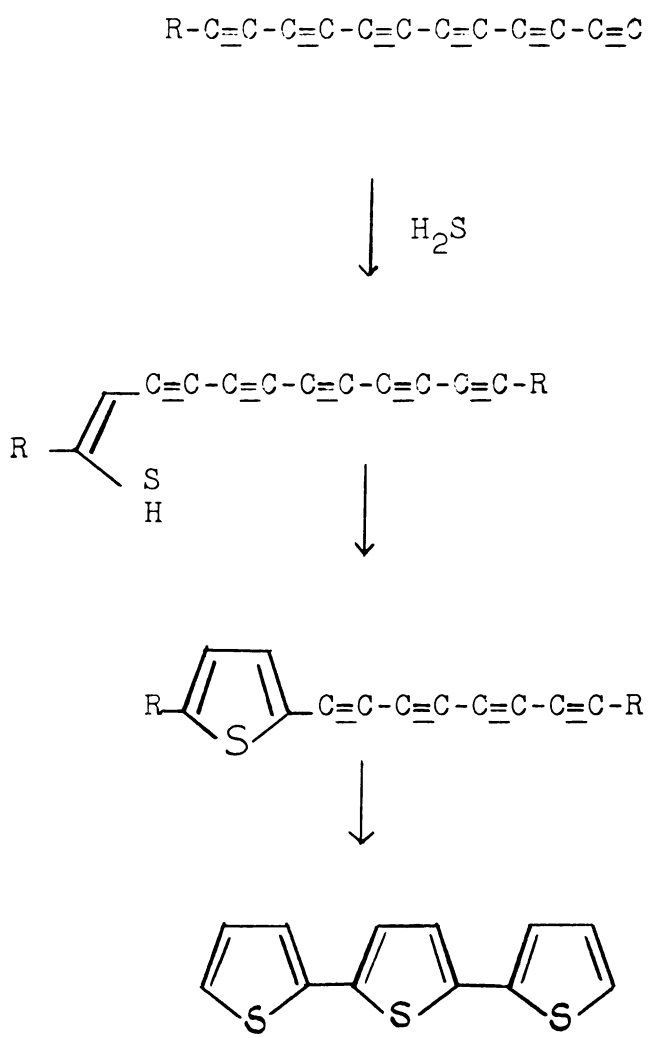
Botanical Tribus		Common Genus	Trivial Tribus Designation	Approximate No. of Genera
I	Vernonieae	Veronia	Ironweeds	41
II	Eupatorieae	Eupatorium	Thoroughworts	42
III	Astereae	Aster (Solidago) (Bellis)	Aster (Goldenrods) (Daisy)	99
IV	Inuleae	Gnaphalium Antennaria (Inula)	Everlastings	152
V	Heliantheae	Helianthus (Dahlia) (Cosmos) (Bidens) (Coreopsis)	Sunflowers	144
VI	Helenieae	Helenium (Tagetes)	Sneezeweeds (African marigolds)	55
VII	Anthemideae	Chrysanthemum (Artemisia) (Matricaria)	Chrysanthemum	49
VIII	Senecioneae	Senecio	Groundsel	51
IX	Calenduleae	Calendula	Marigolds	8
X	Arctotideae	Arctotis		11
XI	Cynareae	Centaurea	Thistles	34
XII	Mutisieae	Gerbera	Gerbera	57
XIII	Cichorieae	Taraxacum (Hieracium)	Dandelions	63
			Total	806

pathway is represented in Scheme 1. As a starting point the long carbon chain could have its origin a long chain paraffin or fatty acid. Hydrogen sulfide could possibly arise from cysteine. In Scheme 1 as well as the others to be mentioned it is assumed that other processes, very probably enzymic, such as oxidation, reduction, decarboxylation, etc., are involved. Scheme 1 would also be facilitated by the presence of olefinic and acetylenic linkages which could aid in ring closure. A specific example of such a pathway is the addition of hydrogen sulfide to 1-phenylheptatriyne to give compound IV (Table 1).

Sorensen and Sorensen (4) have considered the suggestion of Challenger and speculated that hexaacetylene would be required as a precursor to terthienyl; however, since polyacetylenes higher than triacetylene leads to carbonaceous materials, the existence of hexaacetylene even in dilute solution in the plant cells in the presence of possible stabilizers seemed highly unlikely. They suggested that some polyketo precursors were more probable, and these could serve both as the common source of polyacetylenes and naturally occurring thiophenes. This method of formation is represented in Scheme 2. The original compound consists of a 2,4-diketo grouping with dehydration and thiophene formation following in a stepwise manner. Scheme 2 represents a speculative pathway for the

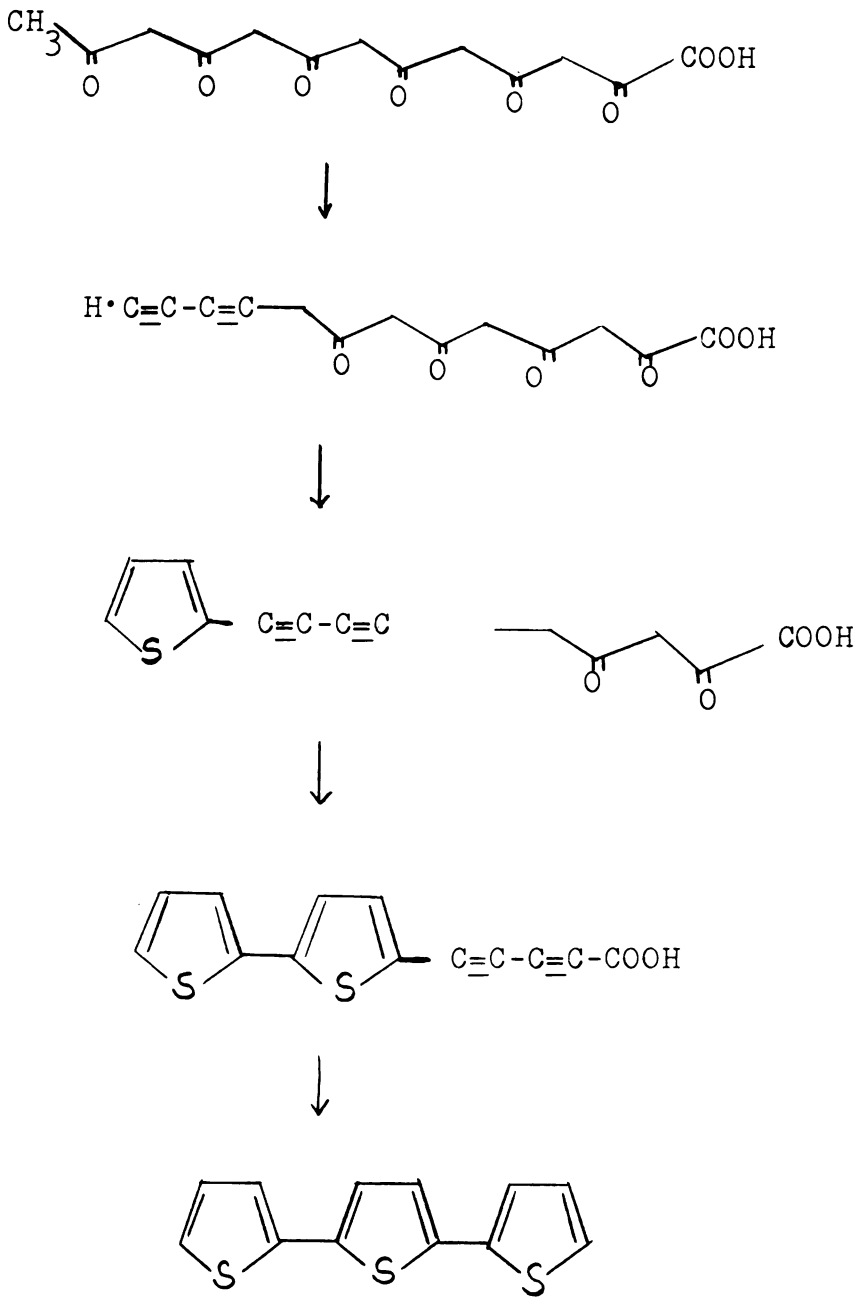
Scheme 1

Challenger's Biogenetic Pathway to Terthienyl

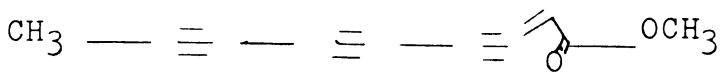


Scheme 2

Sorensen and Sorensen's Suggested Biogenetic Pathway to Terthienyl



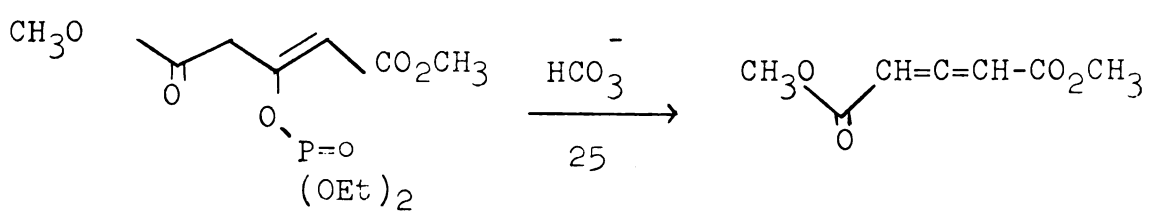
formation of terthienyl. A specific example of their scheme is the conversion to compound V (Table 1) from the addition of hydrogen sulfide to the dehydromatricaria ester, XV, which is found to occurr in a closely related species (4).



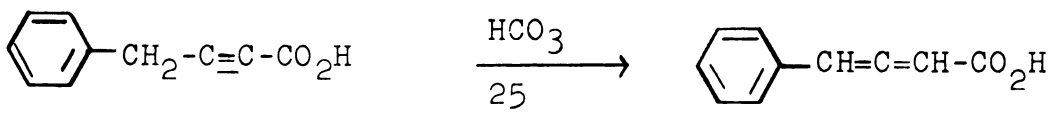
XV Dehydromatricaria ester

Recent evidence for the transformation of a methylene carbon to an acetylene linkage was obtained by Craig and Moyle (14). The model compound in their experiments was the methyl ester of acetone dicarboxylic acid. It was converted to its enol phosphate, and the conversion of the phosphate to an allene under mild conditions was achieved (equations 1 and 2). The results shown in equation 2 indicate that acetylene-allene conversion occurs with the allene being the stable product. Craig and Moyle suggested that in nature the triple bond

Equation 1



Equation 2.



is accompanied by intramolecular transphosphorylation giving an intermediate, the hemiketal phosphate. The dehydration could give two different enol phosphates leading to a stable diyne, found in nature, or a diethynylmethane, unknown in nature. Thus, Scheme 2 is supported by this biogenetic evidence. The formation of the triple bond as indicated by Craig and Moyle is represented by Scheme 3.

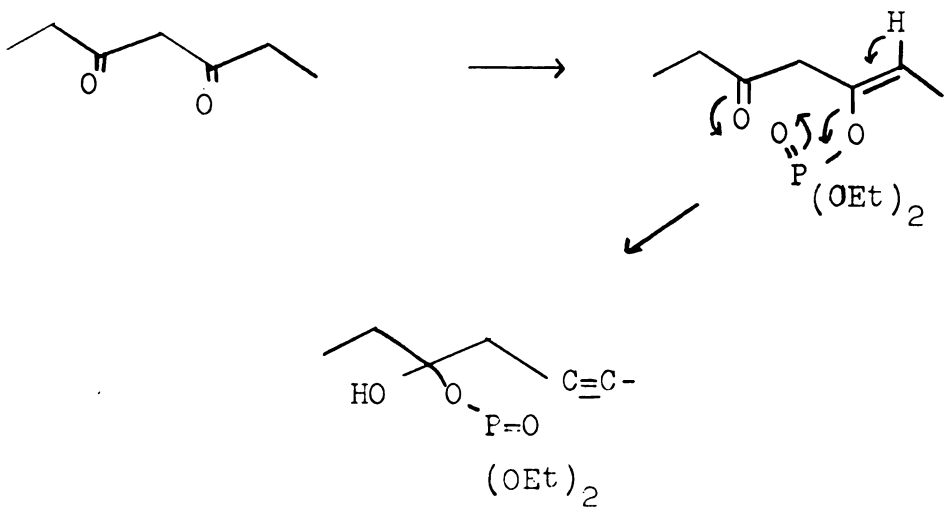
Sorensen and Sorensen have reported that their preliminary investigations revealed about a dozen new compounds from members of the Compositae as being thiophene derivatives and in part acetylenic thiophenes. Four of these new compounds have been tentatively identified by Sorensen and are listed in Table 1 as compounds VIII-XI (11). No other published results are available with respect to these new compounds.

In addition to the above discussion Sorensen implied that the occurrence of thiophene compounds in the Compositae may indicate thiophenes to be precursors to polyacetylenes rather than end products of polyacetylene metabolism.

