AN INVESTIGATION OF THE BIOSYNTHESIS OF POLYTHIENYLS IN *TAGETES ERECTA* L.

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
S. M. de Paul Palaszek, R. S. M.
1965

THESIS

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ABSTRACT

AN INVESTIGATION OF THE BIOSYNTHESIS OF POLYTHIENYLS IN TAGETES ERECTA L.

by S. M. de Paul Palaszek, R. S. M.

The intent of this investigation was to examine the role of acetogenesis in the formation of the thiophene ring of terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl in Tagetes erecta L., to discover the physiological precursor of the sulfur atom of the thiophene ring, to ascertain the relationship between the polythienyls of Tagetes, and to explore a possible route to the systematic degradation of terthienyl.

Sodium sulfate-^{35}S, DL-glucose-UL-^{14}C, DL-ornithine-2-^{14}C, sodium malonate-2-^{14}C, DL-methionine-2-^{14}C, sodium pyruvate-3-^{14}C, DL-cysteine-^{35}S, DL-cystine-1-^{14}C, DL-serine-3-^{14}C, sodium pimelate-7-^{14}C and malonic-2-^{14}C acid were examined for their relative efficiency as precursors to terthienyl in Tagetes erecta L. The radioisotope form was administered to the plants hydroponically. Terthienyl (and 5-(3-buten-1-ynyl)-2,2'-bithienyl in some experiments) was isolated from the roots at specific times from the initial feeding, and its radioactivity was determined.

The least dilution of the carbon-14 radioisotope in terthienyl occurred with DL-methionine-2-^{14}C, malonic-2-^{14}C acid, and pimelate-7-^{14}C. Dilution factors of 1385, 1865, and 445, respectively, were calculated. Organic acids and malonyl CoA, therefore, have been shown to have a role in the biosynthesis of naturally occurring thiophenic compounds in Tagetes, and preformed polyacetylenes are not necessarily required in the synthesis.

DL-Cysteine-^{35}S has clearly been shown to be the precursor to the sulfur atom in the polythienyls of Tagetes. A time dependent study of the incorporation of DL-cysteine-^{35}S into terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl suggested that these polythienyls are independently metabolized at some point in their biosynthesis.

Isolation of terthienyl after various metabolic intervals for sulfate-^{35}S and DL-methionine-2-^{14}C indicated that 8 to 10-week-old plants were optimal for this work.

Fungal and bacterial uptake of the radioisotope forms administered was shown to be insignificant. The absorption by root tissues, however, of DL-cysteine-^{35}S and DL-cystine-1-^{14}C from aqueous solution has revealed a selectivity to an equilibrium specie.

The attempt to obtain terthienyldicarboxylic acid by a metal-hydrogen exchange reaction between terthienyl and

n-butyllithium, formylation, and subsequent oxidation has led to a difficultly separable mixture of products. Acetylation of terthienyl has, on the other hand, provided a 37-47% yield of the monoacetylated derivative. Unreacted terthienyl, 25-31%, was recovered in the acetylation reactions.

Desulfurization of a crude sample of terthienyldicarboxylic acid to a long chain acid was accomplished with slightly basic Raney nickel. The Beckman Monoxime degradation of long chain acids and the Barbier-Wieland procedure were examined. The latter gave 52 and 53% yields of the degradation products. The Beckman Monoxime procedure was unsuccessful.

This complete examination of possible precursors to the polythienyls in Tagetes decidedly indicates that an organic acid (pimelic acid) of high specific activity and known labeling pattern ought to be the precursor used in a degradation study of terthienyl. The scheme suggested by this work consists of conversion of 5-acetylterthienyl to the carboxylic acid by the haloform reaction (already known), desulfurization with slightly basic Raney nickel, and Barbier-Wieland degradation of the long chain acid.

AN INVESTIGATION OF THE BIOSYNTHESIS OF POLYTHIENYLS IN TAGETES ERECTA L.

By

Sister Mary de Paul Palaszek, R. S. M.

A THESIS

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES - Continued

LIST OF FIGURES

LIST OF CHARTS

CHART Page

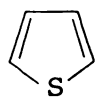
LIST OF APPENDICES

APPENDIX Page

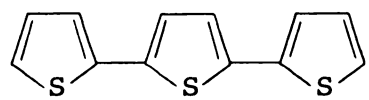
INTRODUCTION

The divergence between plant and animal chemistry is reflected in the discovery of thiophenic compounds in the tissues of higher plants. Biotin, a tetrahydrothiophene, is the only known simple thiophene, I, derivative common to plant and animal tissues.

The polythienyl, terthienyl, II*, discovered by Zechmeister and Sease (1) in 1947 in extracts of Tagetes erecta L., is the first representative of thiophenic natural products of higher plants. Of the forty-four known thiophenic compounds, Tables 1 to 3 and 7, sixteen are discoveries of the current year. All these compounds are the constituents of species in the Compositae Family except junipal, a fungal product.



I
Thiophene



II
Terthienyl

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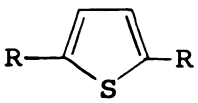
*2,2';5',2"-Terthienyl is the nomenclature approved by the I.U.P.A.C. 1957 rules on Organic Nomenclature. Terthienyl is used throughout this work for simplicity and the unambiguity of the term in this context.

Table 1. Naturally Occurring Thiophenic Compounds Isolated 1947-1962 (38), 

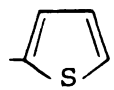
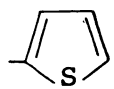
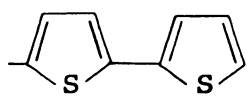
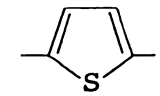
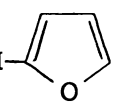
Substituents R	Substituents R'	Absorption Bands (mμ)	Species	Ref.
$H_3CC{\equiv}C-$	$-CHO$	216,286,320(ethanol)	Daedelia juniperina(fungus)	6
$H_3CC{\equiv}C-$	$-C_6H_5$	310(hexane)	Coreopsis grandiflora Hogg ex Sweet	14
$H_3CC{\equiv}C-$	$-CH{=}CHCOOCH_3$	235,341	Chrysanthemum vulgare	34
H	$-C{\equiv}C(CH{=}CH)_3H$		Matricaria inodora	35
H	$-C{\equiv}C(CH{=}CH)_2COCH_3CH_3$		Matricaria inodora	35
H	$-C{\equiv}CCH{=}CH(CH_2)_2COCH_2CH_3$		Matricaria inodora	35
H_3C-		258,369(hexane)	Bidens radiata Bidens ferulaefolia	36
H		251,340(isooctane)	Tagetes erecta	15
H		252,350(hexane, ethanol)	Tagetes erecta Tagetes glanderlifera	1,37

Table 2. Recently Isolated Thiophenes of the Structure  R'

Substituents R	Substituents R'	Absorption Bands (mμ)	Quantity (weight %)	Species	Ref.
$H_3CC{\equiv}C-$	$-C{\equiv}CC{\equiv}CCH{=}CH_2$	258,264,274,(324), 338,357(ether)	0.01	Schkuhria senecioids	11
$H_3CC{\equiv}C-$	$-C{\equiv}CCH{=}CHCH{=}CH_2$	253,334,357(ether)	1.0	Rudbeckia amplexicaulis	11
$H_2C{=}CHC{\equiv}C-$	$-C{\equiv}CCH{=}CHCHO$	268,360,(380)(ether)	0.0004	Baeria aristata	11
$H_2C{=}CHC{\equiv}C-$	$-C{\equiv}CCH{=}CHCH_2OH$ acetate	335(ether) 335(ether)	0.0004	Baeria aristata	11
$H_2C{=}CHC{\equiv}C-$	$-C{\equiv}CCH{=}CHCH_3$	333,(355)(ether)	0.0003	Serratula radiata(whole plant)	11,39
H	$-C{\equiv}CCH{=}CHCH{=}CHCH{=}CH_2$	257,332,335(ether)	0.01	Xeranthemun cylindraceum	11
				Matricaria oreades	40
H	$-CH_2CH{=}CHCH{=}CHCONHCH_2CH(CH_3)_2$			Chrysanthemum foeniculaceum, fructescens	41,42
H	$-C{\equiv}CCH{=}CH-$ 	260,339(356)(hexane)	0.03(cis) 0.00003(trans)	Santolina pinnata vv	43

continued

Table 2 - Continued

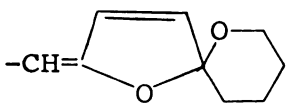
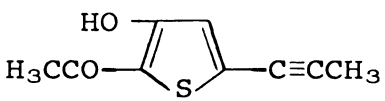
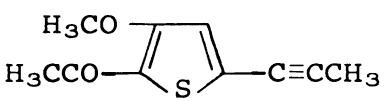
Substituents R	Substituents R'	Absorption Bands (mμ)	Quantity (weight %)	Species	Ref.
H		(315),327,(342)(ether)	0.02	Artemisia ludoviciana	43
H_3C-	$-C{\equiv}CCH{=}CHCOOCH_3$	338(ether	0.004	Anthemis nobilis L.	43
H	$-C{\equiv}CCH{=}CHCOOCH_3$	(335),320,262(ether)	0.004(trans)	Anthemis fruscata B.	44
				Artemisia arborescens	60
				Artemisia arborescens	60

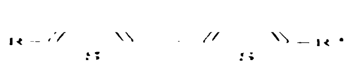
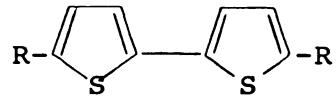
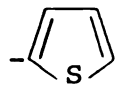
Table 3. Recently Isolated Polythienyls of the Structure 

Table 3. Recently Isolated Polythienyls of the Structure 

Substituents R	Substituents R'	Absorption Bands (mμ)	Quantity (weight %)	Species	Ref.
H		350(hexane)	0.01	Tagetes minuta	37 45
H	$-C\equiv CCH=CH_2$	251,341 251,345(ether)	0.09 0.015	Tagetes minuta Tagetes erecta	37 46
H	$-C\equiv CCH_2CH_2OH$	242,328,334		Tagetes minuta	45
H	$-C\equiv CCH_2CH_2OCOCH_3$		0.026	Tagetes erecta Tagetes minuta	46 45
H	$-C\equiv CCH(OH)CH_2Cl$	245,336		Tagetes minuta	45
$H_3COCOCH_2-$	$-C\equiv CCH=CH_2$		0.0006	Bidens dahlioides (whole plant)	39
$H_2(OH)C-$	$-C\equiv CCH=CH_2$		0.0002	Bidens dahlioides (whole plant)	39
$H_2C=CH-$	$-CH=CHCH_3$	254,362,366(ether)	0.0018	Bidens connatus (whole plant)	39

The biosynthetic origin and mode of formation of the thiophene ring in natural products have not been definitively established. Several biological pathways to terthienyl have been proposed; schemes A to D (Table 4) are based on considerations of biogenetic* evidence and practical laboratory syntheses. Schemes E and F are the results of biosynthetic* investigations with Tagetes erecta L. (2,3), Chrysanthemum segetum (4), and Echinops sphaerocephalus (5).

A favored origin of the carbon skeleton of the thiophenic compounds from higher plants is a long chain polyacetylene. The biogenetic support of this hypothesis cites the similarity in structure and source between terthienyl and polyacetylenes.

Since the thiophenic natural products reported except junipal (6) are constituents of one taxonomical group of plants, Compositae, identical biosynthetic pathways among the thiophenic compounds appear to be operative. All natural thiophenic compounds possess an unbranched chain of ten to thirteen carbon atoms and are generally isolable from plant root tissue.

The polyacetylenes, compounds containing conjugated triple bonds, are widely distributed in the Compositae Family (7,8,9,10). Their formulae range from eight to

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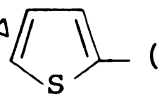
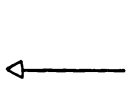
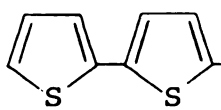
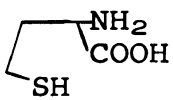
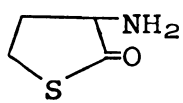
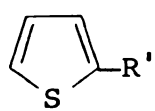
*Biogenetic denotes an evolutionary development from pre-existing biological material. Biosynthetic is the term used in referring to the production of a chemical compound by a living organism utilizing synthesis or degradation.

Table 4. Proposed Biological Pathways to Terthienyl

Scheme	Proposed Precursor	Intermediates	Reference
A	$R(C{\equiv}C)_6R'$ + H_2S	Unknown intermediate	18
B	$H_3CCO(CH_2CO)_4CH_2COOH$	$HC{\equiv}CC{\equiv}C(CH_2CO)_3CH_2COOH$ → $HC{\equiv}C(C{\equiv}C)_nR$ +  $(CH_2CO)_nR$	14
C	$HC{\equiv}CC{\equiv}CR$	 ← R ← Unknown	9
D	$HC{\equiv}C(C{\equiv}C)_nR$ + H_2S	 $-C{\equiv}CCH{=}CH_2$	15
E			3
F	$R(C{\equiv}C)_2R$ + CH_3SH	$H_3CS\text{-}CR{=}CHC{\equiv}CR'$ →  $-R'$	4,14

eighteen carbon atoms in a continuous chain with some preference for chains of ten and thirteen carbon atoms. The polyyne III occurs abundantly among one-hundred forty genera of the Compositae Family,* and IV is typical in ten of twelve tribes of this Family (11,12). Derivatives of acids, methyl esters and isobutylamides, are the simpler acetylene compounds found in the root and aerial parts of the plants.

$H_3C(C{\equiv}C)_5CH{=}CH_2$ III

$H_3CCH{=}CH(C{\equiv}C)_4CH{=}CH_2$ IV

The well-known instability of compounds with a system of extended conjugated triple bonds (13) is the basis of Sorensen's (14) proposed biological pathway, Scheme B (Table 4). This pathway assumes that the dehydration and the dehydrogenation of two oxo-functions of a polyketone produces an acetylenic ketone. A monothienyl derivative is formed by addition of hydrogen sulfide across the conjugated triple bonds with cyclization. Repetition of this sequence of reactions yields terthienyl according to this Scheme, while the omission of the reaction with hydrogen sulfide produces the polyacetylenes of the Compositae Family. The discovery of 5-(3-buten-1-ynyl)-2,2'-bithienyl as a component of the root extract from Tagetes erecta in which

*One classification of the Natural Order, Compositae, designates 806 genera for thirteen tribes (7).

terthienyl had been isolated is taken as biogenetic support for the sequential formation of the thiophene ring (15). The structures of all known thiophenic natural products are suggestive of this mode of formation.

The requirement for a polyketonic or polyacetylenic structure is ultimately supplied by acetyl CoA* in biological systems. Chart 1 summarizes the experimentally demonstrated and hypothesized pathways to polyacetylenes and polyketones. Nemotinic acid (V) and matricaria ester (VI) from fungi (16) and matricarianol (VII) from seedlings of Santalum acuminatum (10,17) show an alternate labeling pattern when grown in the presence of malonate-2-^{14}C and acetate-1-^{14}C respectively. Thus, these polyynes, as acetate-derived are established.

$HC{\equiv}CC{\equiv}CCH{=}C{=}CHCH(OH)CH_2CH_2COOH$

V

$H_3CCH{=}CHC{\equiv}CC{\equiv}CCH{=}CHCOOCH_3$

VI

$H_3CC{\equiv}CC{\equiv}CC{\equiv}CCH{=}CHCH_2OH$

VII

Laboratory syntheses justifying the biogenetic arguments that the thiophene ring in biological systems could be formed by the addition of hydrogen sulfide to an acetylenic

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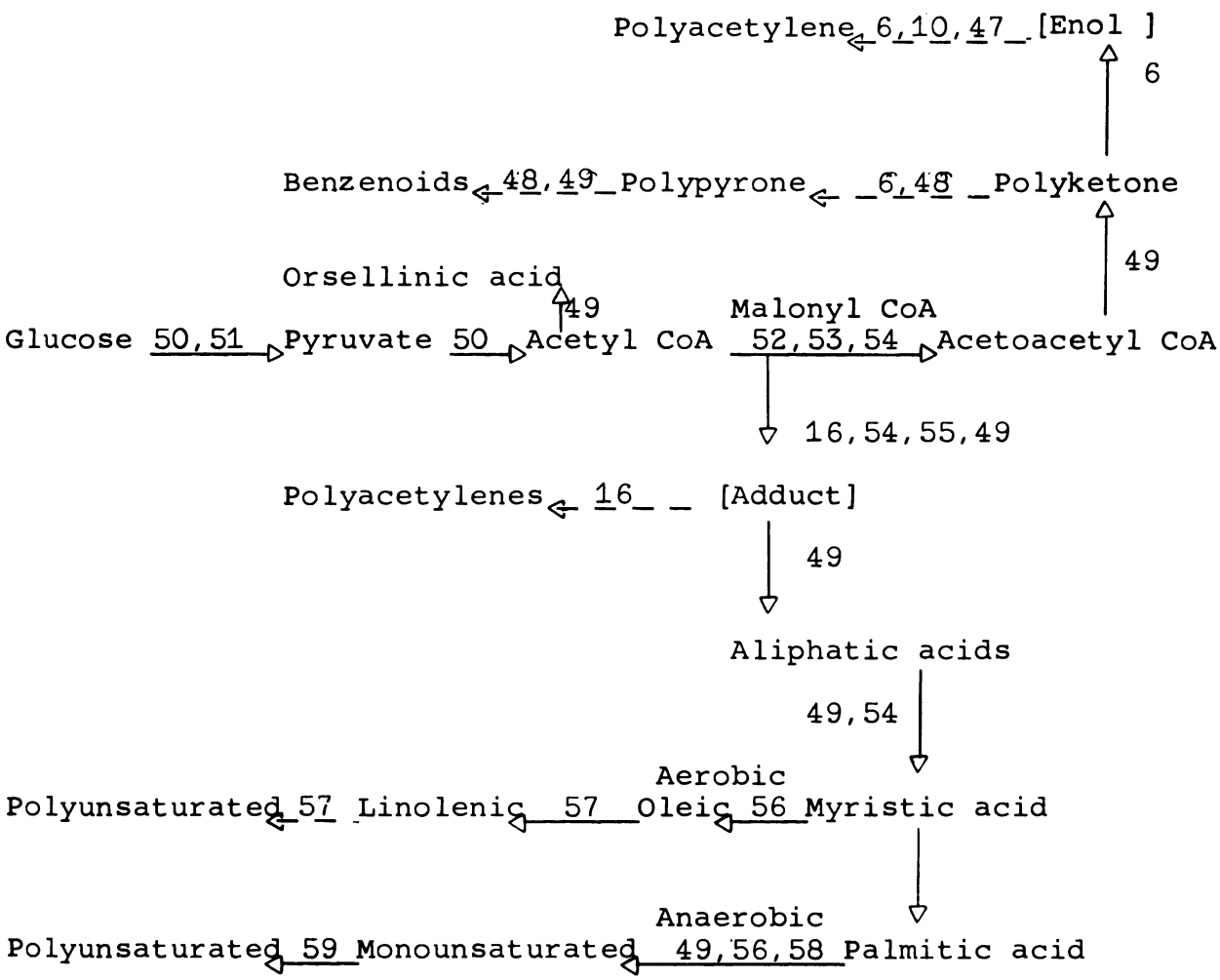
*Acetyl Co-enzyme A is an enzyme thiol ester of acetic acid.