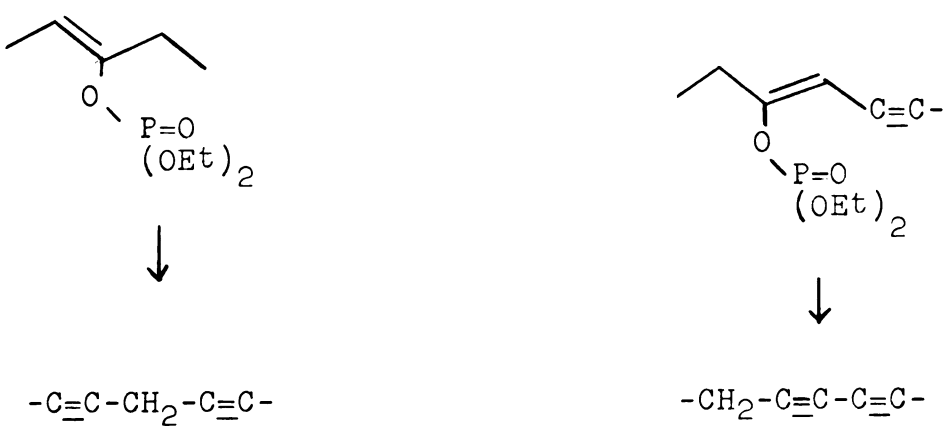
There is some indirect evidence concerning the biosynthesis of terthienyl. Uhlenbroek and Bijloo

Scheme 3

Biogenetic Formation of the Triple Bond



Hemiketal - $PO(OEt)_2$



$-C\equiv C-CH_2-C\equiv C-$

Unknown

$-CH_2-C\equiv C-C\equiv C-$

Known

$-CH=C=CH-C\equiv C-$

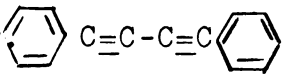
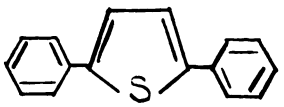
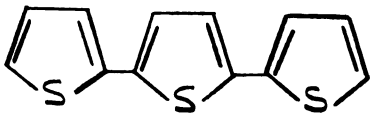
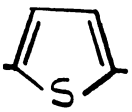
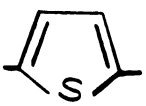
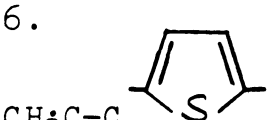
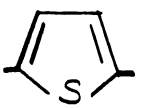
Known

identified not only terthienyl but compound VI (Table 1) as being produced by the roots of the African variety of the common marigold (Tagetes erecta). They suspected that terthienyl might arise from the addition of hydrogen sulfide to compound VI followed by dehydrogenative ring closure (6). Obviously, this theoretical reasoning only suggests that compound VI could possibly beaprecursor of terthienyl.

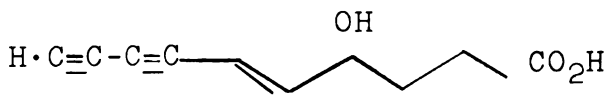
Terthienyl was claimed to have been obtained from the reaction of hydrogen sulfide and 1,4-dithienylbutadiene in a weakly alkaline medium at 20-60 degrees (13). Other 2,5-disubstituted thiophenes were also prepared under mild conditions. The reactions are shown in Table 3. The products obtained in reactions 3, 4, and 6 (Table 3) are those found in nature. This biogenetic relationship may be of some significance. If the assumption is made that polyacetylenes are precursors to terthienyl, the work of J. D. Bu'Lock and his co-workers represents a major contribution to the resolving of the biosynthetic problem.

Observing fungus cultures of Basidiomycetes B. 841, Bu'Lock and Leadbeater (8) concluded that glucose was metabolized via two different routes, and that polyacetylenes were further converted to breakdown products. Besides the reactions producing "normal" metabolites, glucose was also converted to polyacetylenes under appropriate conditions with as much as 7 per cent conversion by

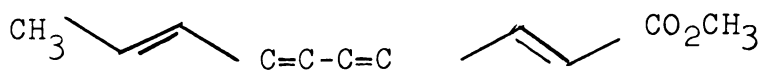
TABLE 3.--Preparation of 2,5-Disubstituted Thiophenes Under Biogenetic Conditions

m	Reactants	Products
1.	$CH_3-C\equiv C-C\equiv C-CH_3 + H_2S$	
2		
3.		
4.	$CH_3C\equiv C-C\equiv C-C\equiv C-CH=CH-CO_2H + H_2S$	$CH_3C\equiv C$  $CH=CH-CO_2H$
5.	$CH_3C\equiv C-C\equiv C-C\equiv C-Ch_2OH + H_2S$	$CH_3C\equiv C$  Ch_2OH
6.	 $CH_2OH + MnO_2$	$CH_3C\equiv C$  CHO

this "alternate" pathway. Bu'Lock and Gregory (9) fed acetate-1-C-14 to the fungus cultures and observed that 15-20 per cent of the original radioactivity was incorporated into one particular polyacetylene, nemotinic acid, XVI. The stepwise degradation of nemotinic acid revealed that the carboxyl carbon atom was labelled as well as alternate carbon atoms in the chain. Thus, the ten carbon acid was formed by head-to-tail linkage of five acetate units. Bu'Lock, Allport, and Turner further showed that acetate-1-C-14 was incorporated in a head-to-tail fashion into the matricaria ester, XVII, which is found both in Compositae and the fungus, Polyporus anthracophilus, a Basidiomycete which was actually used in the experiments.



XVI Nemotinic acid



XVII Matricaria ester

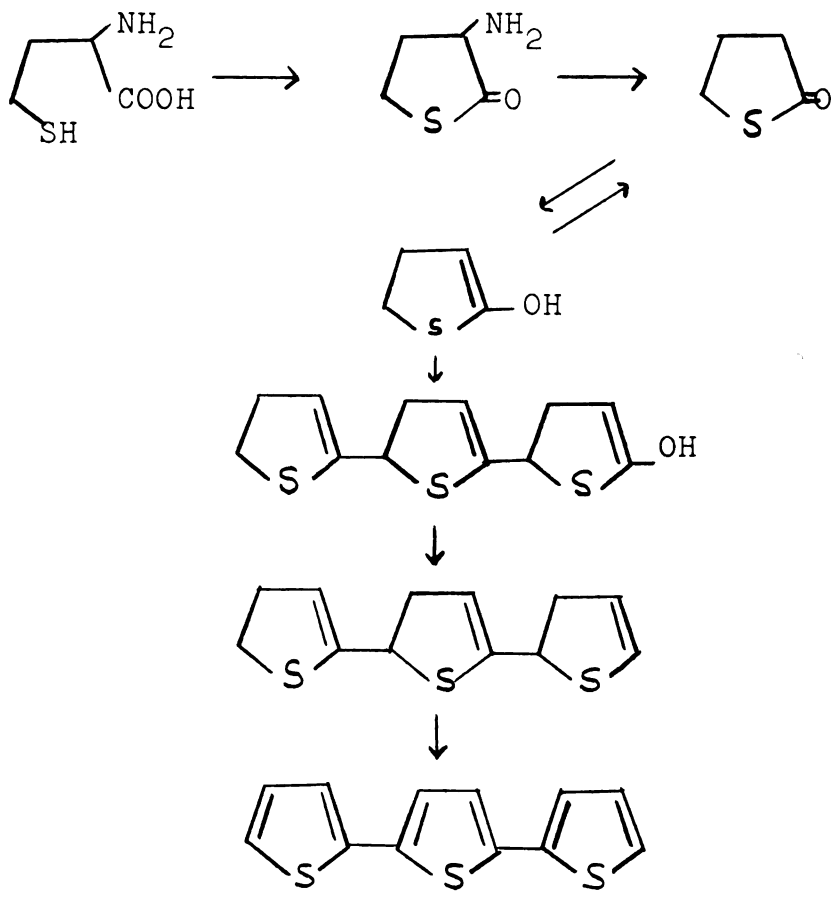
The research reported here was undertaken to attempt to elucidate the biosynthesis of terthienyl in the African variety of marigold. It was hoped that an organo sulfur compound could be discovered which was a precursor to terthienyl in the plant. The relationships of organo sulfur compounds in plants was considered with respect to the known metabolic conversions of sulfur compounds in higher animals.

Initially consideration was given to the possibility of a four carbon unit as a precursor, namely homocysteine. This compound contains not only four carbons but also a sulfur atom, the ingredients of a thiophene ring system. Such a pathway involving homocysteine is represented in Scheme 4. Because of the uncertainty of the reactions leading to terthienyl, the pathway cannot include details of such a conversion.

The existence of Schemes 1, 2, and 4 was also investigated. Compounds containing sulfur-35 or carbon-14 which were suspected as likely precursors to terthienyl were fed to marigold plants. The work reported here represents the first biosynthetic study of terthienyl or of any of the known naturally occurring thiophene derivatives other than biotin.

Scheme 4

Proposed Biogenetic Pathway to Terthienyl from Homocysteine



EXPERIMENTAL

Plants

The seeds of Tagetes erecta variety were purchased from W. Atlee Burpee Company, Fordhood Farms, Doylestown, Pennsylvania. The Tagetes were used in all experiments except two. A petite variety of marigolds with orange blooms was studied in Experiments 3 and 4. Generally, four plants were utilized, unless some expired during the course of the experiment. The actual weight of roots obtained varied from 0.3 to 4.0 g. with most yields in the range of 2.0 g. The plant age at the time of the administration of the radiosotope was 2 to 5 months. Each plant was transferred from the soil to the nutrient solution two to four weeks before dispensing the radioisotope. This period in the nutrient solution was necessary in order for a large enough root system to develop, since about 80% of the roots were cut away after the plant was removed from the soil. In later experiments (25-26) the plants were seeded and grown in sand prior to the transfer to the nutrient solution. The roots did not die after the transfer under these conditions, and therefore were not cut away. Each plant was placed in a separate Erlenmeyer flask containing enough nutrient solution to cover the roots.

Additional solution was required while the new root system was developing.

Preparation of the Nutrient Solution

Hoagland's #2 solution was utilized as the nutrient solution (18). Stock solutions of 1N concentration were prepared: 19.2 g. ammonium dihydrogen phosphate, 50.5 g. potassium nitrate, 41.0 g. calcium nitrate tetrahydrate, and 30.0 g. magnesium sulfate were dissolved in separate volumes of 500 ml. each of distilled water and stored until needed. For the preparation of 1 l. of nutrient solution, aliquots of 1, 6, 4, and 2 ml., respectively, were diluted to 1 l. with distilled water. The solution was then ready for use.

Administration of Radioisotopes

Method A: The plants were removed from the nutrient solution, and the roots were dried by blotting them with absorbent paper. The roots were further air-dried for an additional hour. At this time the solution of radioisotope (approximately 20 microcuries/ml.) was added with the aid of a pipette to each plant, so that each one received 5 microcuries distributed over the roots. Nutrient solution was again added to each plant in one hour following the initial dispensing of the radioisotope. The above procedure was based upon that of an earlier publication (21).

Method B: It was not required under these circumstances to remove the plants from the nutrient solution. A white cotton thread (Coats and Clark's) was inserted with the aid of a sewing needle through the stem of each plant about three inches above the roots. The two ends of the thread functioned as the wick. A five ml. beaker containing 0.25 ml. (approximately 5 microcuries) of radioactive solution was attached below the junction of the thread and stem. The solution was taken up in approximately two hours, and 0.5 ml. of distilled water was added to the beaker in order to "wash" as much radioisotope as possible into the plant. At the conclusion of the feeding time the thread was extracted twice with 100 ml. of distilled water. The amount of radioactivity extracted from the thread was subtracted from that amount originally dispensed. The difference was assumed to be the amount actually taken up by the plant.

Method C: An attempt to inject the radioactive solution directly into the stem with the aid of a needle and syringe was unsatisfactory. A syringe was situated in a vertical position with the end of the needle inserted into the stem, and a weight was applied to the plunger. None of the solution was taken up by the plant after forty-eight hours of application.

Harvest of the Roots

The plants were removed from the nutrient solution at the appropriate times. Only the roots were investigated,

and they were separated from the remainder of the plant, washed with 95% ethanol, and weighed after they were blotted dry with adsorbent paper. The roots were then disintegrated in 100 ml. of 95% ethanol for five minutes with a Waring blendor. A sample of "cold" terthienyl was added (if desired) to the ethanol mixture immediately before the blending step. The mixture was filtered through a soxhlet thimble, and extraction of the residue with the use of the filtrate was continued for twenty-four hours in the soxhlet apparatus. The filtrate was evaporated to dryness under reduced pressure, and the residue was redissolved in petroleum ether (30-60). The petroleum ether solution of crude terthienyl was chromatographed over activated alumina. The specific activity of terthienyl was determined. Further purification was carried out until a constant specific activity of terthienyl was attained.