Chart 1. Partial Acetyl CoA Flow Chart for the Plant Kingdom



*References associated with each transformation are given as numbers above the arrow. Transformations shown by in vitro enzyme study or carbon-14 in vivo study are indicated by a solid line and routes proposed from biogenetic considerations and laboratory syntheses are indicated by a broken arrow.

bond generated in a stepwise fashion from a polyketone or, as Challenger suggested (18), by addition of hydrogen sulfide to a preformed polyacetylene are the contribution of Schulte and co-workers (19). The formation of thiophenic compounds is effected in Schulte's syntheses by the interaction of polyacetylenes with sodium sulfide at the boiling point of the methanol-water solvent system (Table 5). It should be noted, however, that in no instance was more than one heterocyclic ring formed. Bohlmann's (20) attempt to synthesize 5-(3-buten-1-ynyl)-2,2'-bithienyl by the addition of hydrogen sulfide to 8-(2-thienyl)-3,5,7-octatriyne-2-ol reveals the thermodynamic stability of the acetylenic bond adjacent to the thienyl function.

Jones (9) is notably alone in proposing that the thiophenic natural products may be the source of polyacetylenes in nature rather than metabolic products of polyacetylenes (Scheme C of Table 4). It is well-known that Raney nickel desulfurization of thiophene compounds is utilized synthetically to obtain fatty acids of a desired length and with a specific carbon-14 labeling pattern (21).

The first biosynthetic investigation of the origin of terthienyl in Tagetes erecta was a study of the incorporation of sulfur and carbon radioisotopic forms (2,3). The data, Table 6, show a high incorporation of inorganic sulfate, low incorporation of methionine-2-^{14}C and -^{35}S, and negligible incorporation of acetate-1-^{14}C and bisulfide-^{35}S. All feedings of likely precursors to the plants were by hydroponic

Table 5. Products of Hydrogen Sulfide Addition to Polyacetylenes

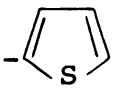
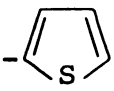
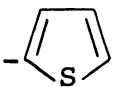
Reactant	Product	Reference
$H_3CC{\equiv}CC{\equiv}CCH_3$	H_3C-  -CH_3	19
$C_6H_5C{\equiv}CC{\equiv}CC_6H_5$	C_6H_5-  -C_6H_5	19
C_4H_3S $C{\equiv}CC{\equiv}C$ SC_4H_3	C_4H_3S-  -SC_4H_3	19
$H_3C(C{\equiv}C)_3CH{=}CHCOOH$	$H_3CC{\equiv}C$-  -$CH{=}CHCOOH$	19
$H_3C(C{\equiv}C)_3CH_2OH$	$H_3CC{\equiv}C$-  -CH_2OH	19
C_4H_3S $(C{\equiv}C)_3CH(OH)CH_3$	C_4H_3S $C{\equiv}C$-  -$CH(OH)CH_3$	20
$C_6H_5(C{\equiv}C)_3C_6H_5$	C_6H_5-  -$C{\equiv}C$ C_6H_5	19
$C_6H_5(C{\equiv}C)_4C_6H_5$	C_6H_5-  -$(C{\equiv}C)_2C_6H_5$	19

Table 6. Biosynthetic Data for Terthienyl

Radioisotope Form	Incubation Period (days)	Dilution Factor
Bisulfide-^{35}S	1.3	∞
Sulfate-^{35}S	2	294
Methionine-2-^{14}C	2	1430
Methionine-^{35}S	1.3	4420
Acetate-1-^{14}C	2	∞
Acetate-1-^{14}C	10	35100

administration since stem feedings showed no incorporation even with inorganic sulfate. The tenuous suggestion that the thiophene rings of terthienyl arise from the cyclization of a four carbon unit, condensation of the cyclized unit with another carbon fragment, cyclization, and a repetition of these reactions is one reasonable interpretation of these data (Scheme E of Table 4). The intact methionine molecule is not likely to be the precursor to terthienyl since the labeled carbon and sulfur methionine are incorporated into terthienyl to different degrees. Cystathionine is related to the sulfur containing amino acids and may be an alternative precursor in this Scheme.

The value of this type of investigation of likely precursors to any biological material is directly related to the value accorded to <u>in vivo</u> evidence as contrasted to

in vitro and biogenetic arguments. Meinwald (22) recently employed a study of likely precursors to refute the supposition that preformed terpenes are required for the biosynthesis of citral and citronella in arthropods.

The biosynthetic origin and mode of formation of the thiophene ring in natural products is still nebulous. The intent of the investigation described here was to investigate the role of acetogenesis in the formation of the thiophene ring, to discover the physiological precursor of the sulfur atom of the thiophene ring, and to ascertain the relationship of terthienyl to 5-(3-buten-1-ynyl)-2,2'-bithienyl in the specie *Tagetes erecta*. This plant is a favorable choice for this study since historically it was the first plant in which terthienyl was discovered; it is a sturdy plant; and, it is easily grown from seed.

Concurrently with this study, investigations reported by Bohlmann and his co-workers are creditable with biogenetic and biosynthetic contributions to the mode of formation and origin of the thiophene ring. The intermediate between the biogenetically proposed conjugated triple bonds and a thiophenic structure is suggested by the addition of methyl mercaptan or its biological equivalent to the terminal triple bond of VIII (4). The polyacetylenes VIII and IX

$C_6H_5COC{\equiv}CC{\equiv}CH$ $C_6H_5COC{\equiv}CCH{=}CHSCH_3$

VIII IX

are isolable from Chrysanthemum segetum L. The former, labeled with carbon-14 at the oxo-carbon atom, was administered to Chrysanthemum segetum L. plants. The degradation of the sulfonyl derivative (96000 cpm/mM) of IX to benzoic acid (84000 cpm/mM) and confirms the transformation of VIII to IX in a biological system.

Additional biogenetic evidence for the metabolic pathway in Scheme F, Table 4, is cited in Table 7. Table 7 is a compilation of polyacetylenes and comparable thiophenic materials present in the same genera. The tribe Anthemidea contains the genera Chrysanthemum and Anthemis. The tribe Helenieae contains the genus Tagetes; and thistles, for example, Echinops sphaerocephalus L., are part of the tribe Cynareae (7).

A tedious separation by repeated column and vapor phase chromatography was reported by Bohlmann and co-workers (23) to yield twelve new thiophene compounds and two polyacetylenes from the same extract of roots. The structures and quantities of the compounds obtained from the roots of Echinops sphaerocephalus L. plants are summarized in Table 8. The compounds are biogenetically related to a penta-yne, X. Compound X, although never isolated from this specie, is found widely distributed in the Compositae Family. The results of an in vivo investigation with 1,2-tritium labeled X and Echinops sphaerocephalus L. is diagrammatically shown in Chart 2 (5).

$$H_3C(C{\equiv}C)_5CH{=}CH_2$$

X

Table 7. Some Thiophenic Natural Products and Related Polyacetylenes

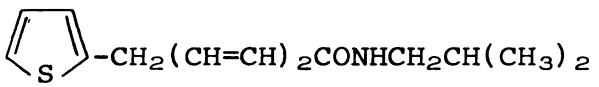
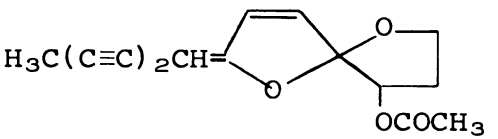
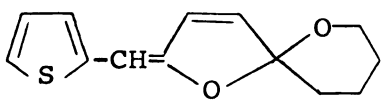
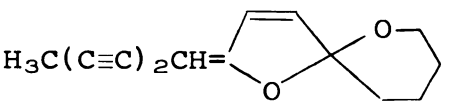
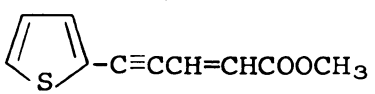
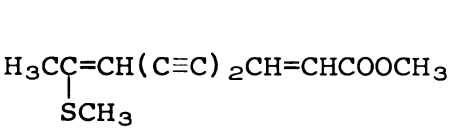
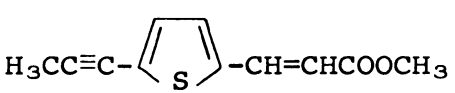
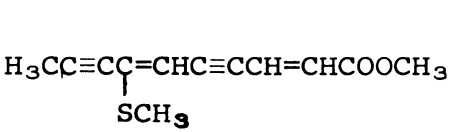
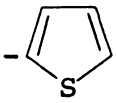
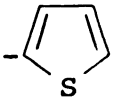
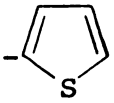
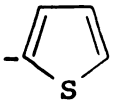
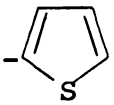
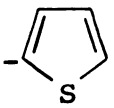
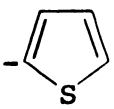
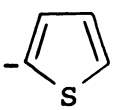
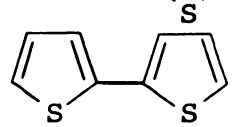
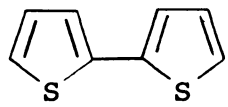
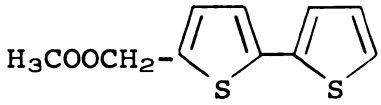
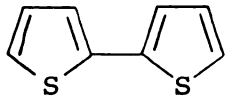
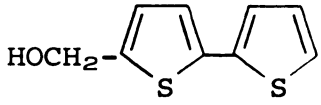
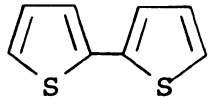
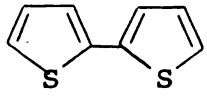
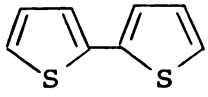
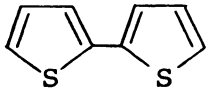
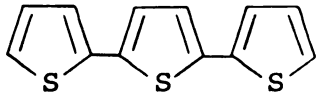
Thiophene Derivative	Source	Polyacetylene	Source
	Roots, Chrysanthemum foeniculaceum (41)		Roots, Chrysanthemum foeniculaceum (41)
	Leaves, Chrysanthemum viscido-hirtum Thell (41)		Leaves, Chrysanthemum viscido-hirtum Thell (41)
	Roots, Anthemis fruscata B. (44)		Roots, Anthemis tinctoria L. (59)
	Roots, Chrysanthemum vulgare (34)		Roots, Anthemis brachycentos F. (59)

Table 8. Thiophenic Compounds from Echinops sphaerocephalus L. (23)

Structure	Column Fraction[a]	Quantity (weight %)
$H_3C(C\equiv C)_3-CH=CHCOOR$	2	0.000083
$H_3C(C\equiv C)_3-(CH=CH)_2-CH(OH)CH_2CH_2OH$	4	0.0083
$H_3C(C\equiv C)_2-$  $-C\equiv CCH=CH_2$	1	0.00041
$H_3C(C\equiv C)_2-$  $-C\equiv CCHCH_2$	2	0.000083
$H_3C(C\equiv C)_2-$  $-C\equiv CCH_2CH_2OCOCH_3$	2	0.00025
$H_3C(C\equiv C)_2-$  $-C\equiv CCHClCH_2OCOCH_3$	2	0.0016
$H_3C(C\equiv)_2$  $-C\equiv CCH(OCOCH_3)CH_2OCOCH_3$	3	0.00016
$H_3C(C\equiv C)_2-$  $-C\equiv CCHClCH_2OH$	3	0.0020
$H_3C(C\equiv C)_2-$  $-C\equiv CCH_2CH_2OH$	3	0.00041
$H_3C(C\equiv C)_2-$  $-C\equiv CCH(OH)CH_2OH$	4	0.00041
b  $-C\equiv CCH=CH_2$	1	0.025

continued

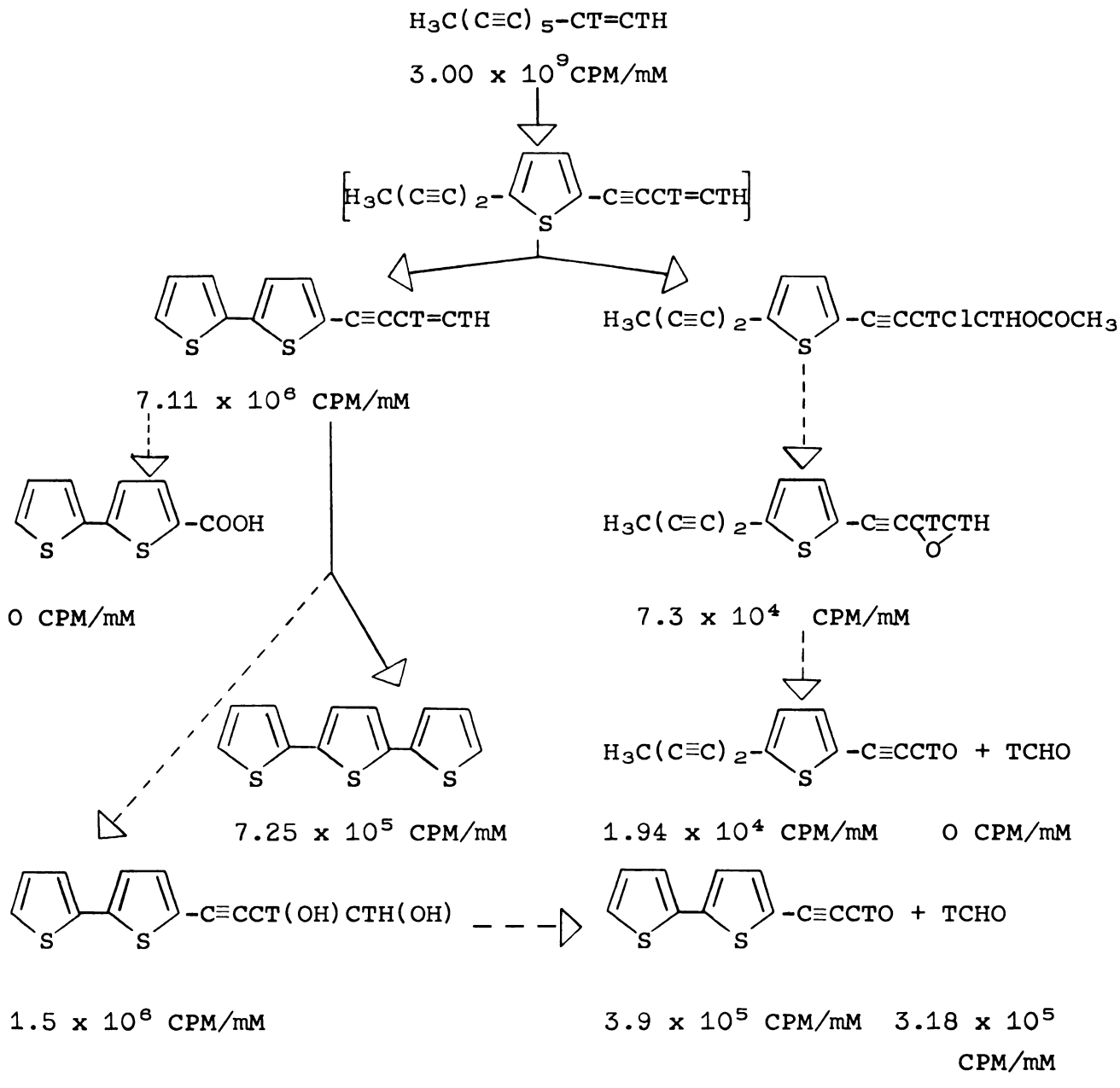
Table 8 - Continued

Structure	Column Fraction[a]	Quantity (weight %)
b 	2	0.00025
	2	0.00041
	3	0.0005
	3	0.00016
b 	3	0.00041
	4	0.00016
	4	0.00025
	4	0.00062
b 	1	0.012

[a]Column fractions: petroleum ether (1), petroleum ether-10% ethyl ether (2), petroleum ether-50% ethyl ether (3), and ethyl ether-10% methanol (4).

[b]Also from Tagetes erecta and/or minuta.

Chart 2. Tritium Labeling Experiment with Echinops sphaerocephalus L.



——— Biological material isolated by column chromatography and assayed.

– – – Chemical conversion produced the product which was assayed.

The pathway which is shown in Chart 2 is consistent with the specific activities and demonstrates the existence of an enzyme system for the conversion of triple bonds to thiophene rings, but it is not sufficient to distinguish between a preformed polyacetylene and another structure as the physiological precursor to terthienyl.

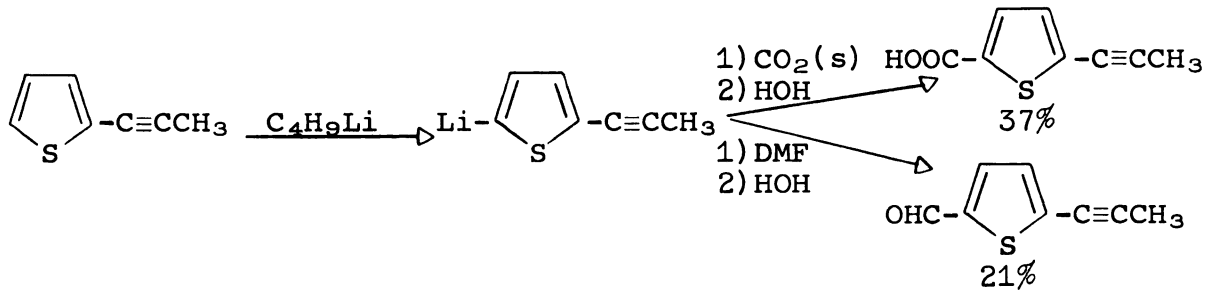
The study of precursors to biological material can provide evidence of the biosynthetic pathway, but a systematic degradation of the labeled biological material is required to ascertain the mode in which the precursor is incorporated into the biological product. The preliminary investigation of the degradation of terthienyl is one aspect, therefore, of this investigation of polythienyls from Tagetes erecta.

The reductive removal of sulfur is a reaction which has been known since 1940 (24). The first desulfurization of a polythienyl is the work of Wynberg and Logothetis (25). 5,5'-Diacetyl-2,2'-bithienyl and Raney nickel interact to form the simple structure, 2,11-dodecanediol. The diacetyl derivative of terthienyl is desulfurized with Raney nickel to form the corresponding long chain diol in 80% yield according to Wynberg (26). This is the only reported desulfurization of a terthienyl. The insolubility of the starting materials was particularly noted by these investigators. Numerous thiophene derivatives may be desulfurized in good yield (27,28).

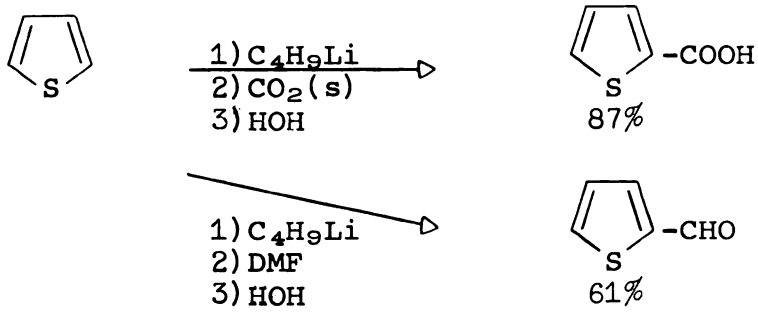
The straight chain bifunctional structure obtained by desulfurization of 5,5'' —diacetylterthienyl is suitable for conversion to a dicarboxylic acid (26). Several procedures for the degradation of fatty acids are available (29,30).

The direct acetylation of terthienyl produces a mixture of the mono- and the diacetylated material. The maximum yields of the diacetylated and monoacetylated terthienyl are 51% and 40 to 50% respectively (26). The comparatively small amount of product which is obtained in this first step of the degradation sequence proposed above is undesirable. The direct introduction of a carboxyl function into terthienyl has not been reported in literature. Metal-hydrogen exchange does occur with thiophene and n-butyllithium (31). The addition of dimethylformamide to thienyllithium followed by hydrolysis yields the 2-thiophenecarboxaldehyde in 61%. Metal-hydrogen exchange with thiophene and thiophene derivatives, followed by direct carbonation to give the carboxylic acid, or followed by the interaction with dimethylformamide to give the carboxaldehyde, are reactions which have been known for more than a decade (32,33). Equations 1 and 2 illustrate these reactions.

1 (33):

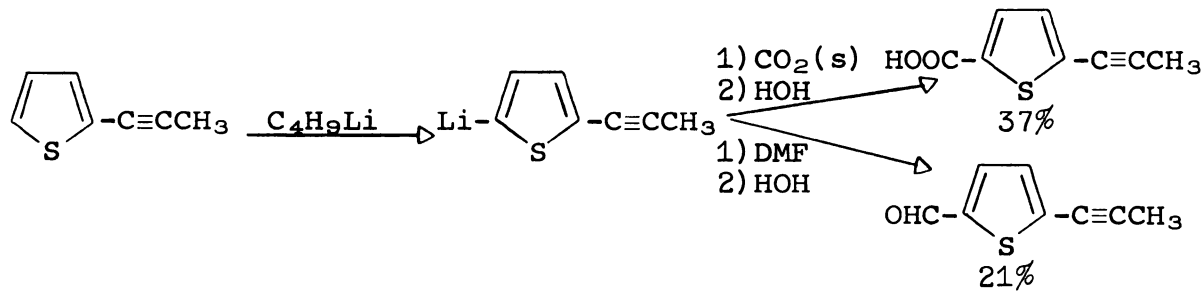


2 (31):

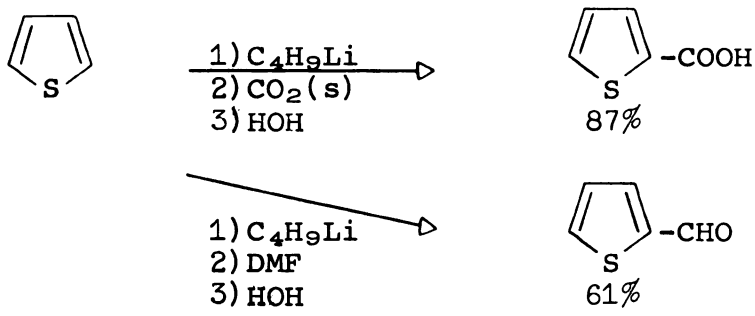


The metal-hydrogen exchange reaction with terthienyl and n-butyllithium desulfurization with Raney nickel, and the degradation of a long chain carboxylic acid constitute the preliminary study of a degradation sequence undertaken in the present study.

1 (33):



2 (31):



The metal-hydrogen exchange reaction with terthienyl and n-butyllithium desulfurization with Raney nickel, and the degradation of a long chain carboxylic acid constitute the preliminary study of a degradation sequence undertaken in the present study.

EXPERIMENTAL AND RESULTS

Preparation of the Plants

Seeds of the Tagetes erecta L. variety of marigold were obtained from W. A. Burpee Company, Fordhood Farms, Doylestown, Pennsylvania. Plants were grown from seeds in a greenhouse for six weeks, and then transferred to the laboratory. There, they were grown in nutrient solution with appropriate lighting, ambient temperature and humidity for 1 to 5 weeks. Growth conditions for all experiments are summarized in Table 9.

The nutrient solution was prepared from stock solutions (1 N) of ammonium dihydrogen phosphate, potassium nitrate, calcium nitrate tetrahydrate, and magnesium sulfate. For the preparation of 1 liter of nutrient solution, 1, 6, 4, and 2 ml, respectively, of the stock solutions were combined and diluted with 987 ml. water.

Plant roots initially grown in sandy soil were generally 80% removed in transferring the plants to beakers or Erlenmeyer flasks for hydroponic growth. The daily addition of 5 to 10 ml. of water or nutrient solution (water and nutrient solution were added on alternate days) supplied the oxygen and nutrient requirements for the regeneration and growth of the plants' root systems. Tap water and continuous aeration enhanced the volume of root growth. The aerial portion of

Table 9. Growth Conditions

Experiment Number	Age of the Plants (wks)	Period in Nutrient Solution (wks)	Season
1	8.8	2.8	Spring
2	8.8	2.8	Spring
3	6.0	0.1	Winter
4	6.0	0.1	Winter
5	7.0	2.0	Winter
6	10	1.0	Fall
7	10	1.0	Fall
8	9.0	2.5	Spring
9	9.0	2.5	Spring
10	9.0	2.5	Spring
11	9.0	2.5	Spring
12	8.0	0.1	Spring
13	10	5.0	Spring
14	10	5.0	Spring
15	3.6	2.2	Spring
16	11	4.0	Fall
17	11	4.0	Fall
18	11	4.0	Fall
21	10	4.0	Fall
22	10	4.0	Fall
23	10	4.0	Fall
24	9.5	5.0	Winter
25	9.5	5.0	Winter
26	10	3.4	Winter
27	10	3.4	Winter
28	10	3.4	Winter
29	9	3.0	Summer
30	9	3.0	Summer
31	11	5.0	Summer
32	11	5.0	Summer
33	11	5.0	Summer

the plants grew rapidly and appeared healthy. Some development of the bud region occurred when the plants reached the age of 10 weeks. Plants of this age were generally used for the hydroponic administration of the isotopically labeled compounds.

Administration of Labeled Compounds

A stock solution of the radioisotope labeled compound under investigation was prepared in distilled water. The carbon-14 and sulfur-35 labeled compounds listed in Table 10 were commercially available (see Appendix) and chromatographically pure.

The plants used in a given experiment were removed from the nutrient solution, the roots were rinsed well in distilled water, and the plants were placed in clean beakers. The number of plants used in a given experiment was determined by the number of plants available, and the total quantity of roots of the plants. The amount of activity dispensed (Table 10) was assumed to be sufficient to flood all the biological pathways available to the radioisotope form administered. The calculated amount of stock solution (2 to 5 microcuries per plant) was administered by spreading the solution by pipet over the roots of the plants. After an absorption period of an hour, nutrient solution was poured into the beaker, and the plants were treated as described above until the roots were harvested.

Table 10. Radioisotope Forms Administered

Experiment Number	Isotope Form	Total CPMx10^{-6} Administered	Specific Activity (DPM/μm x 10^{-6})
L	Sodium sulfate-^{35}S	0.672	76.2
2	Sodium sulfate-^{35}S	0.672	76.2
3	DL-Glucose-UL-^{14}C	1.99	193
4	DL-Glucose-UL-^{14}C	1.99	193
5	DL-Ornithine-2-^{14}C	31.5	4.28
6	DL-Glucose-UL-^{14}C	16.9	9.99
7	DL-Glucose-UL-^{14}C	16.9	9.99
8	Sodium malonate-2-^{14}C	35.5	3.62
9	Sodium malonate-2-^{14}C	35.5	3.62
10	Sodium malonate-2-^{14}C	53.5	3.62
11	Sodium malonate-2-^{14}C	35.5	3.63
12	Sodium malonate-2-^{14}C	44.4	3.62
13	DL-Methionine-2-^{14}C	16.0	2.33
14	DL-Methionine-2-^{14}C	16.0	2.33
15	DL-Methionine-2-^{14}C	24.0	2.33
16	Sodium pyruvate-3-^{14}C	21.0	14.4
17	Sodium pyruvate-3-^{14}C	15.8	14.4
18	Sodium pyruvate-3-^{14}C	14.7	14.4
21	DL-Cysteine-^{35}S	178	4.63
22	DL-Cysteine-^{35}S	125	4.63
23	DL-Cysteine-^{35}S	125	4.63
24	DL-Serine-3-^{14}C	36.3	11.6
25	DL-Serine-3-^{14}C	30.3	11.6
26	DL-Cystine-1-^{14}C	47.5	17.2
27	DL-Cystine-1-^{14}C	32.5	17.2
28	DL-Cystine-1-^{14}C	30.0	17.2
29	Sodium Pimelate-7-^{14}C	28.5	4.44
30	Sodium Pimelate-7-^{14}C	28.5	4.44
31	DL-Methionine-2-^{14}C	65.4	9.26
32	DL-Methionine-2-^{14}C	65.4	9.26
33	Malonic-2-^{14}C Acid	66.7	8.88

The possibility of fungal growth over the long period allowed for root growth and development in nutrient solution was considered. Test and control solutions (experiments 19 and 20) using root fragments of equal length and sodium pyruvate-3-^{14}C revealed a 2% difference in the radioactivity recovered from the nutrient solutions prepared with and without fungicide. An experiment with whole plants showed the relative uptake of sodium pyruvate-3-^{14}C in the presence and absence of fungicide (Table 11). Experiments 31 and 32 tested the effect of an antibacterial agent on the uptake of activity dispensed.

Harvest of Roots and Extraction of Polythienyls

The plants were allowed to metabolize the administered compound 0.1-10 days. The plants were then removed from the nutrient solution, and the roots were well rinsed, first with water and then with 95% ethanol. The nutrient solution and washings were assayed for activity. Paper chromatograms of a qualitative amount of the concentrated nutrient solutions examined gave no blue fluorescent areas under ultraviolet irradiation. Therefore, the polythienyls were not exuded by the plant roots in a detectable amount. The roots were air-dried one hour, weighed, and macerated with 40 ml. of 95% ethanol in a Waring blendor. The macerated roots were extracted with about 200 ml. of refluxing ethanol (95%)

Table 11. Study of Fungal and Bacterial Uptake

Experiment Number	Fungicide[a] (ppm x 10^{-3})	Antibacterial[b] (ppm x 10^{-3})	Administered[c] (CPM x 10^{-7})	Percent Activity[d] per Gram Root	
				Ethanol Extract	Nutrient Solution
16	0		2.10	25.4	23.4
17	1.6		1.57	19.4	12.2
31		1.5	6.54	3.27	1.85
32		0	6.54	3.40	1.67

[a]Phaltan is a commercially available fungicide (California Chemical Company, San Francisco).

[b]The antibacterial was a mixture of cetylpyridinium chloride and decamethylene-bis(4-amino-quinaldinium) chloride in a 3:1 weight ratio.

[c]The activity administered was dispensed as described above.

[d]The percent activity found represented all radioisotope forms present.

in a Soxhlet extractor for approximately 20 hours. The ethanolic extract was assayed for radioactivity. The uptake of the radioactivity dispensed is shown in Table 12. Paper chromatography was successfully used to monitor the presence of the fluorescent polythienyls in the concentrated extract. The solvent was removed under reduced pressure and the residue was extracted with petroleum ether (30-60°) or alternately dissolved in 30 ml. of 2% methanolic KOH and saponified. Saponification was carried out by gentle reflux on a steam bath for 30 minutes. An investigation by paper chromatography of the residue which was not immediately soluble in petroleum ether showed the residue to contain about one-third (32.2%) of the total terthienyl isolated (experiment 5). For this reason and because Horn (37) had shown by n.m.r. that triglycerides complicate the purification of terthienyl, the immediate saponification of the residue from the root extract was preferred. The saponification mixture was diluted with 200 ml. water and extracted with approximately 150 ml. of hexane or petroleum ether (30-60°). The extract was dried one hour over anhydrous magnesium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in 10 ml. of hexane, and the solution was chromatographed over a column of alumina (Alcoa, F-20).

Table 12. Uptake of Radioactivity

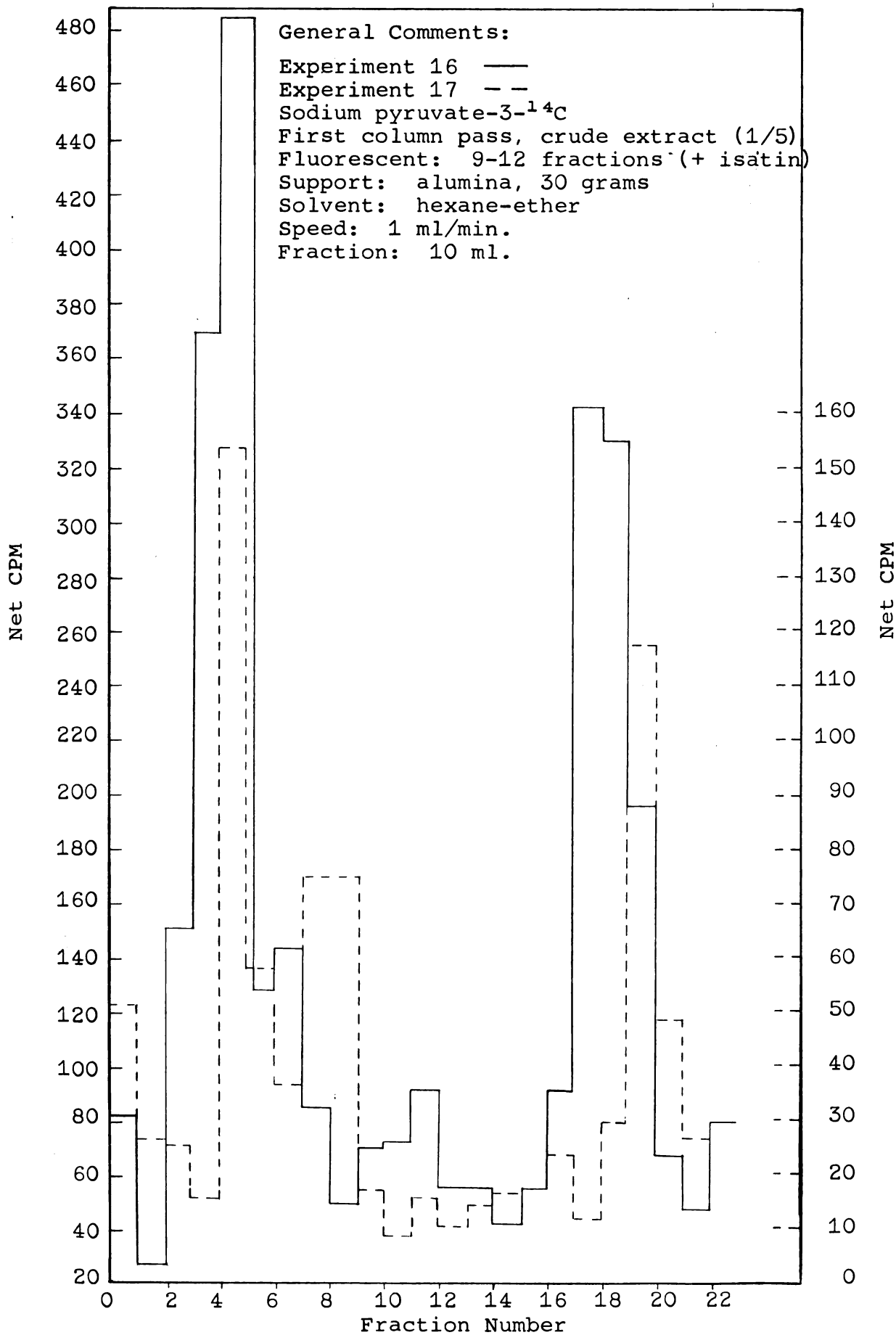
Experiment Number	Weight of Roots (g)	Incubation Period (days)	Percent of Activity Recovered From	
			Ethanol Extract	Nutrient Solution
1	3.2	1.0	46.0	-
2	2.8	10.6	52.6	-
3	0.9	1.0	-	-
4	0.9	2.0	3.77	-
5	0.8	2.0	0.83	-
6	1.8	2.0	-	2.83
7	1.3	10.0	3.21	1.77
8	0.9	2.0	0.94	0.58
9	1.0	2.0	1.11	0.33
10	1.4	2.0	1.61	0.18
11	0.5	8.7	-	0.21
12	4.0	2.0	0.17	0.84
13	1.5	1.9	21.2	2.08
14	1.7	1.9	9.82	0.15
15	0.5	2.0	4.46	0.10
16	2.9	2.0	8.76	7.43
17	1.6	2.0	11.5	6.96
18	0.5	9.8	3.98	7.42
21	3.1	8.9	1.98	3.21
22	2.2	0.8	2.91	26.4
23	1.5	1.8	1.89	25.2
24	1.7	0.8	-	3.58
25	4.7	1.9	-	4.00
26	1.3	0.1	0.31	49.5
27	0.9	1.0	0.76	23.6
28	1.1	7.8	1.03	0.80
29	1.8	2.0	2.65	82.7
30	1.5	2.0	4.75	2.65
31	2.0	2.0	6.48	3.66
32	2.0	2.0	6.83	5.16
33	3.0	2.0	3.98	1.60

Purification Procedures

The purification of biosynthetic terthienyl to constant specific activity was accomplished by one of three procedures: (a) repeated column chromatography of the crude biosynthetic terthienyl diluted with nonradioactive terthienyl, (b) the synthesis and isolation of the 5-acetyl derivative of the diluted sample of terthienyl, or (c) the fractionation of the marigold root extract over an alumina column followed by successive thin layer chromatograms of the column eluant fractions showing fluorescence under ultraviolet irradiation. The heterocyclic, 5-(3-buten-1-ynyl)-2,2'-bithienyl, was isolated for assay only by the latter method.