Preparation of the Samples for Counting

Aliquots of 0.1 to 1.0 ml. of the desired solutions were evaporated to dryness in aluminum planchets using an infrared lamp as a source of heat. The length of counting time for each sample was determined by the accuracy desired and the activity of the sample. All counting was conducted in a windowless, gas-flow proportional counter: Baird Associatates-Atomic Instrument Company, Cambridge 39, Massachusettes. The solid samples of terthienyl and its acetyl

derivates were obtained from the evaporation of their ethanol solutions. The addition of several drops of water to the solution of sample in the planchet enabled a more uniform layer of the solid deposited. A thin uniform layer resulted with no self-absorption by the sample being observed. The lack of self-absorption is shown by the data represented in Figure 1 and Tables 4 and 5.

Self-absorption by Terthienyl-and 5-Acetylterthienyl-S-35

Terthienyl-S-35 from Experiment 23 was purified by alumina chromatography and diluted to 25 ml. (concentration = 18.6 mg./ml.). Aliquots of the solution were added to each of ten aluminum planchets. Enough ethanol was added to bring the total volume to 1.0 ml. The solvent was evaporated under an infrared lamp, and the activity was determined with a SD of 2%. The net activity (cpm) was plotted against the density of the sample. The same procedure was followed with the 5-acetyl derivative (concentration = 1.84 mg./ml.) also taken from Experiment 23. The results are summarized in Tables 4 and 5. The data obtained from each compound were plotted. A straight line was constructed for each set of points using the method of least squares. The results are shown graphically in Figure 1.

TABLE 4.--Self-absorption by 2,2'-5',2"-Terthienyl-S-35

Planchet Area = 7.07 $cm.^2$

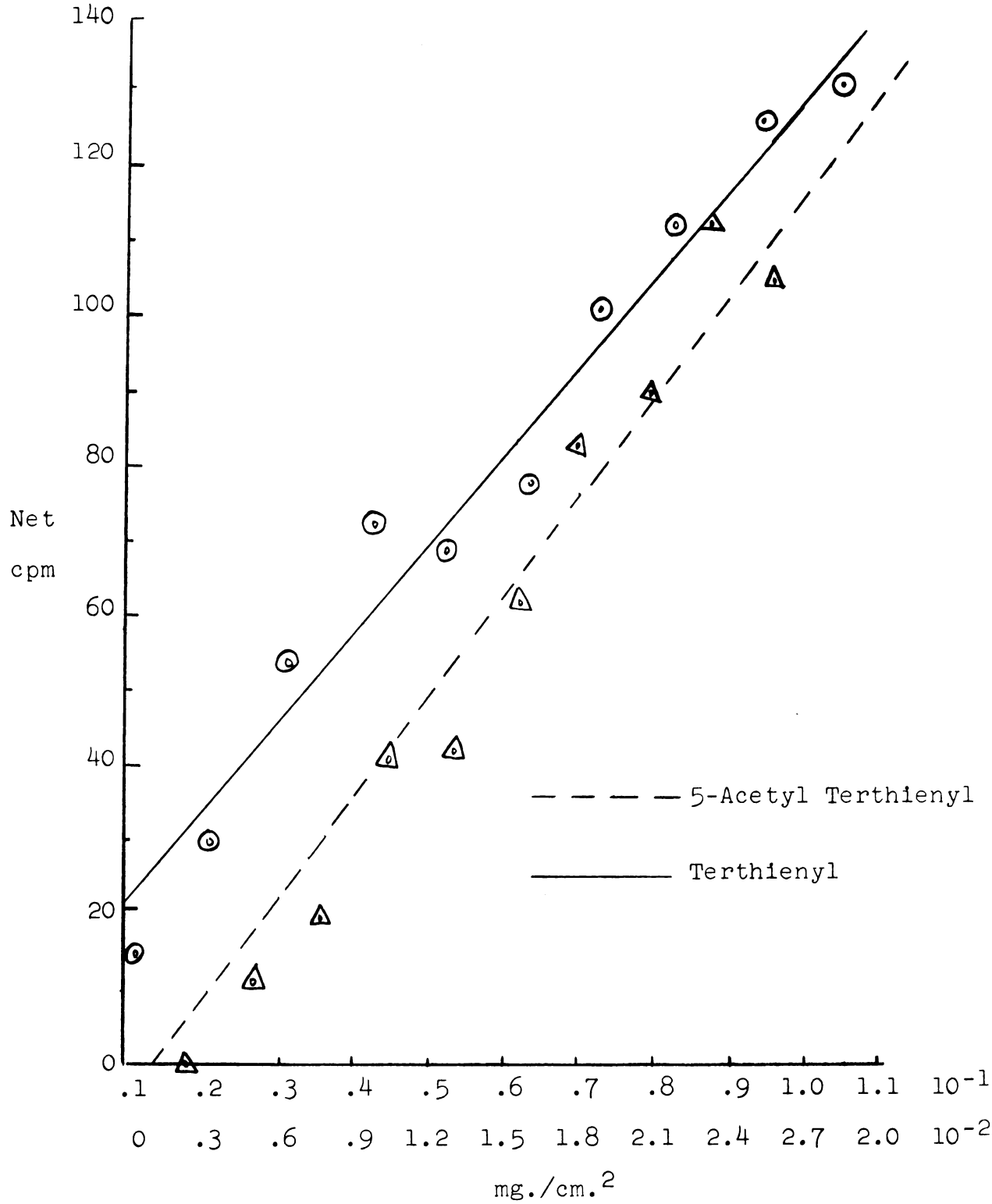
Sample	Terthienyl Solution-ml.	Ethanol Volume-ml.	Terthienyl Weight-mg.	Density-$mg./cm.^2$	Net cpm
1	0.1	0.9	0.0744	0.0105	15
2	0.2	0.8	0.149	0.0211	30
3	0.3	0.7	0.223	0.0316	54
4	0.4	0.6	0.297	0.0420	73
5	0.5	0.5	0.372	0.0525	69
6	0.6	0.4	0.446	0.0631	77
7	0.7	0.3	0.520	0.0735	101
8	0.8	0.2	0.595	0.0841	113
9	0.9	0.1	0.669	0.0946	126
10	1.0	0.0	0.744	0.105	130

TABLE 5.--Self-absorption by 5-Acetyl-2,2'-5',2"-Terthienyl-S-35

Planchet Area = 7.07 $cm.^2$

Sample	Terthienyl Solution-ml.	Ethanol Volume-ml.	Terthienyl Weight-mg.	Density-$mg./cm.^2$	Net cmp
1	0.1	0.9	0.0184	0.00260	None
2	0.2	0.8	0.0368	0.00520	7
3	0.3	0.7	0.0552	0.00781	20
4	0.4	0.6	0.0736	0.0104	41
5	0.5	0.5	0.0920	0.0130	42
6	0.6	0.4	0.110	0.0156	63
7	0.7	0.3	0.128	0.0181	82
8	0.8	0.2	0.147	0.0208	90
9	0.9	0.1	0.165	0.0233	112
10	1.0	0.0	0.184	0.0260	105

Figure 1.--Data from Self-absorption Measurements of 2,2'-5',2"-Terthienyl-S-35 and 5-Acetyl-2,2'-5',2"-terthienyl-S-35



Determination of Specific Activities

Quantitative determination of terthienyl was obtained by measuring its absorption at 350 millimicrons (ϵ =24,100). The radioactivity measurements were carried out by planchet counting of solid samples. Constant specific activities resulted after several rechromatographic operations over alumina. Another method of purification was the conversion of terthienyl to its acetyl derivatives, 5-acetyl-2,2,2';5',2"-terthienyl and 5,5"-diacetyl-2,2';5',2"-terthienyl. The acetyl derivatives were purified to constant specific activity by adsorption chromatography over alumina. The 5-acetyl compound was easier to handle of the two. Its elution from the alumina column was faster. Qualitative measurements were taken by its absorption at 391 millimicrons (ϵ = 30,500). The radioactivity was determined in the same manner as that for terthienyl. The purity of the above compounds was checked by paper chromatography and thin layer chromatography. The UV absorptions were obtained with a Beckman DU spectrophotometer. The statistical treatment of the counting data was based upon the discussions of Overman and Clark (19).

Isotope Dilution Analysis

The procedure was essentially that which was reported by Mayor and Collins (20). To the crude terthienyl fraction from the alumina column was added a known quantity

of "cold" terthienyl (0.8-10 mg.). The diluted mixture was divided, and a second quantity of terthienyl, twice that of the first dilution, was added to one of the divided portions. The terthienyl from each dilution was converted to its 5-acetyl derivative and purified to constant specific activity. The original terthienyl isolated from the plant and its original specific activity were calculated from the following relationships:

$$X = A_2D_2/A_1-A_2-(D_1-S) \qquad A_o = A_1D_1/X$$

X = Weight of terthienyl isolated

A_o= Specific activity of X

D_1= Dilution 1

D_2= Dilution 2

S = Weight of diluted terthienyl taken for analysis

A_1= Specific activity of D_1

A_2= Specific activity of D_2

Isotope Dilution Calculations of DL-Methionine-S-35, Experiment 23:

A_1* = Actual sample counted

A_2* = Actual sample counted

R_s = Net counting rate in cpm

R_b = Background rate in cpm

R_{s+b}= Rate of sample and background in cpm

A_1* = Two measurements were observed with counting intervals of 20 minutes each.

R_b (1) 2560 c/20 m = 123 cpm

(2) 2310 c/20 m = 115 cpm

R_b (average) = 119 cpm

R_{s+b} (1) 4250 c/20 m = 212 cpm

(2) 3800 c/20 m = 190 cpm

R_s (1) 212-119 = 93 $\pm$ 4 cpm SD = 4%

(2) 190-119 = 71 $\pm$ 4 cpm SD = 6%

Standard deviation of R_s was calculated from the equation:

$$SD = (R_{s+b}/t + R_b/t)^{1/2}$$

Total R_s = R_s x Dilution factor

R_s (1) 93 x 5 = 465 cpm

(2) 73 x 5 = 365 cpm

A_1 = Total R_s/.805 mg.

(1) 465 cpm/.805 mg. = 578 cpm/ mg.

(2) 365 cpm/.805 mg. = 453 cpm/ mg.

A_2*: Two measurements were observed with counting intervals of 20 minutes each.

R_b (1) 1470 c/20 m = 74 cpm

(2) 1400 c/20 m = 70 cpm

R_b (average) = 72 cpm

R_{s+b} (1) 2110 c/20 m = 105 cpm

(2) 2230 c/20 m = 111 cpm

R_s (1) 105-72 = 33 $\pm$ 3 cpm SD = 9%

(2) 111-72 = 39 $\pm$ 3 cpm SD = 8%

Total R_s = R_sx Dilution factor

R_s (1) 33 x 2 = 66 cpm

(2) 39 x 2 = 78 cpm

Since these samples are assayed 7 days later than those of A_1, a correction was necessary for the radioactive decay during this interval.

$-2.3 (\log 66 - \log R_s) = .00778 (7)$

Corrected R_s (1) 70 cpm

(2) 83 cpm

A_2 = Total R_s/.705 mg.

A_2 (1) 70 cpm/.705 mg. = 99 cpm/mg.

(2) 83 cpm/.705 mg. = 113 cpm/mg.

From $X = A_2D_2/A_1-A_2- D_1+ S$ and using the combination of the two different values of A_1 and A_2, four values of X were calculated :

X = 99 x 20/578-99 - 5.0 = 1980/479 - 5.0 = 4.14-5.0 = -.86

X = 99 x 20/441-99 - 5.0 = 1980/342 - 5.0 = 5.80-5.0 = +.80

X = 113 x 20/578-113 - 5.0 = 2260/465 - 5.0 = 4.86-5.0 = -.14

X = 113 x 20/441-113 - 5.0 = 2260/328 - 5.0 = 6.90-5.0 = +1.90

X (average) = 1.5 mg. which is equivalent to 5.14 Micromoles

Since $A_o = A_1D_1/X$, then

A_o = (1) 578 x 10/1.5 = 3380 cpm/mg. = 1.12 x 10^6 cpm/mM

(2) 441 x 10/1.5 = 2960 cpm/mg. = 0.86 x 10^6 cpm/mM

A_o (average) = 0.99 x 10^6 cpm/mM

Adsorption Chromatography

Terthienyl was best separated from the petroleum ether solution of the roots by chromatography of the crude sample over 7.5 g. of alumina (Alcoa, F-20) in a 25 x 1.5 cm.

glass column fitted with a stopcock. The sample size was approximately 15 ml. Fractions of 10 ml. were collected. Terthienyl was eluted with petroleum ether (30-60) after 40 to 60 ml. of eluant had been collected. A 2% diethyl ether solution hastened its movement through the column. The 5-(3-buten-1-ynyl)-2,2'-dithienyl fraction was always collected prior to the terthienyl fraction. Both fractions were eluted together if caution was not taken when using 2% diethyl ether as the eluant. The terthienyl fraction was identified from its blue fluorescence and instant violet color with isatin dye in concentrated sulfuric acid. The dithienyl compound also displayed a positive reaction with isatin, but the color was wine in appearance compared to that with terthienyl. Both compounds were distinguished by their behavior on a paper chromatogram. A further purification of terthienyl over 5 g. of alumina resulted in only a partial purification in some cases as shown by the data presented below.