Column packings of cellulose, silica gel, and activated alumina (Alcoa, F-20) were examined to determine the most efficient column packing. The columns were prepared in the usual manner. Methanol-water (60-40), hexane and hexane-diethyl ether (95-5), respectively, were the developing solvents employed. The eluant fractions containing terthienyl were identified by the isatin test (61). Columns of alumina, 10 and 30 g., were found superior, based on the fractionation of the root extract effected by adsorption on alumina, the time required, and the ease of recovery of terthienyl by removal of the solvent. The frequency distribution of activity in the eluant fractions of experiments 16 and 17 when one-fifth of the total root extract was chromatographed is shown in the chart on page 32. The eluant fractions

Chart 3. Frequency Distribution of Radioactivity



4 to 6 from a 10 g. column of alumina through which the solvent flow was 0.7 to 1 ml. per minute, and the volume/fraction was 10 ml. showed a blue fluorescence under ultraviolet irradiation.

The synthesis of the 5-acetyl derivative of terthienyl is reported in Table 13. The maximum recovery of 5-acetyl-2,2';5',2''-terthienyl was 47.3%. The reaction mixture in carbon tetrachloride was chromatographed over a column of alumina. Carbon tetrachloride-diethyl ether was the eluting solvent. Thin layer chromatography using silica gel as the support was also applied.

Table 13. Acetylation of Terthienyl

Experiment Number	Terthienyl Acetylated (micromole)	Yield of 5-Acetyl Derivative (%)	Specific Activity	
			Terthienyl (CPM/μM)	5-Acetyl Derivative (CPM/μM)
2	46.8[a]	47.3	544000	520000
17	0.995[a]	28.0	422	(c)
18	6.65[b]	40.7	(d)	(d)

[a]Reaction conditions: 80° for 18 hours in benzene with stannic chloride catalyst.

[b]Reaction conditions: 25° for 19 hours in benzene with stannic chloride catalyst.

[c]The expected activity calculated on the basis of yield was within the standard error of the backround.

[d]No activity was observed in the 5-acetyl derivative isolated from a thin layer chromatogram. Activity was observed in a 358 mμ component.

Thin layers of silica gel H (Brinkman Company) or adsorbosil-2 (Applied Science Laboratories) were prepared on 8" x 8" glass plates. The silica gel support was activated by heating at 100-110°. The eluant fractions from an alumina column, which were fluorescent under ultraviolet irradiation, were concentrated to approximately 0.01 ml. A 3 to 5 drop quantity of purified benzene was added. The solutions were applied either by syringe or capillary bore pipet to a region about one-half inch from the base of the chromatoplate. The ideal fine line at the point of application was difficult to obtain. The thin layer chromatogram was developed with purified hexane in a sealed glass tank by ascending chromatography. The fluorescent areas on the thin layer chromatogram which were observed under an ultraviolet lamp were removed from the plate by an all-glass vacuum apparatus. The fluorescent materials, 5-(3-buten-1-ynyl)-2,2'-bithienyl and terthienyl, were eluted from the silica gel with reagent grade diethyl ether. A solution of the polythienyl in 95% ethanol was prepared for assay, as was a blank. The recovery of terthienyl from thin layers of silica gel was limited by technique and not by significant retention or decomposition. A yellow coloration remained on the silica gel after elution of 5-(3-buten-1-ynyl)-2,2'-bithienyl with diethyl ether. Horn (37) observed the same phenomenon in his studies.

Table 14. Thin Layer Chromatography of Terthienyl

Total micromoles plated	Percent Recovered	Total micromoles plated	Percent Recovered
0.0157	0	0.297	87.2
0.0296	53.2	2.38	95.4
0.0592	94.6	3.18	83.6
0.148	76.4	9.52	83.9

The polythienyls isolated from the roots of the marigolds were identified by the observed chemical and physical properties (Table 15). A component absorbing at 332-334 mμ was present in the polythienyl fractions in experiment 26 after the undiluted material was passed through a column of alumina once and chromatographed once on a thin layer of silica gel. The concentration of this component appeared greater in the bithienyl fraction. A contaminant absorbing at 336 mμ was present in the polythienyl fractions of experiments 31 and 33 on the second thin layer chromatogram. No absorption at 336 mμ was observed on the third chromatogram, nor was absorption in this region observed in experiments 24, 25, and 32.

Autoradiograms were made from the first thin layer chromatograms of the column fractions from the D_1 sample in experiments 21, 22, and 23, and of the column fractions of the undiluted sample in experiments 24 and 25. Fast medical x-ray film was laid directly on the surface of the

Table 15. Identification of Biosynthetic Polythienyls

Observation	Terthienyl	5-(3-Buten-1-ynyl)-2,2'-bithienyl	Rf Ratio
Isatin test	violet to blue-green	wine red[a]	
Permanganate test	negative	positive	
Rf (paper)	0.63	0.75	1.19[b]
Rf (silica gel)	0.49	0.58	1.17±1[c]
λ_{max} (mμ)	350[d]	344[e]	

[a]This material was oxidized to 2,5-thiophene dicarboxylic acid (3,15).

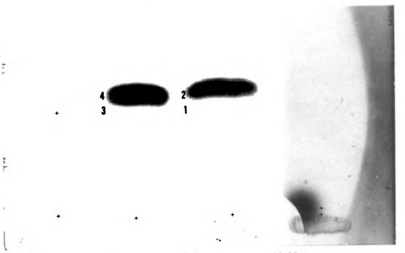
[b]Rf's already reported give a ratio of 1.18 (3).

[c]This is an average of two observations.

[d]A maximum of 350 mμ has been reported in ethanol and synthetic material has been observed to absorb at 352 mμ in ethanol (2).

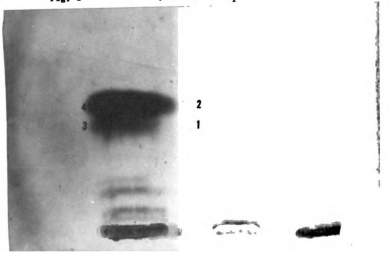
[e]Bohlman has reported a maximum of 345 mμ in ether (46).

chromatoplate. A glass plate held the film firmly against the silica gel surface for 92 hours. The x-ray film was developed by automatic processing. Figures 1, 2, and 3 are photographs of the autoradiograms of experiments 21, 22, and 23. The pertinent data are reported in Table 16. The positive (+) marks on figures 1 and 3 were areas on which phosphomolybdic acid was reduced (62). Terthienyl on silica gel did not affect this reagent; however, 5-(3-buten-1-ynyl)-2,2'-bithienyl readily reduced a dilute solution of permanganate. There was no reduction of the x-ray film



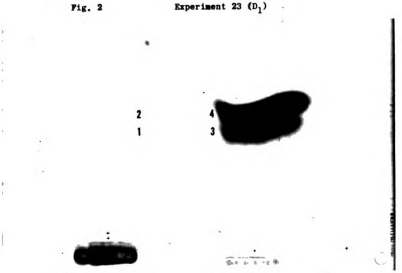
Column Fractions
Experiment 22 (D_1)

Fig. 1



Column Fractions
Experiment 23 (D_1)

Fig. 2



Column Fractions
Experiment 21 (D_1)

Fig. 3

Table 16. Autoradiogram Data

Experiment Number	Area Number	Wavelength (mμ)	Concentration (micromoles)	Activity (CPM)
22	1	352	0.288	6920
	2	340±4	0.0457	10000
	3	336sh	-	2550
	4	340	0.0730	13700
23	1	336±4sh	-	2790
	2	332sh	-	2660
	3	350	0.304	18900
	4	343	0.164	52800
21	1	-	-	1160
	2	-	-	1920
	3	352	0.282	24600
	4	344	0.260	76500
24	3	352	0.0670	60
	4	343	0.0705	92
25	3	352	0.135	89
	4	344	0.0555	51

emulsion in area number 3 on the autoradiograms of experiments 24 and 25 and only slight reduction in area 4. The phosphomolybdic acid reagent (62) is sufficiently sensitive to detect 10-0.1 μg. of reductants, but these substances did not reduce the film in experiments 21 and 22. The radioactivity present in the polythienyl areas reduced the silver emulsion of the film; however, 5-(3-buten-1-ynyl)-2,2'-bithienyl also caused some chemical reduction of the film.

Paper chromatography was employed to monitor the purity of terthienyl samples from repeated column chromatography.

A qualitative amount of sample was spotted about 1" from the bottom of a Whatman #1 filter paper strip, 8" x 3", and developed by ascending chromatography in a cylindrical specimen jar. Methanol-water, 60-40, was the solvent system. The bithienyl component (Rf 0.75) was distinct from terthienyl (Rf 0.63). Quantitative paper chromatography was examined using a tank, 24" x 12", fitted for ascending chromatography. The latter technique, however, was not suitable over a wide range of concentration.

Measurement and Determination of Concentration

Concentrations of terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl were obtained by the measurement of absorbance at 350 mμ ($e = 2.41 \times 10^4$) and 344 mμ ($e = 2.78 \times 10^4$)* respectively. The solvent used was 95% ethanol. Absorbancies were determined with a Beckman DB recording spectrophotometer.

The concentration of 5-(3-buten-1-ynyl)-2,2'-bithienyl calculated from the absorbance at 344 mμ was the concentration of the biosynthetic material directly isolated and purified by column and thin layer chromatography.

The determination of biosynthetic terthienyl was accomplished by isotope dilution analysis and by the direct observation of the material isolated by chromatography.

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*The molar absorbancy index at 341 mμ calculated in hexane is 2.81×10^4 (37).

The double dilution method of Mayor and Collins (63) is an application of isotope dilution analysis to the determination of yield and activity of radioactive compounds. A known amount (D_1) of nonradioactive terthienyl was added to the ethanolic root extract after saponification, and it was diluted to 10 ml. Half of this solution was removed and a second and larger quantity of nonradioactive terthienyl (D_2) was added to one five-milliliter portion. Both portions, D_1 and D_2, were purified to constant specific activity by procedures already described. From the relationships

$$x = \frac{A_2 D_2}{A_1 - A_2} - \frac{D_1}{2} \quad \text{and} \quad A_o = \frac{A_1 D_1}{x}$$

the amount of biosynthetic terthienyl isolated (x) and its specific activity (A_o) were calculated. Successful application of this method was found to depend on three factors: (a) the selection of D_1 such that D_1 closely approximated x, (b) that D_2 be five times greater than D_1, and (c) that A_1 be larger than A_2. Negative or unreasonably large values of x were obtained when these requirements were not met. Two sample experiments are reported in Table 17.

The direct observation of the amount of purified biosynthetic terthienyl was made possible by the selection of a satisfactory thin layer support and a solvent system for chromatographic separation. The accuracy of the calculated concentration of terthienyl was not limited by any

Table 17. Double Dilution Method

Experiment Number	Weight[a] of roots (g.)	Estimated μmoles[b] of terthienyl	Double Dilution D_1 (μM)	Double Dilution D_2 (μM)	Calculated X (μM)
16	2.9	1.02	0.96	4.80	0.905
17	1.6	0.56	0.96	4.80	-0.248

[a]The roots were air-dried one hour before being weighed.
[b]This estimation was made by assuming 0.35 μM of terthienyl isolated per gram of root.

estimations but did involve the direct measurement and processing of small concentrations. Nonetheless, this method provided the most reliable values of specific activities.

For experiments in which the double dilution method and the direct observation of concentration had not yet been implemented or were inapplicable, a third means of arriving at a concentration of biosynthetic terthienyl was employed. This was based on the observation that marigold roots, 0.5-1.5 g., which were rinsed in 95% ethanol and air-dried one hour at room temperature averaged 0.35 μM terthienyl per gram of root on one fractionation of the root extract over a column of alumina (Alcoa, F-20). The root extracts to which this method was applied were diluted with a large amount of nonradioactive terthienyl relative to the estimated

amount of biosynthetic material and purified by chromatography to constant specific activity.

Measurement of Radioactivity

Radioactivity measurements (64,65) of purified terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl were made on thin layers of the compounds. Analytical solutions of the polythienyls were prepared in 95% ethanol and an aliquot of each was plated on an aluminum planchet. No correction of the counting data to infinite thinness was required for the samples of terthienyl (2,3). All samples prepared for counting were 3.03-0.02 $\mu M/cm^2$. It was assumed on the basis of sample size that no significant correction factor was required for thin layers of 5-(3-buten-1-ynyl)-2,2'-bithienyl of 0.03-0.02 micromole. If geometric efficiency for the radiation has been determined, errors of the order of 5% can be expected for essentially weightless sources and may be as large as 20% according to Snell (66). The uniformity of composition of the samples of terthienyl was shown by the stability of dilute solutions of terthienyl over a five-month period, of the solid protected from direct light, and of the solution irradiated at 220 mμ for 40 minutes and 350 mμ for 3 hours.

All planchets were counted in a windowless gas flow proportional counter. A Baird-Atomic model 135 scaler and argon -methane gas were used. The planchets were generally observed to a 2% probable error in the observed activity.

Decay losses for sulfur-35 samples were corrected. No correction for decay was required for carbon-14 samples. A secondary standard was used to monitor the daily performance of the counting arrangement. The observed efficiency for $BaCO_3$-^{14}C was calculated as 24.9% (Tracerlab standard, 6070 dps).

In addition to thin layer counting, purified samples of terthienyl were assyed as $BaCO_3$-^{14}C for experiment 11. The biosynthetic material was diluted with two quantities of nonradioactive terthienyl according to the double dilution method previously described. The samples were purified to constant specific activity as assayed by thin layer counting. An aliquot of the ethanolic solution of each dilution sample (D_1 and D_2) was evaporated to dryness in a wide mouth standard tapered joint tube and oxidized according to the Van Slyke-Folch method (67). The liberated carbon dioxide was precipitated as $BaCO_3$ from a saturated solution of $Ba(OH)_2$, collected by vacuum filtration, and reprecipitated once. The $BaCO_3$ samples were dried at 110°, cooled in a desiccator (ascarite), mounted on aluminum planchets, and counted in a gas flow windowless proportional counter (Nuclear Chicago model 192). Activity was observed to 10,000 counts at the beta plateau voltage. The observed rates were corrected for self-absorption to zero sample thickness. The correction factor applied was obtained from an empirical calibration curve constructed for the Nuclear

Chicago instrument from the data of the fraction of maximum activity observed and the corresponding density for samples of $BaCO_3$ of known specific activity.

Table 18. Experiment 11: Barium Carbonate Counting

Diluted Samples Terthienyl	Specific Activity[a] $BaCO_3$	Specific Activity[b] Terthienyl
D_1	4,700	3,750
D_2	3,380	3,120

[a]Corrected to zero sample thickness, CPM/mM.

[b]Calculated as CPM/mM by thin layer counting with no correction for self-absorption.

Dilution Factor

The dilution factor has been used to express the efficiency of incorporation of an administered carbon-14 or sulfur-35 labeled compound into biosynthetic terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl. The observed activities of all samples of biosynthetic material were corrected for the efficiency of the counting arrangement to carbon-14 radiation. The efficiency of the counter to sulfur-35 radiation (E_{avg} 0.05 mev) was assumed to be the same as that observed for carbon-14 (E_{avg} 0.05 mev). The dilution factor is defined as the quotient of the specific activity (DPM/μM) of the biosynthetic material and the specific activity (DPM/μM) of the administered radioisotope form. The dilution factors determined are summarized in Tables 19 and 20.