The acetyl derivatives of terthienyl were best purified by adsorption chromatography over alumina using carbon tetrachloride as the solvent. The 5-acetyl derivative was eluted from the column by a 10% solution of diethyl ether in carbon tetrachloride. The percentage of diethyl ether was increased to 60% in order to elute the 5,5"-diacetyl derivative. Terthienyl passed through the column with carbon tetrachloride. The sample size was not

crucial and could vary from 5 to 75 ml. using 5 g. of alumina and a total of 20 mg. of solids in solution.

The results of the purification of terthienyl-S-35 are shown below. This data was taken from Experiments 2 and 3 using sodium sulfate-S-35. "Cold" terthienyl was added during the step in which the roots were disintegrated in ethanol.

Experiment 2:

	Total cpm	Terthienyl MicroM	Specific Activity cmp/MicroM
Ethanol extract	37.2×10^4	274	1350
Petroleum ether solution	6.56×10^4	227	289
First chromatography	1.27×10^4	271	47
5-Acetyl derivative	$.109 \times 10^4$	23.3	47

Experiment 3:

	Total cpm	Terthienyl MicroM	Specific Activity cpm/MicroM
Ethanol extract	65.4×10^4	238	2740
Petroleum ether solution	17.1×10^4	254	674
First chromatography	1.84×10^4	189	97
Second chromatography	1.40×10^4	156	90

The data presented for Experiment 3 represents the actual observed activity, and corrections for the half life of sulfur-35 were not considered here as they were in Table 10. The purification to constant specific activity of added "cold" terthienyl in Experiment 14 is shown as follows

	Total cpm	Terthienyl MicroM	Specific Activity cpm/MicroM
First chromatography	3890	80.7	48
5-Acetyl derivative	130	8.6	15

Oxidation of 5-(3-Buten-1-ynyl)-2,2'-dithienyl

In trial 1 the fraction from the alumina column containing the dithienyl compound was evaporated to dryness. The residue was dissolved in 10 ml. of acetone. Approximately 50 mg. of potassium permanganate and 1 ml. of 2N sulfuric acid were added. The reaction mixture was stirred for 45 minutes at room temperature. The product was isolated according to the procedure of Uhlenbroek and Bijloo (6). In trial 2 the same procedure was followed, except the reaction mixture was heated to reflux for 5 minutes and immediately allowed to cool to room temperature. A gas appeared to be given off.

Preparation of 5-Acetyl-2,2';5',2"-terthienyl and 5,5"-Diacetyl-2,2';5',2"-terthienyl

Several different experimental conditions were investigated in order to obtain the highest yield of the 5-acetyl compound. Using 100 mg. of terthienyl as starting material, yields ranged from 2-42%. A 100 mg. quantity (.4 mM) of terthienyl was dissolved in 50 ml. of dry benzene. The solution was heated to its reflux temperature, and .056 ml. (.8 mM) of acetyl chloride and 8 drops (3mM) of stannic chloride were added. After heating it at reflux temperature for 24 hours the reaction mixture was poured into 50 ml. of 1N hydrochloric acid and ice. The mixture was neutralized with sodium bicarbonate and extracted with diethyl ether. The ether solution was dried over sodium sulfate and evaporated to dryness. The residue was dissolved in carbon tetrachloride and chromatographed over alumina. The unreacted terthienyl was recovered, and the maximum yield of 5-acetyl compound was 42%. The 5-acetyl compound was obtained as fine, yellow crystalline needles, m.p. 168-169 ; UV maximum 391 millimicrons (ϵ = 30,500) and 252 millimicrons. The 5,5"-diacetyl compound was obtained as yellow needles after sublimation (235 , 6mm.), m.p. 243 ; UV maximum 406 millimicrons (ϵ = 39,300) and 253 millimicrons. A yield of 42% was also obtained with 8 mg. of terthienyl as starting matieral dissolved in 20 ml. of dry benzene. Two drops each of stannic chloride and acetyl chloride added, and the reaction mixture was heated at its reflux temperature for 15 hours.

Oxidation of 5-Acetyl-2,2';5',2"-terthienyl

A solution of potassium hypochlorite was prepared following a modified procedure of Newman and Holmes.(24) A 2.5 g. quantity of HTH (calcium hypochlorite) was dissolved in 10 ml. of warm water, and a 3 ml. solution containing 2.1 g. of potassium carbonate and 0.5 g. of potassium hydroxide was added. The gel which formed was stirred until it was liquid. The mixture was filtered, and the filtrate of potassium hypochlorite was saved for the oxidation procedure. A 10 ml. volume of the hypochlorite solution was added to approximately 8 mg. of the 5-acetyl terthienyl previously dissolved in 5 ml. of dioxan. The homogeneous mixture was stirred until the reaction was completed. The extent of reaction was followed by the use of thin layer chromatography. An aliquot of the reaction mixture was taken at 0, 5, 80, and 155 minutes after the addition of potassium hypochlorite. At zero time the 5-acetyl terthienyl was the only compound present (R_f approximately 0.2). After 5 minutes a new compound appeared which did not move from the origin. The new compound at the origin increased in fluorescence (blue), and fluorescence (green) of the 5-acetyl terthienyl decreased after 80 minutes. The reaction was complete at the end of 155 minutes, since the main component was the compound at the origin, and only a trace of fluorescence was present due to the acetyl compound.

The reaction mixture was diluted to 50 ml. with distilled water. The solution was acidified to pH 5 and extracted for 18 hours with diethyl ether in a liquid-liquid extraction apparatus. The yellow, amorphus residue remaining was redissolved in a minimum of ethanol. Paper chromatography of the ethanol solution showed a blue fluorescent spot at R_f of 0.61 (terthienyl R_f of 0.62) and a blue fluorescent spot at the origin. The chromatogram was treated with ammonia and sprayed with a bromothymol blue solution. The compound at the origin exhibited a yellow spot against a blue background. The remaining aqueous layer from the liquid-liquid extraction did not show any acid components when treated in the same manner, but only one blue fluorescent spot at R_f of 0.44 (development solvent-methanol:water, 66:34).

Paper Chromatography of 2,2';5',2"-Terthienyl

The ascending method of chromatography was employed because of its convenience. The development tank consisted of a 10 x 30 cm. battery jar protected with a glass plate. Whatman #1 filter paper was cut into rectangular strips, 7 x 25 cm. and suspended from the glass plate by the use of masking tape. The width of the paper strips was determined by the number of samples to be chromatographed at one time. An aqueous methanol solution was utilized as the development solvent. It was found that a 66-70% methanol

solution (aqueous) offered the best results for terthienyl. The compound was adequately separated from other compounds, and reproducible R_f values were realized as shown by the following experiment results.

A stock solution of terthienyl was prepared by dissolving 25 mg. in 500 ml. of 95% ethanol. Five different aliquots were taken and applied to the origin of the paper. The solvent front moved 13.5 cm. after development for one hour with 66% methanol. The results are shown by sample numbers 1 through 5 in Table 4. Samples A through C in Table 6 were treated in the same manner, except the solvent front traveled 4.0 cm. Six different chromatograms were also prepared using a sample size of 2 micrograms. The movements of the solvent fronts varied from 10.1 to 11.8 cm. yielding R_f values ranging from 0.66 to 0.73. The latter six values are not shown in the table.

The consequence of varying the percentage composition of the solvent was not throughly investigated. Some results of solvent effects are summarized in Table 7. The sample size was from 2 to 4 micrograms, and the solvent front traveled 7.5 cm.

Terthienyl was located on the chromatogram by observing the paper under an ultra violet lamp. The compound was easily distinguished from its brilliant blue fluorescence. As little as 0.1 microgram was detected by this method.

TABLE 6.--Comparison of R_f Values of Terthienyl Against the Amount of Sample

Sample Number	Micrograms of Sample	R_f Value
1	0.11	0.65
2	0.25	0.65
3	0.35	0.67
4	0.44	0.67
5	0.70	0.68
A	approximately 2	0.66
B	approximately 4	0.58
C	approximately 6	0.55

TABLE 7.--Comparison of R_f Values of Terthienyl Against Changes in Solvent Composition

% Methanol	R_f Value
50	0.00
75	0.73
88	0.80
100	1.00

A second solvent system was investigated which involved the use of Whatman #1 filter paper previously treated with a 5% paraffin solution of benzene. The development solvent consisted of a 5% ethyl acetate solution of n-heptane. R_f values were irregular but were mostly in the range of 0.68 to 0.73. The system was not investigated further.

Paper Chromatography of 5-Acetyl-2,2';5',2"-terthienyl and 5,5"-Diacetyl-2,2';5',2"-terthienyl

During the preparation of these compounds by acetylation of terthienyl, it was necessary to check the purity of the products. Both compounds were treated in the same manner, and the development of the chromatograms was identical to that which was followed for terthienyl. The results are summarized in Table 8. The solvent was 66% methanol (aqueous), and development time was one hour. The solvent front traveled 13.6 cm.

Thin Layer Chromatography

Materials:

The adsorbent used for the preparation of all chromatostrips was Fisher Alumina, A-540, 80-200 mesh or Alcoa Activated Alumina, F-20 grade. All solvents were C. P. grade and could be used without further purification. Soluble starch (Nutritional Biochemicals Corporation) or a commercial plaster of paris was mixed as a binder with the adsorbent.

TABLE 8.--R_f Values of 5-Acetyl-2,2';5',2"-terthienyl and 5,5"-Diacetyl-2,2';5',2"-terthienyl from Paper Chromatography

Compound	Ultra Violet Light	R_f Value[a]
Terthienyl	Blue	0.71
5-Acetyl-	Yellow	0.46
5,5"-Diacetyl-	Blue-green	0.16
Mixture:		
Terthienyl	Blue	0.72
5-Acetyl-	Yellow	0.45
5,5"-Diacetyl-	Blue-green	0.15

a Average value of two chromatograms

Preparation of the Chromatostrips:

Two methods were found to be satisfactory for the separation of compounds containing the thiophene nucleus. Method 1--Alumina and starch in a ratio of 19:1 dry weight were thoroughly mixed. For each 20 g. of dry mixture 36 ml. of distilled water were added. The slurry was heated on the steam bath and stirred for 4 minutes. The upper water layer was poured off leaving a thick, smooth mixture. This was spread immediately on previously cleaned microscope slides, 25 x 75 mm. The chromatostrips were dried in an oven for 30 minutes at 110° C. The dried chromatostrips were stored in a vacuum desiccator over potassium hydroxide until needed.

Method 2--Alumina and plaster of paris were thoroughly mixed dry in a ratio of 7:3 dry weight. For each 10 g. of dry mixture 8 ml. of distilled water were added. For increased hardness of the surface 2% sodium hydroxide was substituted in place of the water. For a thinner slurry water was added enabling an easier spreading of the mixture. The chromatostrips were dried from 2-5 hours in an oven at 75° C. The chromatostrips were stored in the same manner as that in Method 1.

For every 10 g. dry weight of adsorbent plus binder, approximately eight chromatostrips were obtained. The surface of the adsorbent was slightly pitted and was smoothed by rubbing it lightly with tissue paper. Immediately

before using each chromatostrip, a border of at least 1mm. in width was made by scraping the adsorbent from the edge of the slide. This enabled an even flow of solvent during development.

Application of Sample:

The sample was applied either as a single spot or as a line at the origin. The origin was one half inch from one end of the chromatostrip. Solutions of sample ranging from 0.01 to 1.0 ml. were applied taking normal precautions in order that spreading of the spot was kept to a minimum. The use of a hair dryer was excellent for this procedure.

Development of the Chromatostrip:

A nonpolar and polar solvent were required for the development of the samples. Most compounds were separated by development with a solvent mixture which was from 1 to 10% composition of the polar solvent. Nonpolar solvents included petroleum ether (30-60), n-hexane, n-heptane, or benzene. The polar solvents comprised diethyl ether, ethyl acetate, or acetone. A n-hexane solution of 5% diethyl ether provided the best over-all separation of the compounds investigated.

After the application of the sample, the chromatostrip was placed in a test tube, 3 x 12 cm., containing 1-2 ml. of the desired development solvent. The solvent

immediately commenced to ascend. The time required for the solvent to travel the distance of the chromatostrip was less than 5 minutes. The chromatostrip was removed from the solvent and dried either at room temperature or in a stream of warm air.

Location of the Compounds:

All the compounds incorporating a thiophene nucleus were detected by very quickly immersing the dried chromatostrip into concentrated sulfuric acid containing 1% isatin dye. Characteristic areas of color were observed for each compound studied with a few exceptions. If the chromatostrip was immersed longer than two seconds in the isatin solution, the surface of the adsorbent was usually destroyed.

The presence of functional groups was also exploited by the use of standard qualitative reagents. Aldehydes were located by immersing the chromatostrip into 2,4-dinitrophenylhydrazine or Tollen's reagent. A few compounds were located by heating the chromatostrip for several minutes at 100° C after it was immersed in an aqueous 2% potassium permanganate solution. The easiest method of locating the compounds was from observing the chromatostrip under an ultra violet light. Most compounds resulted in an easily visible fluorescent spot. A summary of the compounds investigated by thin layer chromatography is given in Table 9.

TABLE 9.--Compounds Investigated by Thin Layer Chromatography

Name	Isatin	Fluorescence
Thiophene	Blue	None
2-Thiophenaldehyde	Yellow	None
2-Acetylthiophene	Red[a]	None
2,2'-Dithienyl	Green	None
5-Formyl-2,2'-dithienyl	Yellow	Blue
2,2'-Dithienyl-5-carboxylic acid	Brown	Trace
5-Thiocarbethoxy-2,2'-dithienyl	Colorless	Blue
2,2';5',2"-Terthienyl	Violet	Blue
2,2';5',2"-Terthienyl-5-carboxylic acid	-	Blue
5-Acetyl-2,2';5',2"-terthienyl	Violet	Green
5,5"-Diacetyl-2,2';5',2"-terthienyl	Red	Green

a 2,4-dinitrophenylhydrazine

RESULTS

Sodium Sulfate-S-35

Experiments 1 through 5 were conducted in order to determine the duration required for maximum incorporation of sulfur-35 into terthienyl as measured from the initial time of administration of the radioisotope. Both feeding methods were investigated, and terthienyl was radioactive only in those experiments where feeding was via roots. The total per cent incorporation, found by dividing the total radioactivity in terthienyl by the total originally fed, ranged from 0.04-0.08%. Dilution factors, found by dividing the original specific activity of terthienyl by the specific activity of the administered radioisotope, varied from 77-294.

With Tagetes erecta the dilution factor varied from 294 at two days duration to 260 at fifteen days duration, while the per cent of activity incorporated was 0.06% and 0.08%, respectively. With the dwarf marigolds (not a Tagetes species) the dilution factor was 161 and decreased to 77 at five and ten days, respectively, while the per cent of activity incorporated increased from 0.04 to 0.06%. The age of the Tagetes and dwarf plants was 5 and 1.5 months. The results of the above experiments are summarized in Table 10.

TABLE 10.--The Incorporation of Sodium Sulfate-S-35 into Terthienyl in Marigold Roots[d]

Experiment Number	Time, Days	Method of Feeding	Weight of Roots g.	Total Terthienyl, mg.	Total Activity Dispensed, cpm
1	2	Stem	0.45	18	3.58×10^7
2	2	Root	1.55	67	1.99×10^7
3[c]	5	Root	2.30	57	8.94×10^7
4[c]	10	Root	2.73	28	11.8×10^7
5	15	Root	3.91	21	4.21×10^7

Experiment Number	Total Activity (cpm)			Terthienyl, cpm	
	Ethanol Extract	Petroleum Ether Extract	Nutrient Solution	Total	/g. Root
1	61200	3800	None	None	--
2	372000	65600	b	12700	8190
3	929000	243000	a	30400	12300
4	1690000	a	2.64×10^6	70400	25800
5	a	252000	6.67×10^6	32300	8260

[a]Not measured for radioactivity.

[b]2ml. aliquot was too "hot" for radioactive assay.

[c]Dwarf variety of plants, exact species unknown.

[d]Experiments 1, 2, and 5 were Tagetes, age 21 weeks; Experiments 3 and 4 were dwarfs, age 7 weeks.

L-Methionine-S-35

The purpose of Experiments 6 through 10 was to see if sulfur from methionine-S-35 was incorporated into terthienyl, to observe the effect of the age of the plant on the incorporation, and to compare the feeding via stems with root feedings.

The older plants took more radioactive sulfur into the roots than the younger plants as shown by the total activity in the ethanol extracts. No radioactivity was found in terthienyl until 17 days had elapsed from the original feeding. This amount of radioactive terthienyl represented an incorporation of 0.003% of the activity, and the diultion factor was 60,200. The results of these experiments are summarized in Table 11.

DL-Methionine-1-C-14

The first carbon compound investigated as a possible precursor to terthienyl was methionine labelled in the C_1 position. These studies are represented in Experiments 11 through 13. The labelled compound was fed via stems. None of the radioactivity was found in terthienyl after 10-19 days from the original feeding. This data is summarized in Table 12.

DL-Methionine-2-C-14

Experiments 14 through 16 represent studies to determine if carbon-14 of methionine located in the C_2 position

TABLE 11.--The Incorporation of L-Methionine-S-35 into Terthienyl in Marigold Roots[a]

Experiment Number	Time, Days	Plants Age, Mo.	Weight of Roots, g.	Terthienyl Added to Ethanol Extract	Total cpm Dispensed
6	2	4	3.14	20 mg.	4.58×10^7
7	2	1.5	2.45	20 mg.	5.03×10^7
8	6	1.5	2.61	25 mg.	4.02×10^7
9	12	1.5	2.22	None	3.02×10^7
10	17	1.5	2.16	None	2.91×10^7

Experiment Number	Total Activity (cpm)			Corrected Activity Dispensed	Total cpm in Terthienyl
	Ethanol Extract	Nutrient Solution	Thread		
6	72200	82800	1.22×10^7	3.36×10^7	None
7	34100	30700	2.72×10^7	2.31×10^7	None
8	131000	11400	2.69×10^7	1.33×10^7	None
9	2390000	26400	1.99×10^7	1.03×10^7	None
10	37500	163000	1.12×10^7	1.79×10^7	600

[a]All experiments were stem feedings.

TABLE 12.--The Incorporation of DL-Methionine-1-C-14 into Terthienyl in Marigold Roots[c]

Experiment Number	Time, Days	Weight of Roots, g.	Total Terthienyl, mg.	Total cpm Dispensed
11	10	4.03	25.5[d]	2.71×10^7
12	15	3.02	20.8[a]	2.07×10^7
13	19	0.35	20.8[a]	2.07×10^7

	Total Activity (cpm)			
Experiment Number	Ethanol Extract	Petroleum Ether Extract	Nutrient Solution	Total cpm in Terthienyl
11	22200	40	8700	None
12	b	b	66100	None
13	47000	2	16800	None

[a]Amount in fraction from alumina chromatography.

[b]Activity not determined.

[c]Stem feedings.

[d]Ethanol extract.

is a precursor to terthienyl when fed via roots instead of the stems. Radioactivity was found in terthienyl only in the two day experiment. All of the activity of terthienyl was eliminated after this time. The dilution factor obtained for methionine-2-C-14 for the two day experiment was 1,430 which represented a total incorporation of 0.01%. The total radioactivity of the roots as shown by the activity in the ethanol extracts, petroleum ether extracts, and the crude terthienyl fractions decreased with time. The results of these experiments are summarized in Table 13.

The diethyl ether extract of the aqueous nutrient solution was investigated further. A portion of the radioactive ether solution was analyzed by chromatostrip chromatography. One blue fluorescent area was extracted from the chromatostrip. UV absorption was detected with the maxima at 335 and 251 millimicrons. No radioactivity was found in this unknown compound. Paper chromatography of the compount in 70% aqueous methanol resulted in an R_f-ratio with terthienyl of 1.12.

Sodium Acetate-1-C-14

Sodium acetate labelled in the carboxyl position was fed to the plants by both feeding methods: Experiments 17 through 19 via stems and Experiments 20 through 22 via roots. Again, it was shown that the accumulated radioactivity in the ethanol extract was less with the stem feedings. Also, from

TABLE 13.--The Incorporation of DL-Methionine-2-C-14 into Terthienyl in Marigold Roots

Experiment Number	Time, Days	Weight of Roots, g.	Crude Terthienyl Isolated, mg.	Total cpm Dispensed[a]
14	2	3.39	0.104	1.78×10^7
15	10	3.22	0.134	1.33×10^7
16	20	3.13	0.032	0.88×10^7

Experiment Number	Total Activity (cpm)			Terthienyl, cpm	
	Ethanol Extract	Nutrient Solution	Crude Terthienyl	Total	/g.Root
14	1600000	37900	10800	1210	357
15	872000	27800	3250	None	--
16	312000	c	200	d	--

[a]The total activity was determined by assuming 40% counting efficiency and indicating the activity fed as cpm.

[b]Diethyl ether extract of the aqueous nutrient solution.

[c]Activity not determined.

[d]The purified sample was lost.

the activity of the ethanol extracts in Experiments 20 through 22, the decrease of activity with time was observed. This is seen in the decreasing amount of activity of the crude terthienyl fraction. Radioactivity in terthienyl was found only with the root feedings and only after ten days from the initial feeding. The results are summarized in Table 14. After ten days duration the amount of radioactivity incorporated was 0.002%, and the dilution factor was 35,100. At the end of 20 days the radioactivity incorporated doubled to 0.004% while the dilution factor decreased to 10,200.

The nature of the radioactivity found in the diethyl ether extract of the nutrient solution in Experiments 20-22 was investigated. Paper chromatography with 70% aqueous methanol showed two compounds which moved from the origin. One compound which showed a yellow fluorescence on paper gave an R_f-ratio with terthienyl which varied from 0.63 to 0.91. The second compound which moved from the origin showed a blue fluorescence on paper and had a relatively constant R_f-ratio with terthienyl of 1.13. A qualitative determination of radioactivity in the ten day experiment indicated that the yellow fluorescent compound contained only a trace, whereas the blue fluorescent compound contained a significant amount of radioactivity (about 100 cpm in an aliquote of the ether). This was determined by eluting the spot from the paper chromatogram into a planchet and counting the residue left from the evaporation of the solvent.

TABLE 14.--The Incorporation of Sodium Acetate-1-C-14 into Terthienyl in Marigold Roots

Experiment Number	Time, Days	Weight of Roots, g.	Crude Terthienyl Isolated, mg.	Total cpm Dispensed[a]
17	2	4.87	0.263	0.95×10^7
18	9	3.61	Unknown	0.98×10^7
19	17	3.82	Unknown	0.95×10^7
20	2	2.80	0.230	1.77×10^7
21	10	2.45	0.032	1.33×10^7
22	20	2.60	0.198	1.33×10^7

Experiment Number	Total Activity (cpm)			Terthienyl, cpm	
	Ethanol Extract	Nutrient Solution	Crude Terthienyl	Total	/g.Root
17	15500	1470000	300	None	--
18	14800	2300	None	None	--
19	4700	7400	300	b	--
20	940000	22400	3600	None	--
21	186000	5400	1250	215	88
22	132000	6500	625[c]	785[d]	302

[a]Correction for activity remaining in the cotton thread in 17-19.

[b]Terthienyl not further purified.

[c]The %SD of the sample counted was ± 40: the value given could range from 375-875 cpm.

[d]The %SD of the sample counted was ± 6.

Isotope Dilution Experiments

Several experiments were conducted in order to determine the original specific activity of terthienyl isolated from the roots. The method chosen was constructed by Mayor and Collins (20) and consisted of a double dilution method. See the experimental section for details of the procedure.

Four different compounds were studied in this investigation: DL-methionine-S-35, sodium hydrogen sulfide-S-35, succinic acid-2,3-C-14, and Dl-glutamic acid-2-C-14. The highest incorporation of activity into the roots, as shown by the activity in the ethanol extracts, occurred with methionine as the source of sulfur. The highest incorporation into the roots with carbon-14 compounds was observed with succinic acid. Only a trace of activity was found in terthienyl from sodium hydrogen sulfide. None of the activity from the carbon-14 compounds was incorporated into terthienyl. The per cent incorporation from methionine amounted to 0.03%, and the dilution factor was 4,420. The results are summarized in Table 15. The calculated specific activity of terthienyl is shown in Table 16.

Investigation of 5-(3-Buten-1-ynyl)-2,2'-dithienyl

Evidence of the presence of another known radioactive compound, 5-(3-Buten-1-ynyl)-2,2'-dithienyl, was observed in Experiment 4. Terthienyl was collected in fractions 3 and 4 from the alumina column, and the dithienyl compound was collected in fraction 2 prior to terthienyl. The properties

TABLE 15.--The Incorporation of Sulfur-35 and Carbon-14 into Terthienyl as Determined by Isotopic Dilution Methods

Experiment Number	Time, Hours	Weight of Roots, g.	Activity (cpm)		Total cpm Dispensed
			Ethanol Extract	Nutrient Solution	
23	32	1.98	1150000	0.50×10^7	3.80×10^7
24[c] (a)	40	1.32	29200	2.42×10^7	3.33×10^7
(b)	30	7.85	5280000	3.00×10^7	5.96×10^7
25	44	0.84	297000	0.37×10^7	2.22×10^7
26	49	1.11	19500	0.58×10^7	0.70×10^7

[c]Two different experiments.

TABLE 16.--Weights and Specific Activities of Isolated Terthienyl in Experiments 23-26

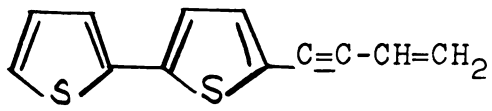
Experiment Number	Compound	Terthienyl MicroM.[c]	Specific Activity cpm/mMole[c]	Terthienyl cpm/g.Root
23	Methionine	5.14	$.99 \times 10^6$	2600
24[d] (a)	Sodium bisulfide	--	--	--
(b)		--	--	--
25	Succinic acid	--	--	--
26	Glutamic acid	--	--	--

[c]Calculated according to the double dilution method.