Table 19. Incorporation Data for Terthienyl

Experiment Number	Isotope Form	Specific Activity CPM/μM	Probable Standard Error of S.A.[c,d]	Dilution Factor
1	Sulfate-^{35}S	161000[a]		118
2	Sulfate-^{35}S	544000[a]		35.1
3	Glucose-^{14}C	1030[a]		46600
4	Glucose-^{14}C	3740[a]		12900
5	Ornithine-^{14}C	[e]		-
6	Glucose-^{14}C	141[a]		17600
7	Glucose-^{14}C	548[a]		4560
8	Malonate-^{14}C	402[a]		2260
9	Malonate-^{14}C	76.9[b]		11800
10	Malonate-^{14}C	112[b]		8060
11	Malonate-^{14}C	79.4[b]		11300
12	Malonate-^{14}C	[e]		-
13	Methionine-^{14}C	557[a]		1040
14	Methionine-^{14}C	380[a]		1520
15	Methionine-^{14}C	[e]		-
16	Pyruvate-^{14}C	174[c]	14.3	20600
17	Pyruvate-^{14}C	74.5[a]		47600
18	Pyruvate-^{14}C	[e]		-
21	Cysteine-^{35}S	80400[c]	5700	14.4
22	Cysteine-^{35}S	19000[c]	2020	60.8
23	Cysteine-^{35}S	300000[c]	14800	3.86
24	Serine-^{14}C	824[d]	20.2	3530
25	Serine-^{14}C	603[d]	88.5	4820
26	Cystine-^{14}C	[e]		-
27	Cystine-^{14}C	1300[d]	314	3300
28	Cystine-^{14}C	[e]		-
29	Pimelate-^{14}C	[e]		-
30	Pimelate-^{14}C	2490[b]	394	445
31	Methionine-^{14}C	1670[d]	544	1385
32	Methionine-^{14}C	[e]		-
33	Malonic-^{14}C acid	1185[b]	141	1865

[a] The method employed the assumption of 0.35 μM terthienyl obtained per gram of root after one fractionation of the root extract over alumina.

[b] The concentration was ascertained by direct observation on two purification processes followed by dilution and further purification.

[c] The double dilution method was applied to obtain the concentration of biosynthetic terthienyl.

[d] The concentration was directly observed on purification to constant specific activity.

[e] No activity was observed on exhaustive purification.

Table 20. Incorporation Data for 5-(3-Buten-1-ynyl)-2,2'-bithienyl

Experiment Number	Isotope Form	Specific Activity CPM/μM	Probable Standard Error of S. A.	Dilution Factor
21	Cystine-^{35}S	317000	4390	3.66
22	Cystine-^{35}S	167000	4250	6.94
23	Cystine-^{35}S	299000	4500	3.87
24	Serine-^{14}C	1300	166	2220
25	Serine-^{14}C	923	33.4	3140
26	Cystine-^{14}C	441	130	9780
27	Cystine-^{14}C	a		-
28	Cystine-^{14}C	b		-
29	Pimelate-^{14}C	b		-
30	Pimelate-^{14}C	2180	486	509
31	Methionine-^{14}C	b		-
32	Methionine-^{14}C	b		-
33	Malonic-^{14}C acid	b		-

[a]The concentration was too small to determine spectrophotometrically.

[b]No activity was observed on exhaustive purification.

3-(2,2'-Bithenoyl)propionic Acid

A solution of 2,2'-bithienyl (25), 3.00 g. (0.018 mole), in 20 ml. of thiophene-free benzene was mixed with 2.71 g. (0.020 mole) of freshly prepared β-carbomethoxypropionyl chloride (68) at room temperature in a 50 ml, round-bottomed flask equipped with a calcium chloride drying tube and magnetic stirring bar. The stirred mixture was cooled to 0°, and 2.1 ml. (0.018 mole) of reagent grade anhydrous stannic chloride in 10 ml. of benzene was added dropwise. The reaction mixture was agitated at room temperature for

one hour after the stannic chloride was added to complete the reaction. A dark green material was observed to collect on the walls of the flask and then dispersed in the reaction medium. The reaction mixture was cooled to 10° and hydrolyzed with dilute hydrochloric acid. The aqueous layer was separated from the organic layer, extracted with diethyl ether, and the aqueous layer was discarded. The combined ether extracts and benzene solution were washed with water and 5% aqueous sodium bicarbonate and dried overnight over magnesium sulfate. The solvent was removed at reduced pressure on a rotary evaporator. The tan residue (4.3 g.) melted at $69-77^{\circ}$. Column chromatography and recrystallization from methanol gave a light yellow solid which melted at $86-87^{\circ}$ (23.3%). The infrared spectrum (KBr pellet, Beckman IR-5) confirmed the presence of an ester function by the carbonyl absorption at 1740 cm^{-1}, the ketone function by a band at 1650 cm^{-1}, and a monosubstituted bithienyl residue by the absorption bands at 840 cm^{-1} and 803 cm^{-1}. The molar absorbancy, 21000, and the absorption maximum, 349 mμ (95% ethanol, Beckman DU), were consistent with a 5-acyl-2,2'-bithienyl.

The ester was hydrolyzed with aqueous sodium hydroxide and the tan product was isolated by precipitation with hydrochloric acid and extraction with ether. Recrystallization from dioxane gave light yellow needles, m.p. $169-70^{\circ}$. The infrared spectrum, Figure 4, (KBr pellet,

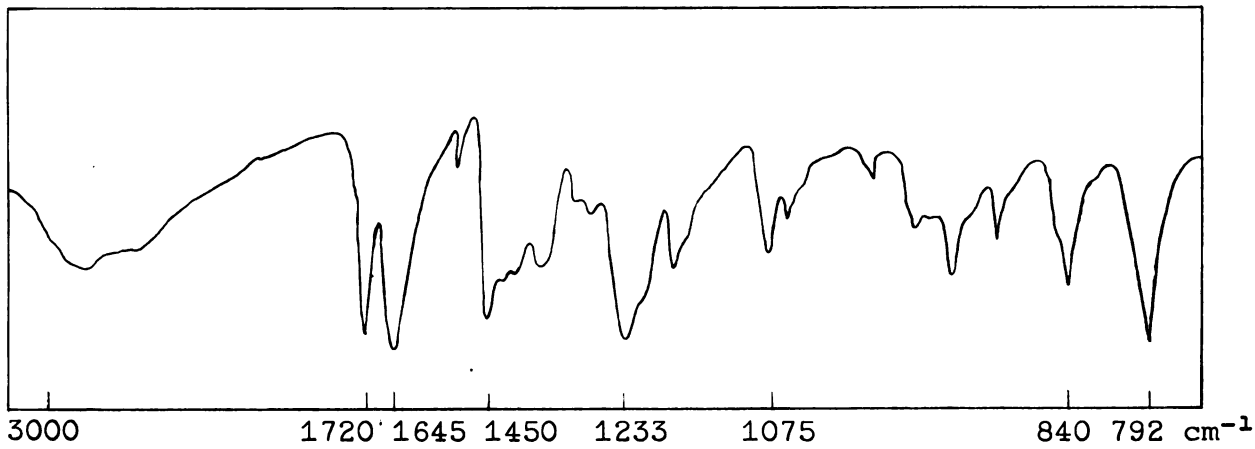


Fig. 4. Infrared Spectrum of 3-(2,2'-Bithenoyl)propionic Acid

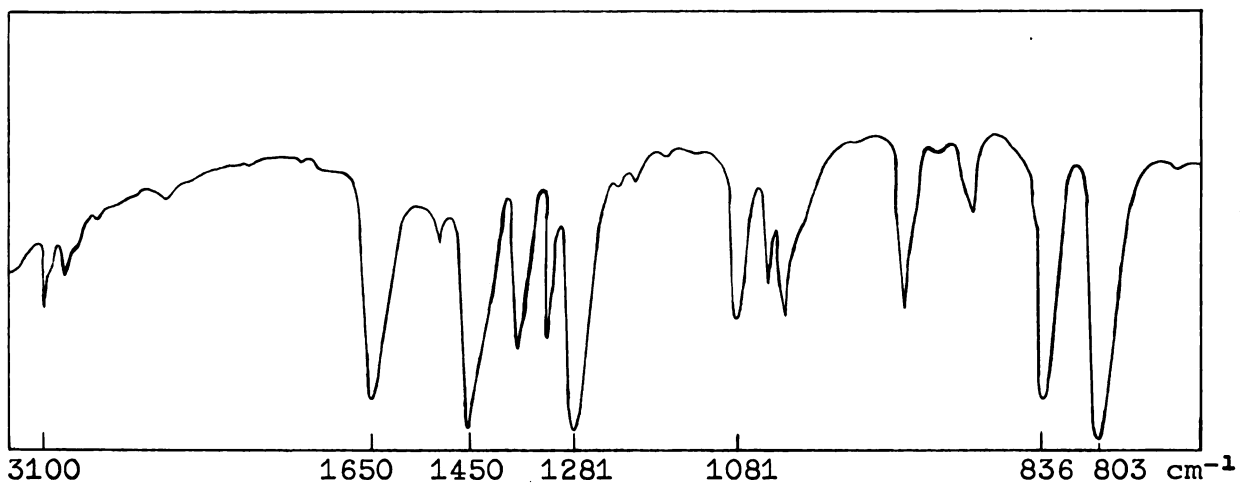


Fig. 5. Infrared Spectrum of 5-Acetyl-2,2'-bithienyl

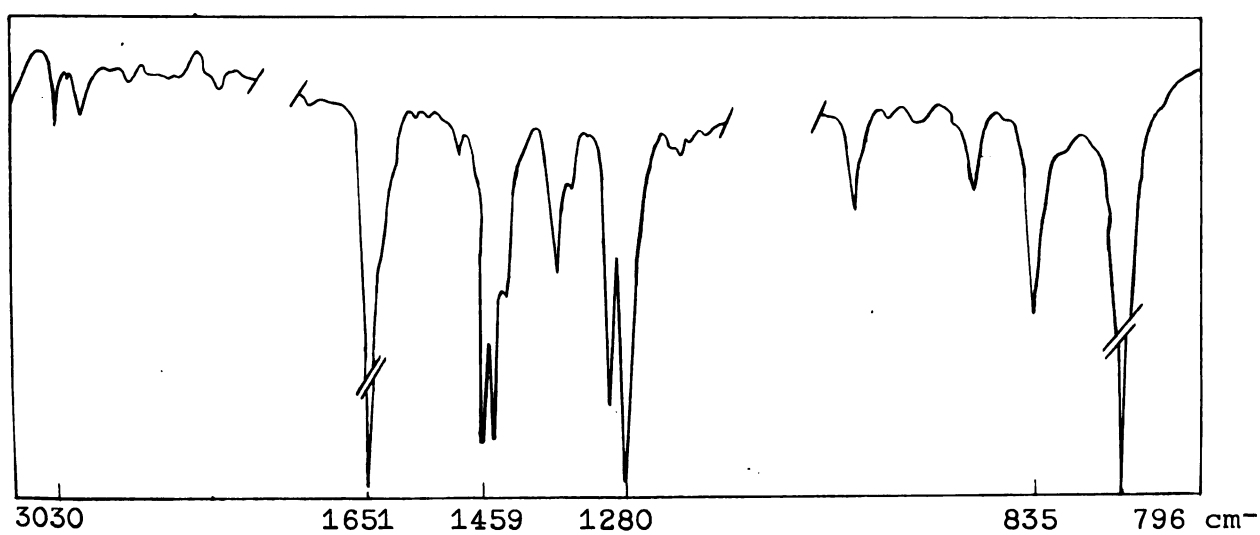


Fig. 6. Infrared Spectrum of 5-Acetyl-2,2';5',2''-terthienyl

Beckman IR-5) showed a broad absorption band at 3340-2500 cm^{-1}, a carbonyl absorption band at 1720 cm^{-1}, and the monosubstituted bithienyl residue absorption bands at 840 cm^{-1} and 792 cm^{-1}. The ultraviolet spectrum displayed the expected 349 mμ band and a band of lesser intensity at 245 mμ (95% ethanol, Beckman DB). The nuclear magnetic resonance spectrum (Varian A-60, dimethylsulfoxide-d_6) exhibited a carboxylic acid proton signal at -2.08 tau and aromatic protons' multiplet centered at 2.45 tau with the low field doublet at 2.04 tau. The calculated coupling constants for the aromatic protons compare to 0.05 c.p.s. with the coupling constants which were measured for 5-acetyl-2,2'-bithienyl (J_{34}=4.10, $J_{3'4'}$=3.75, $J_{4'5'}$=5.10, $J_{3'5'}$=1.20 c.p.s.). Anal. Calcd. for $C_{12}H_{10}S_2O_3$: C, 54.2; H, 3.76. Found: C, 54.5, H, 4.82.

5-Acetyl-2,2'-bithienyl

2,2'-Bithienyl, 3.00 g. (0.018 mole), and reagent grade acetyl chloride, 1.42 g. (0.018 mole), were mixed in 20 ml. of thiophene-free benzene at room temperature in a 50 ml. round-bottomed flask equipped with a magnetic stirring bar and calcium chloride drying tube. Stannic chloride, 2.1 ml. (0.018 mole), was added at 0° and the acylation performed in the manner previously described. Product isolation gave a solid, 2.75 g, m.p. 103-108°. The yellow solid failed to sublime at 100°/11mm. Column chromatography and recrystallization from 95% ethanol raised the melting

point to 114-115° (literature value, 114-115°) (69). The ultraviolet spectrum (95% ethanol, Beckman DB) exhibited an absorption maximum at 350 mμ (19800) and an absorption band of lesser intensity at 245 mμ (70). The nuclear magnetic resonance spectrum (Varian A-60, dimethylsulfoxide-d_6) revealed a multiplet centered at 2.49 tau with a low field doublet at 2.09 tau. The coupling constants were calculated as J_{34}=4.05, $J_{3'4'}$=3.80, $J_{4'5'}$=5.00, $J_{3'5'}$=1.25 c.p.s. The infrared spectrum, Figure 5, was consistent with the known structure and was used to confirm the structure of 3-(2,2'-bithenoyl)propionic acid.

5-Acetyl-2,2';5',2''-terthienyl

Terthienyl (1), 0.319 g. (1.2 mmoles), was dissolved in 100 ml. of thiophene-free benzene in a 250 ml. round-bottomed flask equipped with a calcium chloride drying tube. Reagent grade acetyl chloride, 0.5 ml., was added. Three drops (1.1 mmoles) of reagent grade anhydrous stannic chloride were added by pipet to the stirred reaction mixture. The reaction mixture was heated at its reflux temperature for 18 hours and then hydrolyzed with dilute hydrochloric acid. The benzene solution and ethereal extracts of the aqueous phase were washed with water and saturated sodium bicarbonate and dried in contact with anhydrous sodium sulfate overnight. The residue obtained on evaporation of the solvents was chromatographed over ten grams of alumina (Alcoa, F-20). Unreacted terthienyl, 82.1 mg. (25.6%), was eluted in

fractions 4-5 (10 ml.) with petroleum ether-diethyl ether. The second component eluted from the column with diethyl ether was rechromatographed. The material eluted with carbon tetrachloride-diethyl ether was sublimed. The yellow sublimate, 99.1 mg. (37.2%), melted sharply at 172°. The ultraviolet spectrum revealed the expected absorption maximum at 392 mμ (3) and the infrared spectrum (KBr pellet, Perkin Elmer Model 21) exhibited the diagnostic absorption bands at 1651 cm^{-1}, 835 cm^{-1}, and 796 cm^{-1}. Attempts to implement the procedure of Wynberg (25,26) were unsuccessful. Higher temperature acetylation with a Lewis acid was known to give a lower yield of monoacetylated polythienyl (69). Repetition of this procedure with 1.4 mmoles of terthienyl and two drops of stannic chloride yielded 31.0% of unreacted terthienyl and 40.1% 5-acetylterthienyl.

2,2'-Bithienyl-5-carboxylic Acid

The experimental procedures of Taft (31) and Skatteboel (33) were followed. 2,2'-Bithienyl, 3.11 g. (18.7 mmoles), was dissolved in 40 ml. of dry ether in a three-necked flask fitted with a reflux condenser, a magnesium perchlorate drying tube, a dropping funnel, a nitrogen inlet tube, and a mechanical stirrer. The solution was cooled to -70° by immersion in a dry ice-acetone bath, and n-butyllithium, 13.3 ml. (20 mmoles), in hexane was added dropwise during 10 minutes. The reaction solution was allowed to warm to room temperature by removing the dry ice-acetone cooling bath.

The temperature of the reaction mixture was increased to 26-30° and then cooled again to -70°. Dimethylformamide, 3.0 ml. (39.0 mmoles), was added dropwise. The reaction mixture was slowly brought to room temperature following the addition of the amide and set aside at room temperature for 18 hours. The lithium salt was hydrolyzed with a saturated solution of ammonium chloride. A solid product was insoluble in either phase. The solid obtained from the ethereal solution was oxidized with silver oxide (71). The crude acidic material, m.p. 174-6° (56.7%), was recrystallized from methanol, m.p. 183-4° (72). The solid was soluble in ethanol and methanol and only slightly soluble in hexane.

Desulfurization of 2,2'-Bithienyl-5-carboxylic Acid

The carboxylic acid, 1.6 g. (7.6 mmoles), was dissolved in dimethylformamide, 100 ml., in a Parr pressure bottle, and 12 g. of W-2 Raney nickel was added.* The Parr vessel was filled with hydrogen, 52 p.s.i. (72.5 mmoles) at 25°, and shaken while the reaction temperature was increased by external heating to 85°. The decrease in hydrogen pressure after 20 hours was 1 p.s.i. (1.39 mmoles), and after 40 hours, it was 15 p.s.i. The reaction mixture was allowed to settle, and the solvent was decanted. The Raney nickel was washed with petroleum ether and filtered. The organic

*Private communication from D. Anderson, Department of Chemistry, Michigan State University.

solutions were combined and poured into 250 ml. of water. The dispersion was extracted with four portions of petroleum ether (200 ml.). There was a great deal of sludge remaining in the separatory funnel. The extract was dried over magnesium sulfate, the solvent was evaporated, and the residue was esterified with methanol-sulfuric acid. Distillation of the esterification product after the usual isolation gave a colorless distillate, 0.2 g., distilling at 65-90°/1 mm. of mercury, which fluoresced blue under ultraviolet irradiation. A sodium fusion test showed sulfur to be present. Vapor phase chromatography of 0.1 ml. on a six-foot Apiezon L column at a column temperature of 191°, attenuation of four and helium flow rate of 2 ml. per minute showed the presence of two components having retention times of 6.0 and 28.8 minutes respectively. The relative peak areas were 1:3.8. The retention time of 6.0 minutes corresponded to that of an authentic sample of methyl pelargonate. The sample size was insufficient for collection and characterization by infrared spectra.