[d]Two different experiments.

are listed in Table 17 with terthienyl as a comparison.

The ditheinyl compound from this experiment was believed to correspond in structure to that of compound XVIII (6). Further evidence was



Compound XVIII 5-(3-Buten-1-ynyl)-2,2'-dithienyl

presented from the L-methionine-S-35 experiments, when a compound was eluted from the alumina column of the petroleum ether solution which absorbed in the UV range, 343-344 millimicrons in petroleum ether. The behavior on a paper chromatogram yielded the same relative R_f value to terthienyl as that shown in Table 17.

In the two day experiment of L-methionine-S-35, the total radioactivity found in the fraction containing compound XVIII was 500 cpm. Whether the activity was due to the dithienyl compound or impurities was not determined. The evidence obtained from the sodium sulfate-S-35 experiment, Table 17, suggests the incorporation of sulfur-35 into compound XVIII.

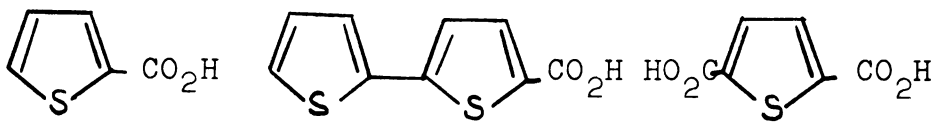
Uhlenbroek and Bijloo previously reported that the oxidation of compound XVIII produced a diacid, XXI, with potassium permanganate in acetone. The fraction believed

TABLE 17.--Properties of the Dithienyl Compound Compared with Terthienyl - Experiment 4

Fraction	Compound	Isatin	Potassium Permanganate	UV, Ethanol millicrons	Rf Value	Activity cpm
2	Dithienyl-	Violet	Positive	346,254[a]	0.84	75300
3&4	Terthienyl	Violet	Negative	350,252	0.71	70400

[a]From another isolation experiment of marigold blooms, the dithienyl compound was purified by chromatostrip chromatography, produced a violet color with isatin, and absorbed in the UV at 347 millimicrons in ethanol and 341 millimicrons in petroleum ether (30-60).

to contain the dithienyl compound was oxidized according to their procedure. Because of the microgram quantity of starting material, the identification of the diacid was shown by its R_f value on paper chromatograms compared to known compounds.(17) Compounds XIX-XXI were chromatographed with the unknown sample. The results are given in Table 18.



XIX XX XXI

The 5-(3-buten-1-ynyl)-2,2'-dithienyl fraction from the alumina chromatography of the petroleum ether solution was identified in experiments 20-22 by a positive isatin test and paper chromatography with known terthienyl. The R_f-ratio with terthienyl was 1.10. No radioactivity was found in the 2 day and 20 day experiments. The dithienyl fraction in the 10 day experiment was not measured for radioactivity. Instead, the oxidation to the known 2,5-thiophenedicarboxylic acid was attempted.

Two trials of the oxidation from two different isolations were completed. The starting material of the first trial absorbed in the UV at 343 and 256 millimicrons (ethanol), produced a violet color with isatin, and showed one spot on a paper chromatogram with an R_f value of 0.83 (terthienyl R_f

TABLE 18.--Paper Chromatography of Carboxylic Acids

Whatman #1 filter paper, ascending method

Reagent- 2,6-dichlorobenzenone-indophenol, pink spots on blue surface

Solvent A: butanol, pyridine, ethanol, and water (3:1:1:1)

Solvent B: methanol and water (7:3)

Compound	Fluorescence	Acid Spot	Solvent	Solvent Front-cm.	R_f
XIX	None	Pink	A	11.5	0.81
XIX	None	Pink	B	12.1	0.89
XX	None	Pink	A	11.5	0.73
XX	Blue	None	B	12.1	0.74
XXI	None	Pink	A	11.5	0.41
XXI	None	Pink	B	12.1	0.14
Unknown					
trial 1	None	Pink	A	12.1	0.42
trial 2	None	Pink	A	11.5	0.05

of 0.75 or R_f-ratio to terthienyl of 1.10). The UV absorption of the crude oxidation product resulted in two shoulders and one broad maxima at 333, 222, and 213 millimicrons, respectively. The starting material from the second trial produced a wine color with isatin and showed one spot on a paper chromatogram with an R_f value of 0.84 (terthienyl R_f of 0.76 or R_f-ratio to terthienyl of 1.10). Paper chromatography of the starting material before oxidation showed no acids to be present as determined from an aliquot. Paper chromatography with solvent A, Table 18, of the crude product of trial 1 showed a very faint pink spot with a R_f value of 0.42 and a blue fluorescent spot at the solvent front. Paper chromatography of the oxidation products from trial 2 showed a faint pink spot with an R_f value of 0.05.

An aliquot of the reaction mixture from trial 1 was applied to a chromatostrip. Development was carried out using 50% diethyl ether in n-hexane. Three different compounds were present as shown by their blue fluorescence: A, at the origin; B, intermediate between the origin and solvent front; C, at the solvent front. Each was eluted from the chromatostrip with ethanol and diluted to 10 ml. The UV absorption for each compound was recorded: A, green fluorescence and gradual absorption from 250-400 millimicrons; B, blue fluorescence and maxima at 243, 248, 254, and 260 millimicrons; C, blue fluorescence and two shoulders at 273 and 255 millimicrons.

In Experiments 14-16 the 5-(3-buten-1-ynyl)-2,2'-dithienyl fraction from the alumina chromatography of the petroleum ether solution was identified by its wine color with isatin, R_f value from paper chromatography, and the UV absorption which varied from 340-344 millimicrons. No radioactivity was detected in the fraction in the 2 day experiment or the 10 day experiment, but a small amount was found after 20 days (400 cpm).

DISCUSSION

A convenient way of representing the incorporation of suspected precursors into terthienyl was the use of a "dilution factor" of specific activity. This was calculated by dividing the specific activity of the compound fed to the plant by the specific activity of the isolated terthienyl. The data in Table 19 show the dilution of sulfur-35 administered either as sodium sulfate or methionine varied from 77 to 4,420. The dilution factors obtained from the carbon-14 compounds were from 1,430 to 35,100. Since the least dilution was received with sodium sulfate-S-35, the sulfur was incorporated near the final step in the biosynthetic pathway as far as the sulfur atom is concerned.

However, the data does not indicate that the carbon-14 precursors have been conclusively demonstrated. The least dilution of methionine-2-C-14 (1,430) showed it to be closer to the final step of the carbon-14 pathway than acetate-1-C-14 (10,200-35,100). The difference in the specific activities of isolated terthienyl from the sulfur-35 compounds as compared to the carbon-14 compounds differed by a factor of 10^2 (sulfur-35 greater than carbon-14), and the dilution factors also differed by 10^2. The similarity in the differences of the dilution factors and specific activities of the isolated

TABLE 19.--Specific Activity of Isolated Radioactive Terthienyl

Assumption: Terthienyl concentration = 0.444 microM/g.Root

Experiment Number	Feeding Method	Isotope	Specific Activity cpm/mM	Time, Days	Terthienyl cpm/mM	Dilution Factor
1	Stem	SO_4	--	2	0	--
2	Root	SO_4	5.42×10^9	2	1.84×10^7	294
3	Root	SO_4	4.46×10^9	5	2.77×10^7	161
4	Root	SO_4	4.46×10^9	10	5.80×10^7	77
5	Root	SO_4	4.83×10^9	15	1.86×10^7	260
10	Stem	Me-S	37.6×10^9	17	6.25×10^5	60,200
13	Stem	Me-C_1	--	19	0	--
14	Root	Me-C_2	1.13×10^9	2	7.88×10^5	1,430
16	Root	Me-C_2	--	20	0	--
19	Stem	Ac-C_1	--	10	0	--
20	Root	Ac-C_1	--	2	0	--
21	Root	Ac-C_1	6.96×10^9	10	1.98×10^5	35,100
22	Root	Ac-C_1	6.96×10^9	20	6.80×10^5	10,200
23	Root	Me-S	4.38×10^9	1.3	$.99 \times 10^6$	4,420[a]

[a]Value from isotopic dilution experiment.

terthienyl indicated that both methods of showing incorporation were giving similar results as to what was actually occurring in the plant.

Dilution factors were given by Griffith and Byerrum (22) from the incorporation studies of acetate-1-C-14, acetate-2-C-14, pyruvate-1-C-14, and pyruvate-3-C-14 into nicotine in tobacco plants. The least dilutions occurred with acetate-2-C-14 and pyruvate-3-C-14 and ranged from 243-550. The highest dilution was received from pyruvate-C-14 (8,370). They stated that incorporation was small for the compound with the dilution factor of 8,370. An intermediate value was obtained with acetate-1-C-14 (911 and 981); thus, acetate was incorporated when labelled at either the first or second carbon position. The significant conclusion to be made from a consideration of the data of Griffith and Byerrum in relation to the present work is that the dilution factors obtained from sodium sulfate-S-35, methionine-S-35, and methionine-2-C-14 indicate incorporation into terthienyl. The high dilution factors of acetate-1-C-14 suggest that no incorporation was achieved, at least as a direct precursor is concerned. The small amount of radioactivity in terthienyl could arise from randomization of the acetate carbon atoms. The summary of data in Table 20 shows that acetate-1-C-14 was incorporated to an extent of 0.002% after 10 days and increased to 0.004% after 20 days. The present data does not conclusively show that randomization has occurred. The question

TABLE 20.--Per Cent Incorporation of Radioactivity in Terthienyl

% Incorporation = Terthienyl cpm / cpm fed x 100

Experiment Number	Compound	Total cpm Dispensed	Total cpm in Terthienyl	Incorporation %
1	$SO_4^=$	3.58×10^7	0	0
2	$SO_4^=$	1.99×10^7	12700	0.06
3	$SO_4^=$	8.94×10^7	30400	0.04
4	$SO_4^=$	11.8×10^7	70400	0.06
5	$SO_4^=$	4.21×10^7	32300	0.08
10	Me-S	1.79×10^7	600	0.003
13	Me-C_1	2.07×10^7	0	0
14	Me-C_2	1.78×10^7	1210	0.007(0.01)[c]
16	Me-C_2	0.88×10^7	a	0
19	Ac-C_1	0.95×10^7	b	0
20	Ac-C_1	1.77×10^7	0	0
21	Ac-C_1	1.33×10^7	215	0.002
22	Ac-C_1	1.33×10^7	785	0.004
23	Me-S	3.80×10^7	5200	0.015(0.03)[c]

[a]See Table 13.

[b]See Table 14.

[c]Per cent incorporation was doubled assuming that the D-form was not utilized.

could be solved by conducting further time studies or by degrading terthienyl-C-14 to locate the position of the labelled carbon atoms when acetate-1-C-14 is fed.

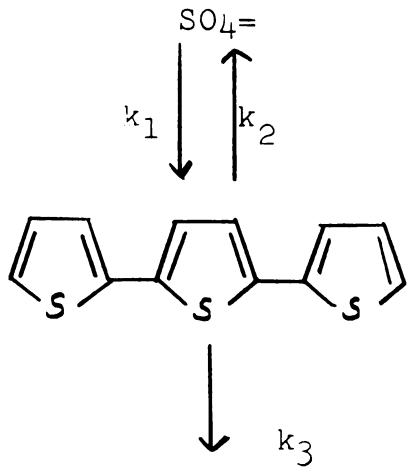
The maximum time of incorporation of sodium sulfate-S-35 into terthienyl was not a simple question to answer as seen from the results summarized in Table 10, 19, and 20. First, using Tagetes plants of age 5 months, the incorporation increased in a period of 2-15 days from 0.06% to 0.08%. The dilution factors of 294 and 260, respectively, represented about equal dilutions of specific activity. If terthienyl were synthesized and stored in the plant, an increase of specific activity would be expected; however, it was found that the value of the specific activity of terthienyl was practically identical after 15 days. One must conclude that de novo synthesis of terthienyl was accompanied by a breakdown to further metabolic products. The rate of synthesis was equal to the rate of breakdown under the experimental conditions, since a constant specific activity was observed at the two time intervals. The conversion is diagramatically represented in Scheme 5.

The symbol, k_1, forward rate of synthesis, may include an indefinite number of steps: k_2, reverse of the forward rate, may also represent the same number of steps: k_3 represents the irreversible breakdown of terthienyl. If radioactive sulfur is readily available for incorporation, after administration of the radioisotope, k_1 is greater than

Scheme 5

Biosynthetic Pathway of

Sodium Sulfate-S-35 to Terthienyl



Metabolic Products

$k_2 + k_3$ with respect to sulfur-35. If sulfur-35 has "saturated" the biosynthetic pathway after the period of "rapid" incorporation, the breakdown of terthienyl-S-35 equals it synthesis, $k_1 = k_2 + k_3$, as shown by equal specific activities in Table 19. This leads to the conclusion that terthienyl is not stored as an inert compound in 5 months old Tagetes plants, and that the time of the maximum peak of incorporation of sodium sulfate-S-35 into terthienyl does not exceed two days.