5,5'' -Terthienyldicarboxylic Acid

To a vigorously stirred solution of 4.04 mmoles of terthienyl in 200 ml. of dry ether at -35° was added 5.3 ml. (8.08 mmoles) of n-butyllithium in hexane. At temperatures below -35°, terthienyl precipitated. Tetrahydrofuran gave better solubility. The dropwise addition of the organolithium reagent required 3 minutes. The yellow reaction

solution became cloudy, and a yellow precipitate formed in 6 to 10 minutes. The dry ice-acetone bath was removed, and the reaction mixture was stirred at room temperature until it reacted (30 minutes). The reaction mixture was again cooled to -40° and dimethylformamide, 1.0 ml. (13.0 mmoles), was added dropwise. An instantaneous deepening of the color of the precipitate to an orange hue occurred. The reaction mixture was stirred overnight at room temperature after the addition of the amide to complete the reaction. Hydrolysis of the lithium salt was effected by the addition of 30 ml. of saturated ammonium chloride solution. The ether was removed from the reaction vessel on a rotary evaporator, and the precipitate was separated from the aqueous phase by vacuum filtration. A positive dinitrophenylhydrazine test was obtained with the red-brown solid. The solid, 1.35 g., was added in portions to a cooled slurry of silver oxide (1.36 g. silver nitrate and 0.54 g. of sodium hydroxide in 10 ml. of water), and the reaction mixture was stirred for 10 minutes. Since particles of the red-brown solid were still visible, 25 ml. of tetrahydrofuran were added. Vigorous stirring of the reaction mixture was continued for 20 minutes. A silver mirror was observed on the walls of the flask. The reaction mixture was filtered, and the residue from the oxidation reaction was extracted overnight with tetrahydrofuran. The solvent was removed under reduced pressure, and the solid was

washed with dilute acid. The filtrate from the oxidation reaction was acidified with dilute acid, the tetrahydrofuran was removed on the rotary evaporator under reduced pressure, and the solid was collected and washed with dilute acid. The amount of total solids recovered was 0.9071 g. The solids were slightly soluble in ether and quite soluble in benzene and glacial acetic acid. Purification on columns of silica gel gave three fractions. The fractions eluted with hexane, hexane-ethyl acetate, and glacial acetic acid were yellow, orange, and red respectively. The yields in relative percent by weight were 15.4, 79.7, and 4.87 respectively. The yellow fraction, m.p. 79-82^{o}, was identified as terthienyl by the Rf (0.588) on chromatostrips and ultraviolet absorption maxima after purification on thin layer preparative chromatograms. The contaminant present in this fraction, a yellow fluorescent substance (Rf 0.12), traveled much slower than terthienyl when hexane was used as the mobile phase. The orange fraction was a mixture of four components in addition to terthienyl. The latter was present in 4.6% by weight. The four components separated by thin layer chromatography on silica gel with hexane to produce yellow fluorescent bands at Rf values 0, 0.068, and 0.125 and an orange band at 0.171. An infrared spectrum (KBr pellet, Unicam SP .200) of the red fraction after sublimation showed no absorption in the carbonyl region and bands at 852 cm^{-1} and 811 cm^{-1}. The absorption band at 1650 cm^{-1} has no analogy in the spectrum of terthienyl (73) and,

therefore, may be a carbon-oxygen stretching frequency in a highly conjugated system. The melting point, 101-105°, distinguished the material from a pure, higher polythienyl (74). Thin layer chromatography (hexane:ether:acetic acid ratio of 90:10:1) showed two major fluorescent areas, red (Rf 0.53) and yellow (Rf 0.31). The fiery red material gave an intense yellow-green fluorescence in 95% ethanol, and absorption maxima were observed (Beckman DB) at 404 mμ, 260 mμ, and 240 mμ. Aqueous sodium hydroxide extracted a material from the orange fraction of the total solids which, after acidification of the aqueous solution, was easily soluble in benzene. The material was identical in behavior on chromatostrips and melting point (187-190°) to the component of the thin layer preparative chromatogram which did not leave the origin (sublimed at a bath temperature of 190° and a pressure of 0.5 mm Hg.). Absorption maxima in 95% ethanol were observed at 368 mμ and 260 mμ (Beckman DB). The infrared spectrum of the yellow sublimate (KBr pellet, Unicam SP .200) showed absorption bands at 3500-2500 cm^{-1}, 1685 cm^{-1}, and 1320-1210 cm^{-1}, and the characteristic 2,5-disubstituted thiophene bands at 855 cm^{-1} and 811 cm^{-1}. The quantity of yellow material isolated from the orange fraction was 23.6% by weight. Thin layer chromatostrips developed in hexane:ether:acetic acid (90:10:1) exhibited a blue (Rf 0.13) and a yellow (Rf 0.31) fluorescent areas, notwithstanding the application of sublimation and chromatography.

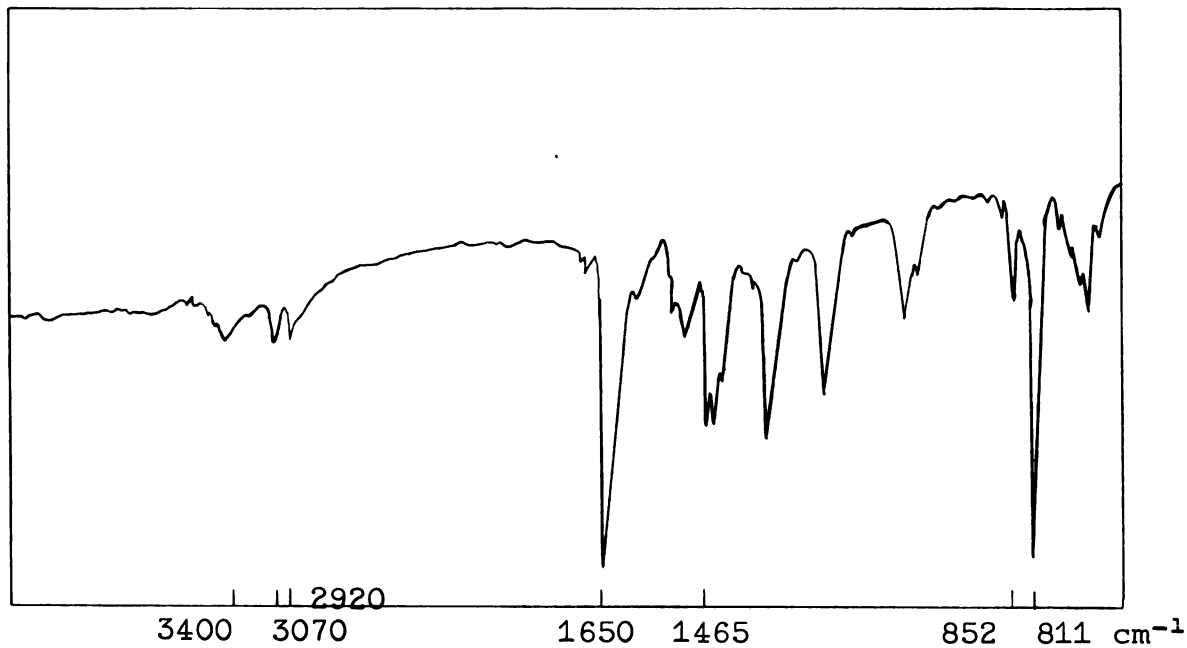


Fig. 7. Infrared Spectrum of the 404 mμ Product

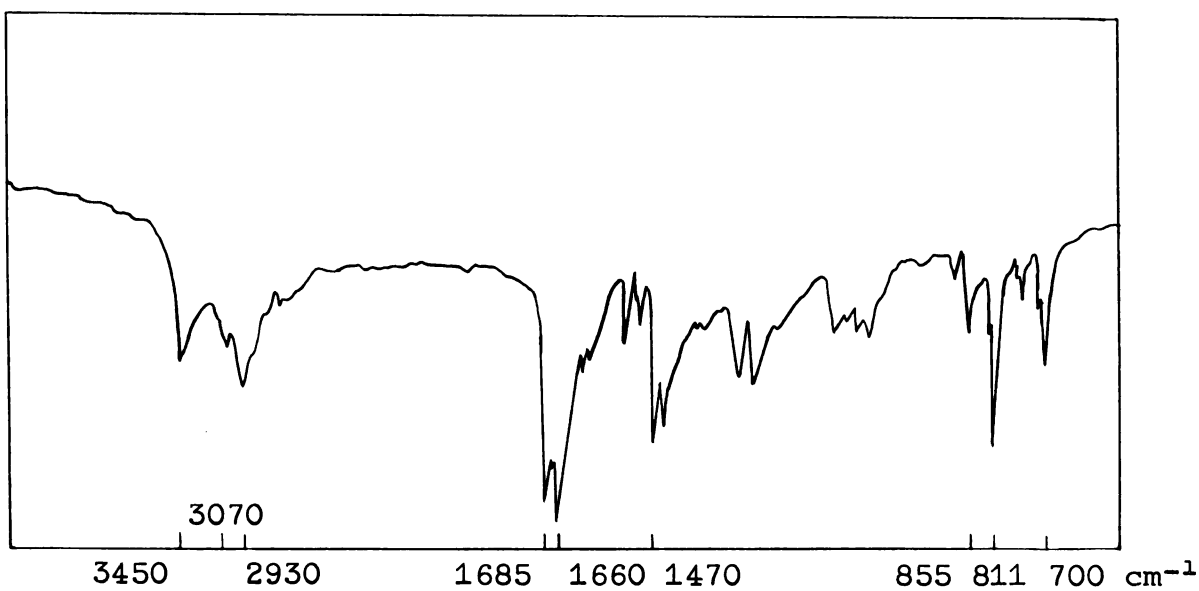


Fig. 8. Infrared Spectrum of the 368 mμ Product

Desulfurization of 5,5'' -Terthienyldicarboxylic Acid

The crude orange solid, 66 mg., obtained from the silver oxide oxidation of the formylated terthienyl was dissolved in 200 ml. of dioxane. Raney nickel prepared from 30 g. of alloy was added (75). The reaction mixture was heated at its reflux temperature. Qualitative thin layer chromatography applied after 18 hours reaction (hexane:diethyl ether:acetic acid in 90:10:1 ratio in silica gel) showed no fluorescent materials under ultraviolet irradiation of the chromatogram. The Raney nickel was separated from the solution by gravitational filtration. The filtrate was concentrated to 100 ml. on a rotary evaporator and 100 ml. of water was added. The slightly basic solution was acidified with hydrochloric acid and extracted with ether. The ethereal extract was washed with water, dried over magnesium sulfate, and evaporated under reduced pressure. The residual oil was esterified with 25 ml. of methanol and 3 ml. of sulfuric acid in the usual manner. The esterification reaction products were distilled at reduced pressure over a short distillation path. The distillate, 0.15 ml., boiled at 130-135^{o}/0.7 mm. (340^{o}/760 mm). The infrared spectrum of the neat sample was simple (Beckman IR-5, Figure 9). Absorption bands at 1740 cm^{-1}, 1200 cm^{-1}, and 1170 cm^{-1} were assigned to the ester carbonyl stretching frequency and methyl ester carbon-oxygen stretching frequency. Vapor phase chromatography on a six-foot

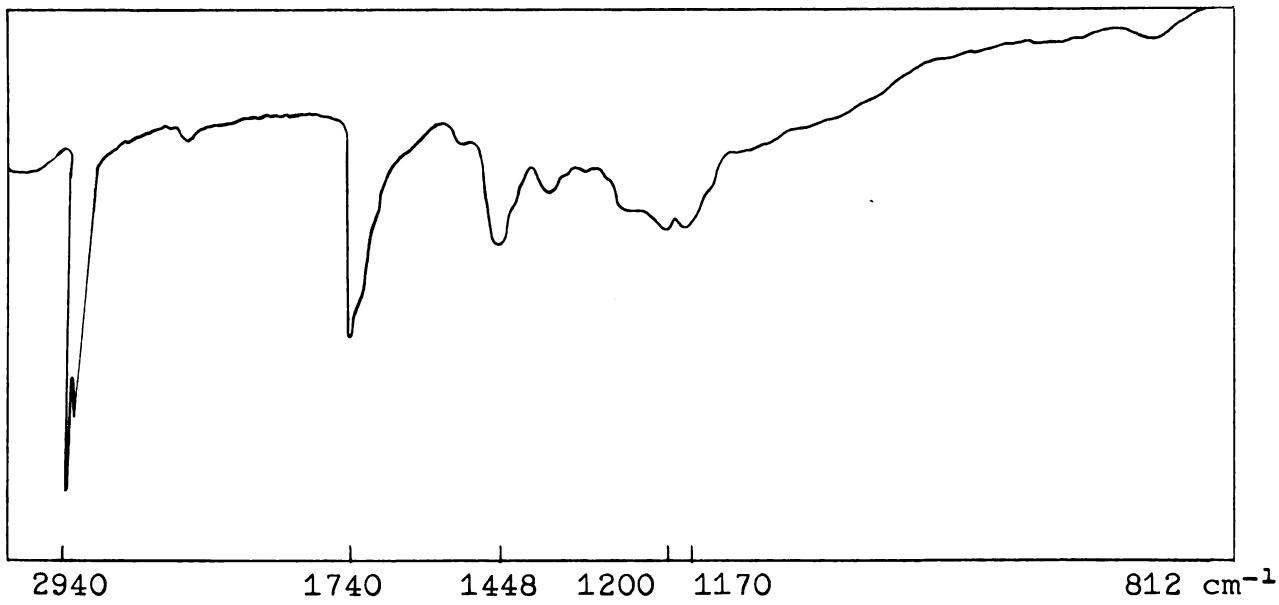


Fig. 9. Infrared Spectrum of Dimethyl Tetradecanedioate

Fig. 10. Infrared Spectrum of 1,1-Diphenyldodec-1-ene (neat)

Apiezon L column at a column temperature of 225° and helium flow rate of 2 ml. per minute showed three major fractions present in the distillate. The retention times of 6.6 minutes, 22.5 minutes, and 27 minutes correspond to relative peak areas of 2.94, 1.08, and 1.00 respectively. Attempted sample collection was unsuccessful because of the small initial sample size. The b.p. of the dimethyl ester of tetradecanedioic acid has been reported as 196°/9.5 mm. (350°/760 mm.) and 202°/14 mm. (342°/760 mm.) (76).

Raney nickel prepared by digestion (77) for 3 hours in concentrated aqueous base was found to be ineffective in the desulfurization of a similar crude sample of terthienylcarboxylic acid.

Barbier-Wieland Degradation Sequence (78)

The esterification of long chain fatty acids was accomplished satisfactorily by two procedures (79a,80).

Table 21. Esterification of Fatty Acids

Acid	Quantity (g.)	Conditions	Reagents	Yield (%)
Lauric	5.09	100°,1 hour	CH_3OH, H_2SO_4	90.7
Hendecanoic	1.19	65°,5 minutes	CH_3OH, BF_3	70.4
Hendecanoic	2.00	64°,5 minutes	CH_3OH, BF_3	87.2
Hendecanoic	3.00	64°,10 minutes	CH_3OH, BF_3	90.0
Hendecanoic	1.00	64°,10 minutes	CH_3, OH, BF_3	83.8

The CH_3OH-BF_3 reagent was prepared by mixing 380 ml. of purified methanol, 0.6 ml. distilled water, and 37 g. of BF_3 at 0-10°. The reagent and carboxylic acid were mixed at room temperature in weight:volume ratios of 1:20, 2:40, and 3:60. The mixtures were heated on a steam bath for the period of time indicated in Table 21. Petroleum ether was added to the cooled reaction mixture; the diluted mixture was poured into a seven-fold excess of water, and the organic phase was separated. Petroleum ether extracts of the aqueous phase, 150 ml., were added to the organic phase separated above, washed with water, and dried over magnesium sulfate. The solvent was removed at reduced pressure, and the residue was distilled. Methyl laurate distilled at 145°/18 mm. over a short distillation path. Methyl n-hendecanoate distilled at 81°/1 mm. and 97°/4.5 mm. over a short distillation path. Boiling points agreed with literature data (81). The ester carbonyl absorption band appeared at 1750 cm^{-1} in the infrared spectra (neat, Beckman IR-5).