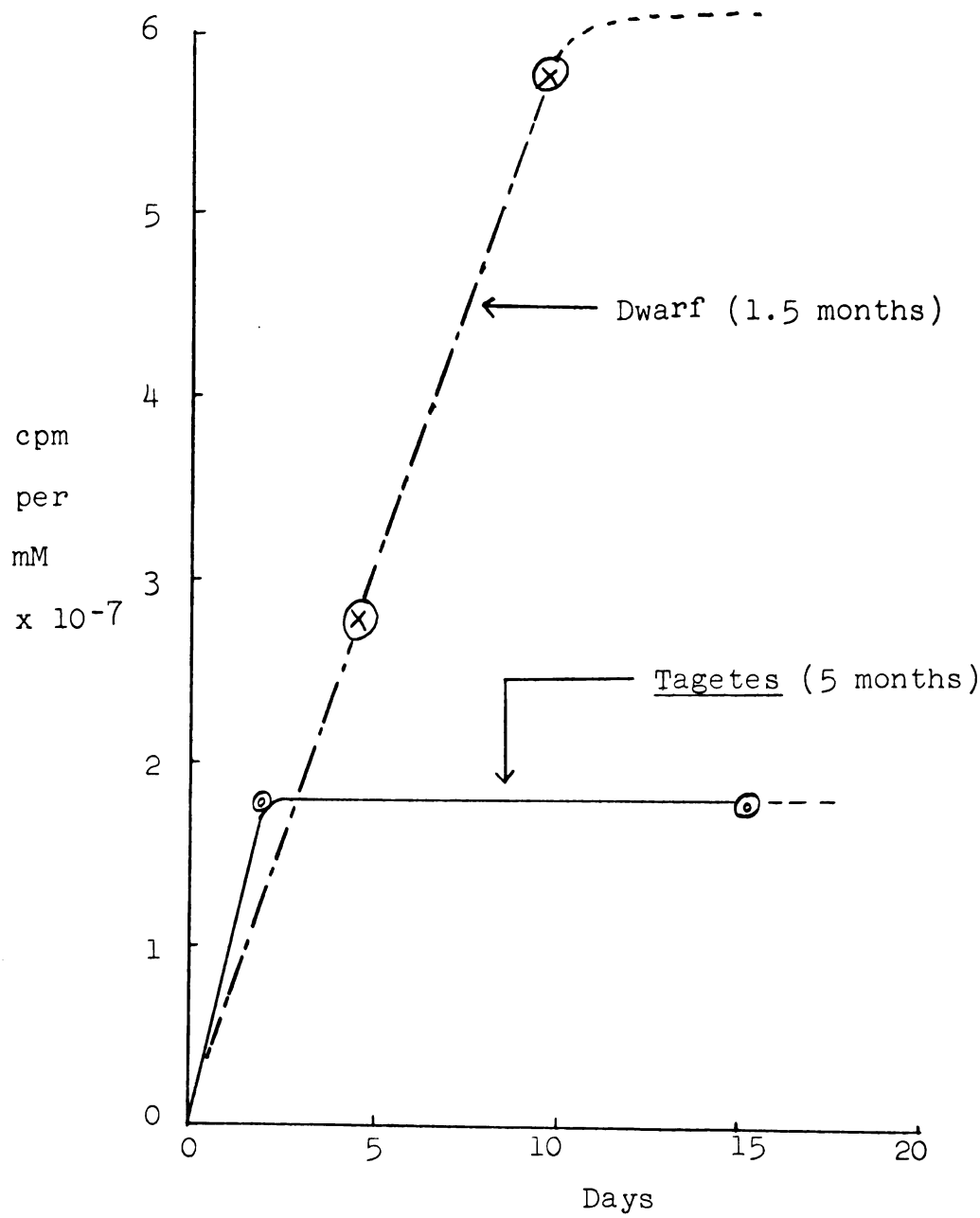
The results of the incorporation using younger plants, but not a Tagetes variety, were quite different from the previous results. At the end of 5 days after feeding, the dilution factor of terthienyl was 161 and decreased approximately by half at the end of ten days to 77. The per cent incorporation increased from 0.04% to 0.06%. These results can be explained by considering Scheme 5. Since the specific activity of terthienyl doubled when the time of incorporation was doubled, k_1 is greater than $k_2 + k_3$. Either terthienyl-S-35 was being stored in the younger plants, or k_3 was considerably slower at this time. The results still are in agreement with the above conclusion that terthienyl is an "active" compound, capable of undergoing further metabolic transformations. The high increase of radioactivity may be due strictly to the changes in rates of over-all synthetic vs. breakdown pathways, k_1 vs k_2 and k_3.

The data does not reveal precisely the ideal conditions for maximum incorporation. Young plants are more suitable because of their metabolic activity as evidenced from the high incorporation of sodium sulfate-S-35 into terthienyl with time.

It is possible that the peak of highest specific activity of terthienyl-S-35 in young plants is near 10 days, and that breakdown of terthienyl-S-35 may begin to equilibrate and attain equilibrium conditions where $k_1 = k_2 + k_3$. Such a condition can be represented by the curves in Figure 2 taken from the results previously described. If the rate of synthesis of terthienyl predominates over the rate of breakdown, a straight line will result as shown in both curves. If the younger plants are synthesizing terthienyl more actively than the older plants as is shown in Figure 2, a higher specific activity would result. As the age of the plants increases, the amount of terthienyl-S-35 should be less at equilibrium, because of k_1 becoming slower and/or k_2 and k_3 increasing, so the equilibrium condition of $k_1 = k_2 + k_3$ is attained.

The feeding method was important, since feeding via stems did not result in any incorporation into terthienyl. Even the amount of activity incorporated into the ethanol extract was less in the 2 day experiments; 13.6×10^4 cpm via stems and 24.0×10^4 cpm via roots per g. of root extracted after feeding sodium sulfate-S-35.

Figure 2.--Incorporation of Sodium Sulfate-S-35 into Terthienyl. Specific Activity of Terthienyl-S-35 vs Time



The importance of the feeding method was demonstrated using L-methionine-S-35. A small incorporation of sulfur-35 into terthienyl was observed; however, the dilution factor was 60,500. This indicated that sulfur-35 from methionine which was present in the upper portion of the plant was not readily available for introduction into the biosynthetic pathway. The higher dilution factor can be compared to that obtained from DL-methionine-S-35, which was administered via roots yielding a value of 4,420. A comparison of plant ages was observed in Experiments 6 and 7 which showed that more than twice the activity was found in the ethanol extract of the roots at the end of 2 days in the 4 month old plants. The activity of the ethanol extract in Experiment 9 cannot be explained, except that an error in the radioactivity assay was possible.

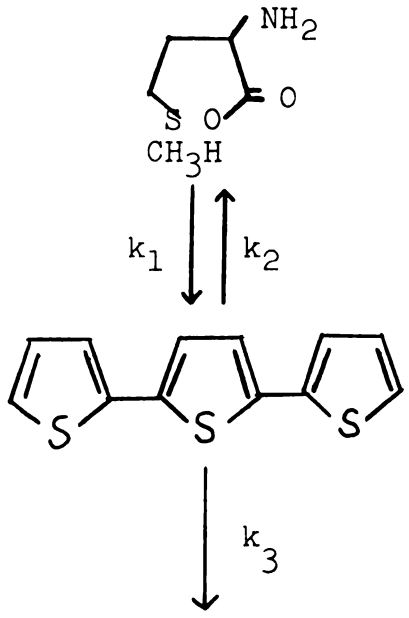
Since root feeding was necessary in order that sulfur-35 could be incorporated into terthienyl, it was concluded that the active site of synthesis of terthienyl was in the roots. Stem feedings in Experiments 1 and 6-10 resulted in essentially none of the sulfur-35 being incorporated regardless of the original form, sodium sulfate or methionine. Incorporation of sulfur-35 was observed from sodium sulfate and methionine from root feedings as seen in Experiments 1-10 and 23, the movement of sulfur-35 from the stems to the roots was less than from the external medium (nutrient solution) into the roots.

A comparison of root vs stem feedings was also observed with carbon-14 which was administered as DL-methionine-1-C-14 via stems and DL-methionine-2-C-14 via roots. None of the radioactivity was incorporated into terthienyl from stem feedings as seen in Experiments 11-13. Little of the carbon-14 was present in the ethanol extract of the roots after 10 days, although the amount of activity approximately doubled at the end of 19 days. Even then, no carbon-14 was detected in terthienyl. This would be expected if the carbon-14 compound did not reach the site of synthesis. In contrast, the results obtained from root feedings was different. After two days carbon-14 was incorporated into terthienyl giving a dilution factor of 1,430. At 10 and 20 day intervals no incorporation was observed, thus, supporting the conclusions from the sulfur-35 experiments.

If one considers a biosynethtic pathway with respect to carbon-14, Scheme 6 can be constructed. The symbols, k_1, k_2, and k_3, have the same definitions as those given for Scheme 5. One must assume that methionine-2-C-14 is metabolized by the plant either to terthienyl-C-14 or other products. The lack of any activity in terthienyl after 10 and 20 days indicates that terthienyl was not stored but actively metabolized. Introduction of methionine-2-C-14 in Scheme 6 results in a "wave" of activity traveling through the entire pathway. The evidence of the "wave" is seen by the specific activity of terthienyl at the end of two days and declining until all

Scheme 6

Biosynthetic Pathway of DL-Methionine-2-C-14 to Terthienyl



Metabolic Products

of the terthienyl-C-14 is further metabolized. If methionine-2-C-14 were available for incorporation all the time, a leveling of specific activity would be seen as discussed above for the sodium sulfate experiments. The assumptions that sulfur-35 is constantly available from sodium sulfate-S-35 and that carbon-14 from methionine is only available during the initial period of incorporation seems reasonable.

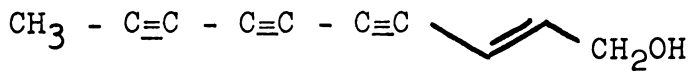
In Experiments 17-22 carbon-14 from sodium acetate-1-C-14 was not incorporated into terthienyl when fed via stems. Some incorporation into terthienyl was observed when administered via roots (0.002-0.004%). The activity of the ethanol extracts of the roots is approximately 10 times higher from the root feedings, indicating that carbon-14 in the stems does not move toward the roots as fast as the carbon-14 from the external medium (nutrient solution). If the assumption is made that acetate-1-C-14 is metabolized quickly as assumed with methionine, then the same pattern of specific activity of terthienyl could be expected. No incorporation after two days and only trace incorporation after 10 and 20 days indicated that acetate-1-C-14 was probably not a direct precursor, and that the activity in terthienyl resulted from a spreading of activity throughout the metabolites of the plant.

However, incorporation of radioactivity from acetate-1-C-14 into the terthienyl fraction is worthy of further discussion. Horn and Lamberton (16) reported the isolation

of glycerides of unsaturated fatty acides in their terthienyl fractions from the roots of marigolds. They eliminated the impurities by a saponification step before purification of terthienyl by chromatography. Saponification was not utilized in the current isolations, and the radioactivity in the crude terthienyl may have resulted from acetate incorporation into the unsaturated glycerides. The disappearance of radioactivity in the 10 and 20 day experiments of the crude terthienyl fraction and the increase of radioactivity in the purified terthienyl may have some correlation to each other. If acetate was a precursor to terthienyl and passed through unsaturated fatty acids in the biosynthetic scheme, such an observation as this is not unreasonable. On the other hand, the maximum amount of radioactivity in terthienyl was 0.004% and randomization of the carbon-14 could be responsible for this small amount of radioactivity which was found.

The results of the acetate experiments suggests further investigations. Malonic acid-C-14 may serve as a better precursor to terthienyl if the true pathway proceeds through an "acetate derived" carbon compound such as a fatty acid or a derivative. Bu'Lock and his coworkers (25) have shown that diethyl malonate-2-C-14 is incorporated by 6% into oleic and palmitic acids and 2-6% into an aromatic, 6-methyl salicylic acid, in fungus cultures of Penicillium urticae. The two fatty acids and the aromatic compound are "acetate derived" and were observed to incorporate the activity from the

malonic acid derivative. Bu'Lock and Smalley have extended their work to the polyacetylenes by feeding diethyl malonate-2-C-14 to fungus cultures of a Basidiomycetes, Tricholoma grammopodium. An incorporation of 0.1% was observed into the compound, dec-2-en-4,6,8-triyne-1-ol.