The Grignard reagent (79b), phenylmagnesium bromide (0.082 mole), was prepared observing the usual precautions. Methyl n-hendecanoate, 0.90-2.90 g. (0.0045-0.014 mole), in 10 ml. of dry ether was added dropwise to the vigorously stirred Grignard reagent at room temperature. A precipitate was observed. The reaction mixture was set aside at room temperature for about 18 hours. Product isolation gave

9.2-8.3% of biphenyl (removed by vacuum distillation at 98°/2.8 mm.) and the crude tertiary alcohol. The alcohol, 100 ml. glacial acetic acid, and 50 ml. of redistilled acetic anhydride were refluxed for an hour. With the distillation head adjusted for delivery, the acetic acid-acetic anhydride solvent was distilled (78-120°) until a volume of 50-60 ml. remained in the reaction flask. The crude olefin and solvent were cooled to 50°. The distilling head was replaced with a parallel side arm connector fitted with a thermometer and dropping funnel. Chromium trioxide, 2-6 g. (0.020-0.060 mole), in 1.5-6 ml. water and 10-40 ml. glacial acetic acid were added in portions to the stirred solution at a rate sufficient to maintain the reaction temperature in the range of 50-60°. After adding the oxidizing agent, the reaction mixture was kept at 50° for an hour and then at room temperature for 18 hours. Excess chromic oxide was destroyed with methanol. The side arm connector was replaced with a Claisen head, and the solvent was removed under reduced pressure until a paste remained in the reaction flask. Water was added, and the mixture was extracted with 300 ml. of diethyl ether in small portions. The ethereal extracts were washed with 10% aqueous sodium hydroxide until the washings were basic to hydrion paper. The combined ethereal extracts were then washed with water and dried over magnesium sulfate. Evaporation of the ethereal solution gave an immiscible

mixture of yellow and colorless oils. The mixture was chromatographed over 20 g. of alumina and column fractions 4 to 7 which were eluted with hexane and 5% ether in hexane gave the crude ketone, benzophenone (m.p. 39-40°, 26%; literature m.p. 45-48°) (82b). The yellow oil from the column which would not crystallize was treated again with chromium trioxide in glacial acetic acid. Product isolation from the neutral organic fraction and one recrystallization from petroleum ether increased the overall yield to 52.2% benzophenone (m.p. 44-46°). Mixed melting point determination with an authentic sample showed no depression.

Basic extracts of the ethereal solution were acidified to a pH of 1 with concentrated hydrochloric acid, extracted with 300 ml. of ether in small portions, and dried over magnesium sulfate. The yellow oil obtained on evaporation of the ether solvent was esterified with methanol-boron trifluoride. The ester, methyl caprate (b.p. 60-66°/1 mm.), was obtained (53.8% overall yield). The boiling point reported in literature was 224°/760 mm. (82a). The infrared spectrum of the neat sample confirmed the ester structure of the distillate. The vapor phase chromatography retention time, 9.9 minutes, of the ester on an Apiezon L column at a column temperature of 188° and helium flow rate of 2 ml. per minute corresponded to an authentic sample of methyl caprate.

Permanganate Oxidation of 1,1-Diphenyldodec-1-ene (83)

1,1-Diphenyldodec-1-ene (Figure 10) was obtained by the dehydration of the tertiary alcohol from the hydrolysis of the appropriate Grignard product. To the olefin in 30 ml. glacial acetic acid was added an excess of solid potassium permanganate in one portion, and the reaction mixture was heated to 75°for a half hour. The reaction mixture was cooled to room temperature and poured into dilute sulfuric acid. The acidic mixture was heated on a steam bath, and the coagulated manganese dioxide was separated by vacuum filtration. The filtrate was cooled and the orange oil was taken up in ether. Extraction of the ethereal solution with aqueous sodium bicarbonate gave a trace amount (not measured) of solid. Product isolation from the ethereal solution gave a colorless solid (m.p. 85-87°, 20%) which analyzed for the 1,2-diol. Anal. Calcd. for $C_{24}H_{34}O_2$: C, 81.20; H, 9.60. Found: C, 80.87; H, 9.53. The infrared spectrum (KBr pellet, Beckman IR-5) exhibited a broad band at 3571-3333 cm^{-1}; the tertiary and secondary carbon-hydroxyl stretching frequencies, at 1176 cm^{-1} and 1093 cm^{-1} and the mono-substituted benzene ring carbon-hydrogen bending absorptions, at 750 cm^{-1} and 697 cm^{-1}. No further study of the products was undertaken.

Attempted Beckmann Monooxime Degradation Sequence (84)

Tridecanoic acid (Eastman Organic Chemicals white label) and a seven-fold excess of thionyl chloride (purified) were

mixed in a round-bottomed flask equipped with a magnetic stirrer and calcium chloride drying tube. The vigorously stirred reaction mixture was heated to 40° for a half hour. Excess thionyl chloride was removed under reduced pressure at room temperature. The yellow-tinted acid chloride was dissolved in a ten-fold excess of dry thiophene-free benzene and cooled to 0°, and a slight excess of reagent grade aluminum chloride was added in portions. The reaction mixture was stirred at room temperature for 18 hours. Dilute hydrochloric acid was added. The organic layer was separated from the aqueous layer. Unreacted carboxylic acid was removed by extraction of the benzene solution with 1N sodium hydroxide. The organic layer was shaken with a 1:2 methanol-water solution to remove traces of the sodium salt of the carboxylic acid. After removal of the solvent from the benzene solution, the white crystalline ketone was recrystallized from hexane (m.p. 39-40.5°). The melting point and infrared spectrum agreed with literature data for the ketone (85). In four experiments using 0.546-2.00 g. of the carboxylic acid, 55-66% yields of the purified ketone were obtained. The ketone, 2.87 mmoles, was dissolved in 10 ml. of purified dioxane and 0.6 ml. concentrated hydrochloric acid (diethyl ether and hydrogen chloride were used with one sample of ketone). The reaction mixture was heated to 50° (34° in the case of diethyl ether). A solution of 0.49 ml. (3.9 mmoles) of freshly distilled isoamylnitrite (purity by v.p.c. analysis, 76%) in 5 ml. of

purified dioxane was added dropwise during 45 minutes. The reaction solution was light brown in color and clear. After an additional 15 minutes heating at 50°, 12 ml. (36 mmoles) of 3N sodium hydroxide were added. (Powdered sodium hydroxide was used with one sample of ketone.) The light brown organic layer separated from the aqueous phase. After cooling to room temperature, 2.0 g. (10 mmoles) of distilled p-toluenesulfonyl chloride were added in portions to the vigorously stirred dispersion during 20 minutes. The dispersion was warmed again to 50° and stirred for an additional 2 hours. The reaction mixture was cooled and poured into 300 ml. of water in a separatory funnel. The organic layer was separated, washed with water, and dried over magnesium sulfate. Lauryl nitrile was not detectable by boiling point range or infrared spectrum (86) in the distillate obtained by reduced pressure distillation. The infrared spectrum had no absorption band between 3330-3000 cm^{-1} and a well defined band at 1730 cm^{-1}. p-Toluenesulfonyl chloride was the only solid isolated from the organic phase. The aqueous phase was acidified with concentrated hydrochloric acid and extracted with ether. The extracts were washed with water and dried over magnesium sulfate. The solvent was removed, and the residue was sublimed. Benzoic acid, m.p. 120-122°, was obtained in yields of 9-19% in four attempted degradations.

DISCUSSION

Biogenetic speculations have the valuable function of providing a focus for the examination of possible pathways for the biosynthesis of naturally occurring compounds. From principles of comparative structural analysis, economy, enzyme catalysis, and modern reaction theory, biogenetic pathways may be formulated. It is convenient to discuss the steps of a pathway in a certain order. The sequence of synthetic steps in the formulation of a natural molecule is impossible to discern from biogenetic considerations alone. Similarly, this inferential approach does not admit the identification of the actual reagents used but only the effective structural element involved. A satisfactory rationalization of the biogenesis of many plant products can be made by assuming an acetate-malonate chain followed by appropriate decarboxylation and condensation. The details of metabolism intermediary between the acetate-malonate primers and the derived natural products require the experimental evidence of radioactive tracer and enzyme studies.

The biogenetic formulation of thiophenic natural products from polyacetylenes conforms to the four criteria for a valid biogenetic hypothesis: comparative structure, economy, enzyme control, and conformity to modern reaction theory.

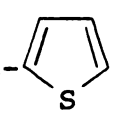
The functional group pattern and size of the polyacetylenes which have been obtained from plants among the Compositae are comparable to the thiophenic compounds found in the same Family. The removal of a terminal methyl group which is formally required in some instances can be expected to proceed by oxidative elimination. Cell free extracts from the fungus, Coprinus quadrifidus, cause the elimination of the terminal functional group carbon of a ten carbon polyacetylenic alcohol to form a nine carbon polyacetylenic alcohol (10).

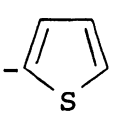
$$HOOCC{\equiv}CC{\equiv}CC{\equiv}CCH{=}CHCH_2OH \longrightarrow HC{\equiv}CC{\equiv}CC{\equiv}CCH{=}CHCH_2OH$$

The polyyne XI and compounds a (11,39) and b (11) are clearly related. Compounds XII, c (43), and d (44) of Chart 4 display a structural similarity.

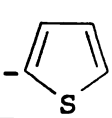
Chart 4. Naturally Occurring Polyynes and Related Thiophenic Compounds

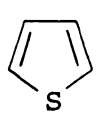
XI. $H_3CCH{=}CHC{\equiv}CC{\equiv}CC{\equiv}CC{\equiv}CCH{=}CH_2$

a. $H_3CCH{=}CHC{\equiv}C-$  $-C{\equiv}CCH{=}CH_2$

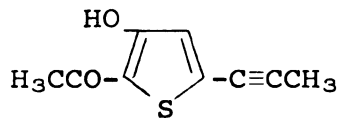
b. $H_2C(OH)CH{=}CHC{\equiv}C-$  $-C{\equiv}CCH{=}CH_2$

XII. $H_3CC{\equiv}CC{\equiv}CC{\equiv}CCH{=}CHCOOCH_3$

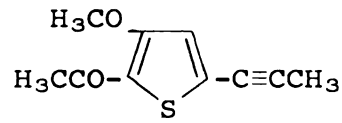
c. H_3C-  $-C{\equiv}CCH{=}CHCOOCH_3$

d.  $-C{\equiv}CCH{=}CHCOOCH_3$

The oxygen functions in the compounds XIII and XIV from Artemisia arborescens (60) support Sorenson's (14) suggestion that polyketones are involved in the biosynthesis of the thiophene ring. Some oxidized sites are known, however,



XIII



XIV

to arise by secondary processes. Nemotinic acid (16) is labeled at alternate carbon atoms in biosynthesis of the acid from acetate-1-^{14}C. The 4-hydroxy substituent of the undeca-5,6-diene-8,10-diynoic acid appears on an unlabeled carbon atom. The genesis of this atom is the methyl group of acetate-1-^{14}C.

Thiophenic compounds are presumably of secondary, if any, metabolic importance to the plants which produce them. The amount of material isolable is generally less than 0.02% by weight of the tissue extracted (Table 8, and references 37, 45, 46). The nematocidal effect of the polythienyl exudates of marigold roots has been observed (73,87), but the function of terthienyl in the blooms of the marigold, Tagetes erecta L., is unknown (1). It is reasonable, therefore, to suppose that the plants use the fewest possible reactions to produce these materials. Economy would also suggest that the enzymes involved in the production of minor products be of a low order of specificity. Enzymes merely

catalyze reactions that are sound mechanistically and are usually known to go *in vitro*.

The predominance of *trans*-addition to an acetylenic bond is mechanistically sound and experimentally demonstrable. Thiolacetone and methyl propiolate in the presence of an equimolar amount of base cyclize to 2-acetyl-3-hydroxythiophene (88). The addition of thiolacetic acid to 1-hexyne at 0° with no initiation produces a total product yield of 55% which consists of the *cis* and *trans* products in 82:18 ratio. A *cis* product is stereoselectively suitable for intramolecular interaction with an electrophilic triple bond for the formation of a five-membered sulfur heterocycle. It has been demonstrated in laboratory syntheses (19) that one thiophene ring may be formed by the addition of the elements of hydrogen sulfide to a triple bond; however, triple bonds adjacent to a thienyl function are unreactive under these laboratory conditions.

Bohlman's work (4,5) unequivocally demonstrates the ability of a biological system to effect the conversion of acetylenic bonds to a vinyl thioether function and a thiophene ring. The formation of XV from XVI is shown

$C_6H_5COC{\equiv}CCH{=}CHSCH_3$ $C_6H_5COC{\equiv}CC{\equiv}CH$

XV XVI

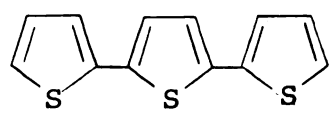
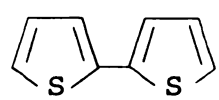
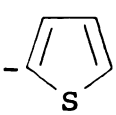
to occur in *Chrysanthemum segetum* by the incorporation of the radioactive oxo-carbon atom of XVI into XV *in vivo*.

The incorporation of the tritium label of XVII, 1,2-^{3}H into XVIII, XIX, and XX in Echinops sphaerocephalus L. (Table 22) illustrates the biosynthesis of these thiophenic compounds from the pentyne.

$$H_3C(C{\equiv}C)_5CH{=}CH_2$$

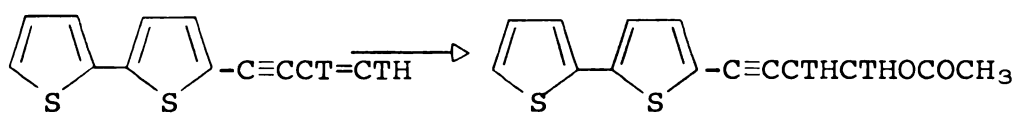
XVII

Table 22. Incorporation Data From Echinops sphaerocephalus L.

Compound	Structure	Dilution Factor
XVIII		4140
XIX	 -C≡CCT=CTH	2000
XX	$H_3C(C{\equiv}C)_2$-  -C≡CCTClCHT($OCOCH_3$)	41000

The data in Table 22 are necessary but not sufficient to establish a preformed polyacetylene of thirteen carbon atoms as an intermediate in the biosynthesis of terthienyl or 5-(3-buten-1-ynyl)-2,2'-bithienyl in Tagetes erecta or of thiophenic natural products in general. The role of

tridec-1-en-3,5,7,9,11-pentyne as an exogenous precursor* cannot be questioned. The dilution factors of Table 22 are large, notwithstanding the incubation period of twelve hours. In this period of time, 6.5% of the total activity administered is incorporated into the substances of the organic extract of the roots of Echinops sphaerocephalus. Very little dilution (dilution factor of 64.6) occurs in Tagetes tenuifolia Cav. with the conversion of XXI to XXII under the same conditions. The dilution factors in Table 22, therefore, can be interpreted in three ways.



XXI XXII

The tritium labeled pentyne XVII may (a) be diluted by the physiological precursor to XVIII, XIX, and XX; (b) be diluted by the pool size of the thiophenic compounds which is not likely; or (c) by the process of discrimination, be transformed only in part to XVIII, XIX, and XX with polymeric decomposition and unknown metabolic routes accounting for the remainder of the twenty-five milligram initial dose.

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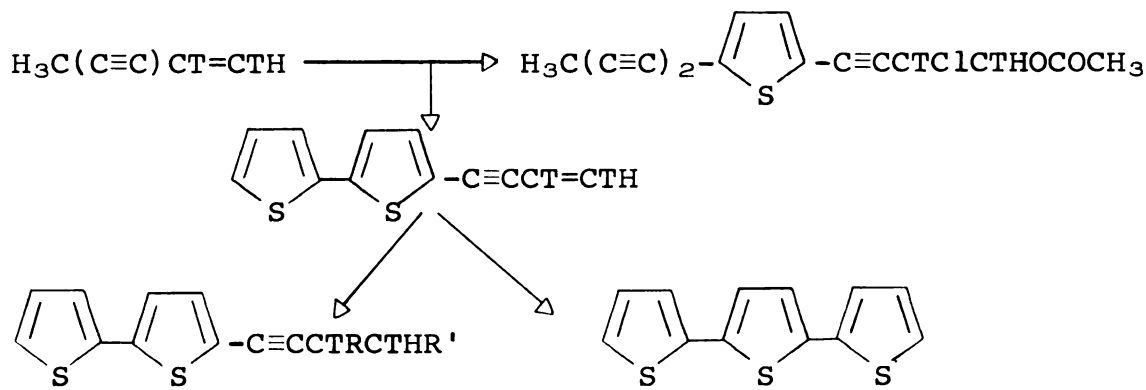
*A precursor is any compound whether exogenous or endogenous that can be converted by an organism into some product. An intermediate is a compound that is both formed and further converted by the organism under identical conditions (90).

The sequence of synthetic steps in the elaboration of a natural thiophenic compound will involve chain elongation of some structural unit presumably by malonyl CoA, desaturation to acetylenic bonds, and the incorporation of a sulfur atom in ring closure to a thienyl moiety. Bohlman (5) has proposed biosynthetic Scheme A, Chart 5, to explain the degree of incorporation of the tritium label reported in Table 22. Bohlman's scheme assumes the requirement of a preformed polyacetylene of thirteen carbon atoms. Whether or not it is possible that chain elongation and/or desaturation occurs after the formation of one thiophene ring is a mute question. Naturally occurring

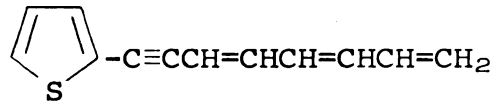
Chart 5. Biosynthetic Scheme A

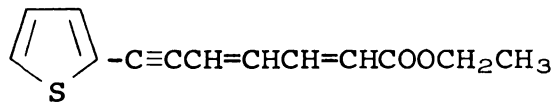


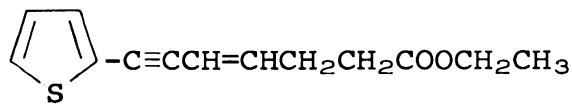
R = H

R' = $OCOCH_3$

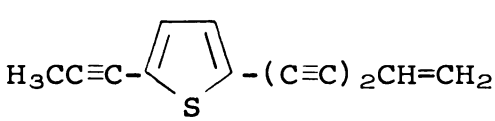
compounds XXIII, XXIV, and XXV which occur in Matricaria inodora (35) are suggestive of this latter mode of synthesis.