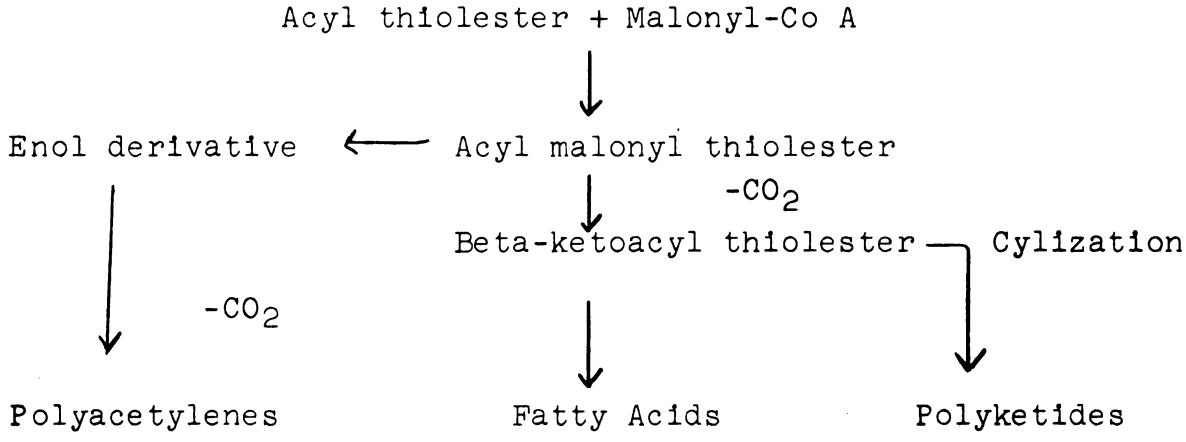
Compound XIX Dec-2-en-4,6,8-triyne-1-ol

The activity located in C_1-C_8 was 97% which indicated that C_9 and C_{10} served as the "starter group" with the remainder of the compound being formed from malonate.(26) A scheme was suggested by Bu'Lock for the formation of the three different "acetate derived" compounds and is shown as Scheme 7. It provides a method of forming triple bonds which has also been postulated by Jones (27) and demonstrated biogenetically in the laboratory by Fleming and Harley-Mason (28, 29). The same type of malonate conversion has been observed by Bentley and Keil (34) in the biosynthesis of penicillic acid in the fungus, Penicillium cyclopium.

Bu'Lock and his workers have followed the rate of synthesis from different precursors of polyacetylene antibiotics in Basidiomycetes B. 841.(30) Using acetate and ethanol as precursors, it was estimated that 90% of the ethanol was utilized to polyacetylenes by the way of acetate. Glucose was also consumed at equivalent rates, but the

Scheme 7

Biosynthesis of "Acetate Derived" Compounds (26)



conversion to polyacetylenes was almost six times less than that of ethanol. They concluded that the conversion of glucose to polyacetylenes consisted mainly of a pathway not involving acetate. Feeding experiments are currently being conducted in these laboratories with marigold plants using uniformly labelled glucose as a suspected precursor to terthienyl.

The isotope dilution experiments were completed only with DL-methionine-S-35. The isolated terthienyl was calculated by this method to establish the original specific activity. The quantity of terthienyl isolated as shown in Table 16 is high in comparison to the previous values, Table 20. Even for this high amount, the specific activity of approximately 10^6 cpm/mM produced a dilution factor of 4,420. It was concluded that sulfur-35 was incorporated when supplied as methionine, but the incorporation is about 1/10 that of sodium sulfate. It was surprising that sulfur-35 was not incorporated when fed as sodium hydorgen sulfide-S-35. One explanation is that sulfur fed in the reduced form was not available at least during the short term experiments (30 hours or less).

No carbon-14 was incprorated from succinic acid-2,3-C-14 or DL-glutamic acid-2-C-14. The lack of incorporation from succinate appears to eliminate compounds of the tricarboxylic acid cycle as precursors to terthienyl. Glutamic acid was investigated because it was readily available.

With respect to Schemes 1 and 2, the results obtained in this investigation do not lend support to either. It is better perhaps to speak of these schemes as involving an "acetate" pathway, since some polyacetylenes have been shown by Bu'Lock to be composed of head-to-tail condensations of acetate. An "acetate" pathway does not distinguish if polyacetylenes or polyketones are involved in the biosynthesis of terthienyl.

There is some support for a type of pathway represented in Scheme 4. The results show that sulfur-35 and carbon-14 when supplied as methionine were not incorporated to the same extent. The dilution factor of methionine-S-35 calculated from the isotope dilution method was 4,420, but was 750 when calculated in the same manner as Experiments 2-5, that is, assuming the concentration of terthienyl as 0.44 micromoles per g. of root. The value of 750 is still about three times greater than that of sodium sulfate-S-35. It could be that sulfur-35 is a precursor to terthienyl through methionine (or homocysteine) and also by another pathway. This would explain the higher dilution factor resulting from methionine-S-35. Experiments 14 and 23 support Scheme 4. Additional evidence is needed and should be obtained by feeding methionine-2-C-14, isolating terthienyl-C-14, and degradating the compound to locate the position of the label.

5-(3-Buten-1-ynyl)-2,2'-dithienyl was identified from its UV absorption, color test with isatin, positive test with

permanganate, and the identification of its oxidation product by treatment with potassium permanganate in acetone. The UV absorption obtained in this work was usually 2-6 millimicrons higher than that reported in the literature. When the dithienyl compound was isolated from several kg. of blooms during the summer, absorption in petroleum ether was 341 millimicrons, and this shifted to 346 millimicrons in ethanol. Two explanations could account for this behavior; first, solvent effects of the polar ethanol compared to the nonpolar petroleum ether, and second, a reaction which occurred such as polymerization was seen by the appearance of small, yellow balls which were present when the petroleum ether was evaporated and replaced with ethanol. These yellow balls, insoluble in ethanol, were also reported by Horn and Lamberton.(16) Therefore, the shift to longer wave-lengths may have resulted from a change of structure and not solvent effects.

The identification of the thiophene 2,5-dicarboxylic acid was not conclusively shown. The oxidation product was shown to be present in trial 1 but could not be detected in trial 2. If the dithienyl compound polymerized before the oxidation procedure, then the acid probably would not be formed. Uhlenbroek and Bijloo previously identified the product from its UV absorption, but could not investigate the compound further because of the small amount available.

Experiment 4 using sodium sulfate-S-35 showed about the same amount of radioactivity in the dithienyl compound as in

terthienyl, Table 14. The specific activity was not determined. A trace of activity, 500 cpm, was observed in the dithienyl fraction in the two day experiment of L-methionine-S-35 when fed through the stems. The activity was not important, because the purity of the fraction was unknown with respect to the radioactivity. A trace of activity, 400 cpm, was found after 20 days from the feeding of DL-methionine-2-C-14 via roots. A randomization of carbon-14 could account for this small amount of radioactivity.

From the results shown in Table 17, it is seen that 5-(3-buten-1-ynyl)-2,2'-dithienyl might be relatively near terthienyl in the metabolic scheme. It could be only one step away. Further work is needed to determine whether it is in the biosynthetic pathway, or if it is a degradative product of terthienyl.

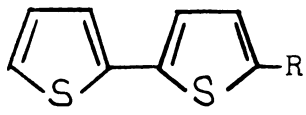
It has been shown by Uhlenbroek and Bijloo (23) that one of the two active nematicidal principles of the Tagetes is terthienyl. They reported that cultivation of Tagetes reduced the populations of the nematode, Pratylenchus spp., and that concentrates from the roots showed higher activity than any other part of the plant. Oostenbrink and his co-workers (31) reported the cultivation of Tagetes for short periods in the spring or autumn had no effect on nematodes in the soil, whereas a period of 3-4 months cultivation was effective against nematodes. These results were confirmed in the laboratory by Omidvar who leached roots of 8-10 week

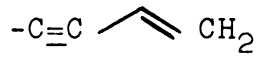
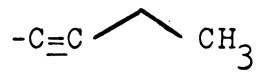
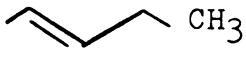
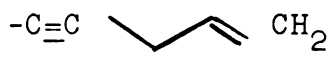
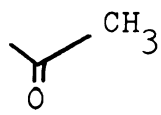
old plants of Tagetes minuta, Tagetes florida, and Tagetes signata. (32) He saved the diffusates and observed no nematocidal effect. His short term experiments were considered ineffective because of the low amounts of terthienyl being produced.

If Tagetes are able to reduce populations of nematodes by the cultivation of the plants, it must be assumed that terthienyl is liberated by the roots into the soil. The presence of terthienyl in the nutrient solution was investigated. If terthienyl was present in the nutrient solution, it was not detected even by paper chromatography. Instead, a blue fluorescent compound was discovered which absorbed in the UV in such a way as to indicate a dithienyl derivative, 335 and 251 millimicrons). The dithienyl type structure was also suspected because of the compound's behavior on a paper chromatogram using 70% methanol as solvent (R_f-ratio with terthienyl of 1.13). The compound was radioactive after 10 days when acetate-1-C-14 was administered.

It is possible that its structure is similar to that of 5 -(3-buten-1-ynyl)-2,2'-dithienyl. In Sorensen's attempt to identify this or a similar compound, a number of derivatives were synthesized, and their UV absorptions were observed, Table 21.(33) The unknown compound in the nutrient solution could have a 4 or 5 carbon chain as a substituent group and unsaturation which might be similar to one of the first 4 R-groups listed in Table 21. When Tagetes are cultivated in the

TABLE 21.--UV Absorption of Some Dithienyl Compounds



R-Group	Millimicrons
	340
	335
	339
	Unknown
	343

soil, it is conceivable that the above compound is converted to terthienyl or some other nematocidal compound by soil bacteria or by the nematodes themselves. Further identification of the compound may prove interesting. The specific action of the effect of Tagetes on nematodes remain speculative according to the literature in that field.

The concentration of terthienyl is variable among difference varieties, ages of plants, and apparently whether isolated from petals or roots as shown by the results in Table 22. Zechmeister noted at the time he first isolated terthienyl in 1947, that none could be obtained from another species of Tagetes. Uhlenbroek and Bijloo suggested that he did not investigate the roots, but only the petals. Concentration differences are seen from the data in Table 22, particularly in the dwarf variety and in the two Tagetes species. Another factor must be considered with respect to the dwarf variety, that is, the time of the year that terthienyl was isolated. It is possible that terthienyl may be stored in the plant as the growing season enters the later maturation of the plant. The data in Table 22 show the higher concentrations of terthienyl in the roots compared to the petals.

The conditions which were present in this work were similar to that of the isolation from 2 month old Tagetes erecta yielding approximately 112 microg. per g. of root as given in Table 22.

TABLE 22.--Weight of Terthienyl Isolated from Various Species of Marigolds

Species	Time Harvested	Weight of Roots, kg.	Terthienyl Microg./g.Root	Reference
Tagetes erecta	Unknown	14, petals	15	(1)
Tagetes erecta	Unknown	24, roots	23	(6)
Tagetes minuta	Unknown	0.9, roots	200	(16)
Tagetes erecta	2 mo.	0.0004,roots	112	This work
Dwarf	June	5.5, petals	0.7	This work
Dwarf	August	6.7, petals	22	This work

The investigation of dandelion blooms was initiated in order to isolate terthienyl. The results showed that terthienyl was present in very low quantities. The low amount in the fraction from the alumina column prevented further identification of the compound suspected to be terthienyl; therefore, the presence of terthienyl in dandelion blooms was not conclusively proven.

APPENDIX

Investigation of Dandelion Blooms

In the spring of 1962, 2.7 kg. of yellow dandelion blooms were picked from the lawns of the Michigan State University campus. They were immediately covered with 20 ℓ. of 95% ethanol in a large crock and allowed to stand for 13 days. The ethanol was evaporated under reduced pressure leaving a dark, vicous residue. The solid was redissolved in 1 ℓ. of methanol with 50 g. of potassium hydroxide, and the solution was refluxed for 60 hours. The saponification mixture was diluted with water and extracted with petroleum ether. The ether solution was concentrated to 50 ml. and dried over sodium sulfate. The dried ether solution was chromatographed over an alumina: celite (25: 2.5 g.) column. Fractions of 25 ml. each were collected.

Fractions 3 and 4 each gave a red color with isatin. Paper chromatography of each fraction with a known sample of terthienyl indicated that two different compounds were present. The first compound was eluted in fraction 3, and a trace of it was seen in fraction 4. The R_f value from 66% methanol was 0.78, terthienyl = 0.64. The second compound in fraction 4 gave an R_f value of 0.64 with paper

chromatography. Both compounds displayed a blue fluroescence on paper under an ultra violet lamp. Further investigation was not continued because of the minute quantities which were available.

Three other compounds were eluted with 5-20% diethyl ether eluant: 1, fraction 10 - colorless crystals, m.p. 73° C; 2, fraction 14 - colorless crystals, m.p. 148-150° C; 3, fraction 16 - yellow needles, m.p. 130-133° C. Compounds 1 and 2 were crystallized from methanol: water.

The compounds in fractions 3 and 4 showed similar characteristics to those of 5-(3-buten-1-ynyl)-2,2'-dithienyl and terthienyl such as the elution time from the alumina column, reaction with isatin, and their behavior on paper chromatograms. Further investigation as to the identification of these two compounds may prove interesting, since Sorensen has stated that polyacetylenes and thiophenes have not been found in members of the Compositae which are "milk containing" as was observed in this dandelion species.

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