XXIII 

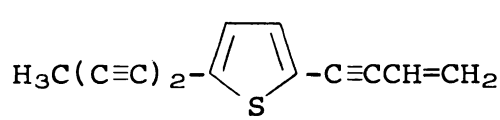
XXIV 

XXV 

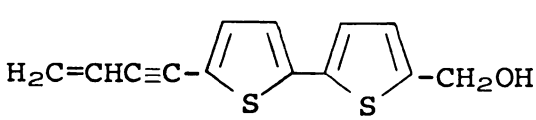
Compounds XXVI and XXVII from Ambrosia (23) and XXVIII and XXIX from Bidens (39) suggest that the formation of the thiophene ring occur across any suitably located triple bonds with preference for the center of a long carbon atom chain. This is consistent with the expectation that the interior triple bonds of a polyacetylene are more electrophilic than perpherial bonds with electron-donating groups and with the desaturation of long chain fatty acids which occurs about the ninth carbon atom (Confer Chart 1). The formation of



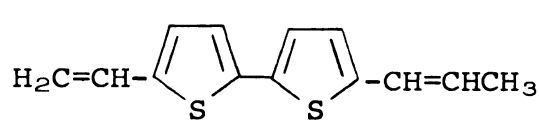
XXVI



XXVII



XXVIII



XXIX

the thiophene ring in biological systems may be construed as a mechanism whereby triple bonds are stabilized.

The investigation of biosynthetic pathways with radioisotopes and using whole plants presents the challenge of microtechniques and a complex dynamic system.

Since a statistical composite is generally admitted to be not less than one-thousand individuals, the appraisal of data collected from less than this number of plants is expected to reveal only the qualitative trends in the organism. Individual plants can, moreover, be expected to display inherent seed and seasonal variation (8,91). It may be noted that Dawson (92) has found a close dependency between root weight and the concentration of nicotine in tobacco roots. A dependency of terthienyl yield on the treatment of the roots has been examined in this work (Table 23).

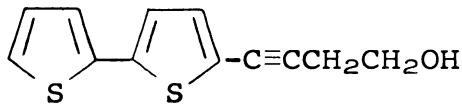
Table 23. Variation in Biosynthetic Terthienyl

Treatment of the Roots	Micromoles per Gram of Root	Reference
Fresh	0.10	73 a
Fresh	0.44	37 b
Blotted dry	0.44	3 c
Air-dried, 1 hour	0.35±.07	d
Desiccated, 18 hours	0.13±.00	e

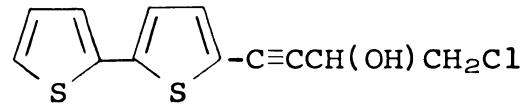
[a] *Tagetes erecta*.
[b] *Tagetes minuta*.
[c] *Tagetes erecta*.
[d] An average of three observations in this work.
[e] An average of two observations in this work.

The small amounts of 5-(3-buten-1-ynyl)-2,2'-bithienyl and terthienyl isolable from Tagetes erecta L. require efficient purification and separation techniques in order to minimize material loss. Material loss was directly observable in experiments 26, 28, 29, and 32. Chromatography is a common technique for the separation of small quantities of substances. This work reports the first procedure for the fast and facile separation of terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl. Silica gel on an 8" x 8" plate effects a satisfactory separation with hexane (purified by treatment with sulfuric acid and distillation from potassium carbonate) as a mobile phase. Autoradiograms of the chromatograms are useful in establishing a permanent record of the separation and the presence of radioactivity. The exposure period of ninety-seven hours is not unusual (93). The intensity of the exposed areas on the autoradiograms is a qualitative index of the activity present. 5-(3-Buten-1-ynyl)-2,2'-bithienyl undoubtedly causes some chemical reduction of the silver emulsion. The autoradiogram data of experiments 24 and 25, Table 16, and the ready oxidation of the bithienyl derivative support this conclusion. The decomposition of the bithienyl derivative by polymerization to a yellow, ether insoluble material has already been reported (37). The physical dispersion of the molecules on the silica gel surface probably retarded any extensive polymerization.

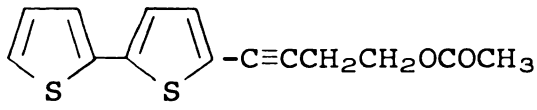
The determination of the ultraviolet and visible spectra of the samples of undiluted biological material has the distinct advantage of revealing trace quantities which absorb in this region. The presence of two contaminants having absorption maxima at 334 mμ and 336 mμ is discernible in some spectra of the polythienyls isolated on thin layers of silica gel. The latter absorption is observed principally in the bithienyl derivative fraction and shows some incorporation of sulfur-35 (experiments 22 and 23). The Rf's and the identification of **XXX** and **XXXI** in root extracts of Tagetes minuta (45) and **XXXII** in Tagetes erecta and minuta (45,46) would suggest these compounds as suspects for the substances described above.



XXX (334 mμ, 328 mμ)



XXXI (336 mμ)



XXXII

Trace quantities of radioactive impurities in the biological samples of terthienyl which had been diluted with synthetic material could only be discerned by determining the constancy of specific activity after successive purification processes or chemical transformation (experiment 2).

The assay of radioactivity in chromatographically pure samples of terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl by the counting of thin layers has precedence in the work of Bu'Lock (51). The density of samples of terthienyl-^{35}S and 5-acetylterthienyl-^{35}S, 0.002-0.105 mg. per cm^2. on aluminum backing, has been shown to be directly related to the amount of activity of the samples (3). The simple equipment and manipulations required and the comparison of the specific activities of samples counted as barium carbonate (experiment 11) and the specific activities calculated from thin layer counting of the corresponding terthienyl samples (Table 18) justify the use of this method.

The determination of the specific activity of the polythienyls isolated from the roots of Tagetes erecta L. requires a knowledge of the concentration of the biological material. The most reliable method to determine the concentration is direct observation. An alternate method, the double dilution method of Mayor and Collins (63), is an application of isotope dilution analysis for the determination of yield and activity of radioactive compounds. The method is satisfactory if one can reliably assume a quantity and dilute the sample with appropriate amounts of the nonradioactive compound.

The radioisotopes' forms examined in this study have been hydroponically administered Tagetes erecta L. An examination of the nutrient solution after harvesting the

plants reveals that 93-99% of the radioactivity dispensed is removed from solution. In the DL-cysteine-^{35}S and DL-cystine-1-^{14}C experiments, 25-50% of the administered activity has been recovered from the nutrient solution (Table 24). One explanation may be the degree of permeability of the root cells to the disulfide, DL-cystine, which exists in solution in equilibrium with the mercapto form, DL-cysteine. Trace quantities of metals such as are present in the nutrient solution are known to catalyze this interconversion.

Table 24. Percent of Radioactivity Recovered in Nutrient Solutions

Experiment Number	Radioisotope Form	Incubation (days)	Percent Recovered
22	DL-Cysteine-^{35}S	0.8	26.4
23	DL-Cysteine-^{35}S	1.8	25.2
26	DL-Cystine-1-^{14}C	0.1	49.5
27	DL-Cystine-1-^{14}C	1.0	23.6
29	Pimelate-7-^{14}C	2.0	82.7

The amount of radioactivity remaining in the nutrient solution after two days incubation in experiment 29 is apparently due to poor absorption by the roots. No radioactivity was found in the polythienyls isolated in this experiment.

Fungal and bacterial removal of available radioactivity in the nutrient solution during the period of incubation cannot be significant. The amount of radioactivity

extracted from the roots of the plants and that recovered from the nutrient solution were unaffected by the presence of a fungicide or an antibacterial (experiments 16, 17, 31, 32). It is reasonably certain, therefore, that the form administered is the form which was absorbed by the plants.

The amount of radioactivity recovered from the ethanol extracts (Table 12) and that recovered from the nutrient solution can be explained by metabolism of the radioisotope form by the plant, the possibility of the radioisotope in root exudates, and the transport of the radioisotope of sulfur or carbon away from the roots, the site of synthesis of terthienyl (2). The rapid transport of a likely precursor to the polythienyls in Tagetes away from the site of synthesis can be expected to result in little or no incorporation of the radioisotope.

The absence of activity in the terthienyl isolated in experiment 5 was expected. The metabolic products of DL-ornithine-2-^{14}C in the plant kingdom are glutamic semialdehyde, citrulline and arginine, and DL-glutamic-2-^{14}C acid was not incorporated into terthienyl by Tagetes erecta L. in earlier work (3).

Remarkably little is known about the metabolism of sulfate in plants. The rapid transport of inorganic ions is generally accepted. Sulfate-^{35}S administered hydroponically to Tagetes erecta L. in this work was incorporated

in essential agreement with an earlier study (3). The sulfate-^{35}S may have been immediately transformed in the root to a form suitable for incorporation into terthienyl. A reductase system for sulfate has been shown to exist in roots of the field pea (94). The reductase is attributed with enabling the conversion of sulfate-^{35}S to a form suitable for incorporation into protein-bound and free sulfur containing amino acids in the roots.

Experiments 21, 22, and 23 examine DL-cysteine-^{35}S as a likely physiological precursor of the sulfur atom to the polythienyls in Tagetes erecta L. The production of hydrogen sulfide and pyruvic acid from cysteine and cystine by microorganisms is a known enzymic process (95). From earlier work (2), hydrogen sulfide as a reagent in the biosynthesis of terthienyl appears to be discredited since bisulfide-^{35}S was not incorporated into terthienyl. The nutrient solution in the bisulfide experiments revealed only a 27-50% decrease in radioactivity after 1.6 and 1.2 days of incubation. The limitation of the root cells to absorb and use bisulfide in that form is one possible explanation for these data. The dilution of DL-methionine-^{35}S by a factor of 4420 in terthienyl-^{35}S from Tagetes (3) suggests methanethiol-^{35}S, homocysteine-^{35}S, or cysteine-^{35}S via cystathionine as possible physiological precursors to the sulfur atom in terthienyl.

The dilution factors for likely sulfur precursors to terthienyl from this work and published data are listed in Table 25. The apparent relationship between the age of the plants and the degree to which inorganic sulfate is diluted in terthienyl correlates with the nematicidal activity of marigolds cultivated for 10 (87) and 12 (96) weeks. The very young plants of experiment 15 and the 20-week-old plants (2) show poor incorporation of likely precursors compared to 8 to 11-week-old plants. De novo synthesis of terthienyl in the bloom of older plants (after 10 weeks) or transport, particularly in the way of root exudates, may be a factor in the high dilution factors obtained with old plants.

Table 25. Incorporation of Sulfur-35 into Terthienyl

Sulfur-35 Form	Plant Age (weeks)	Incubation (days)	Dilution Factor
Sulfate[a]	20	2	294
	6	5	161
	6	10	77
	20	15	260
Methionine[a]	16	1.3	4420
Sulfate[b]	8.8	1	118
	8.8	10.6	35.1
Cysteine[b]	10	0.8	60.8
	10	1.8	3.86
	10	8.9	14.4

[a]Reference 2.
[b]This work.

Clearly, the sulfur atom of DL-cysteine is efficiently incorporated into the heterocyclic nuclei of terthienyl. The dilution factors for the 0.8 day experiment are of the same magnitude as the dilution factor for the *in vivo* conversion of tritium labeled 5-(3-buten-1-ynyl)-2,2'-bithienyl to 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl, Chart 5. The mode of incorporation of cysteine sulfur cannot be stated with certainty. This reaction step in the biosynthetic sequence may proceed by way of a *cis*-vinyl thioether of cysteine or of its metabolic product, β-mercaptopyruvic acid. Alternatively, the *in vivo* generation of hydrogen sulfide and formation of a vinyl mercaptan is not disproven. The failure in laboratory syntheses to form more than one thiophene ring from conjugated acetylenic bonds and the elements of hydrogen sulfide suggests that a driving force such as a good leaving group on sulfur(cysteine or a biological equivalent) or a bond activated towards nucleophilic attack is required for polythienyl formation. The incorporation of methionine sulfur into terthienyl in *Tagetes* can adequately be explained by the formation of cystathionine from methionine by the condensation of homocysteine and serine. The hydrolysis of cystathionine in biological systems generates cysteine. The formation of methanethiol from methionine in mammalian tissue has been verified, but whether is occurs in plants is not clear.

A time dependent study for the incorporation of cysteine sulfur into terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl provides some insight into the biogenetic relationship of these polythienyls. Since the polythienyls were obtained from the same sample, the dilution factors reported in Table 26 are a measure of the metabolism of the respective polythienyls relative to each other. The rapid increase in radioactivity is expected from the flooding of the biological system with radioactive substrate. On the second day of incubation, the initial pulse of radioactivity appears to have been expended. A similar change in the increment of incorporation of inorganic sulfate-^{35}S and glucose-UL-^{14}C (Tables 19 and 25) is also noted. If terthienyl is the product of ring closure on sulfur of 5-(3-buten-1-ynyl)-2,2'-bithienyl, the rate of sulfur-35 appearance in terthienyl would initially be expected to be slightly less than that of 5-(3-buten-1-ynyl)-2,2'-bithienyl. It also follows that on saturation of the bithienyl derivative pool, further incorporation of sulfur-35 and/or nonradioactive sulfur should result in a dilution of sulfur-35 in terthienyl less or equal to that of 5-(3-buten-1-ynyl)-2,2'-bithienyl. This has not been observed. The data of Table 26 are explicable if, at some point in the biosynthesis of the thiophenic compounds, independent metabolism for the polythienyls is postulated.

Table 26. DL-Cysteine-^{35}S Incorporation into Polythienyls of Tagetes erecta L.

Polythienyl	Incubation (days)	Dilution Factors
5-(3-Buten-1-ynyl)-2,2'-bithienyl	0.8	6.94
	1.8	3.87
	8.9	3.66
Terthienyl	0.8	60.8
	1.8	3.86
	8.9	14.4

The variation of dilution factors with time, Figures 11 and 12, can be used to illustrate three types of precursors. DL-Cysteine-^{35}S is rapidly absorbed into the metabolic polythienyl pool and subsequent changes in the extent of incorporation are relatively small. Inorganic sulfate or an intermediary form appears to be available for synthesis over the entire 10-day period of observation. Glucose-UL-^{14}C, although highly diluted, rapidly appears in terthienyl and, seemingly, continues to be incorporated at a different rate after two days' incubation.

Biogenetic hypotheses have classified terthienyl and the naturally occurring thiophenic compounds as acetogenins.* The examination of acetate as a likely precursor of terthienyl in Tagetes (2) does not support this concept. Because the

*The term acetogenin has been defined by Richards and Hendrickson (49) as a generic name for compounds biogenetically derivable by the acetate hypothesis and its several variants and to exclude the terpenes.

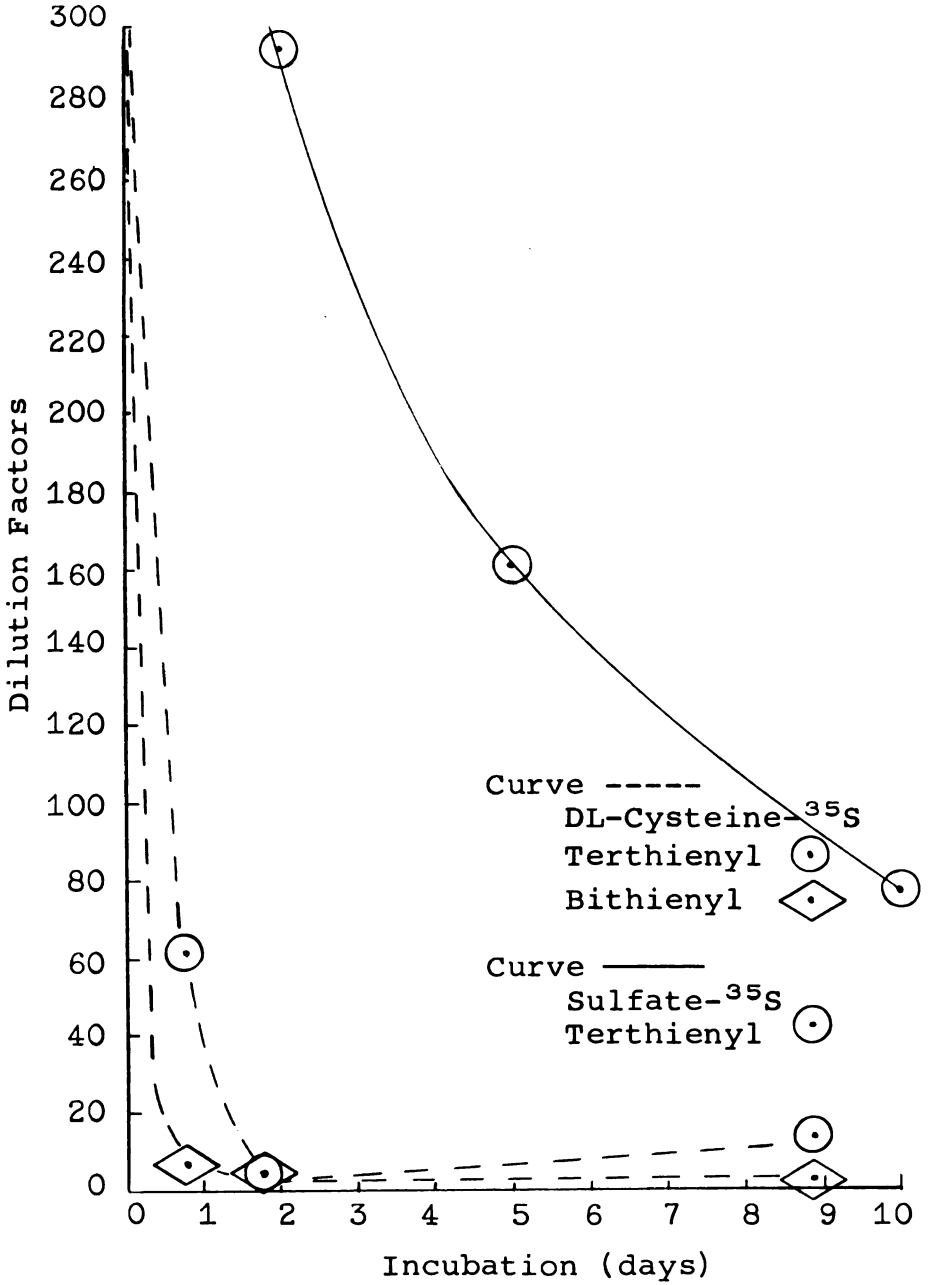


Fig. 11. Time Dependent Study on ^{35}S

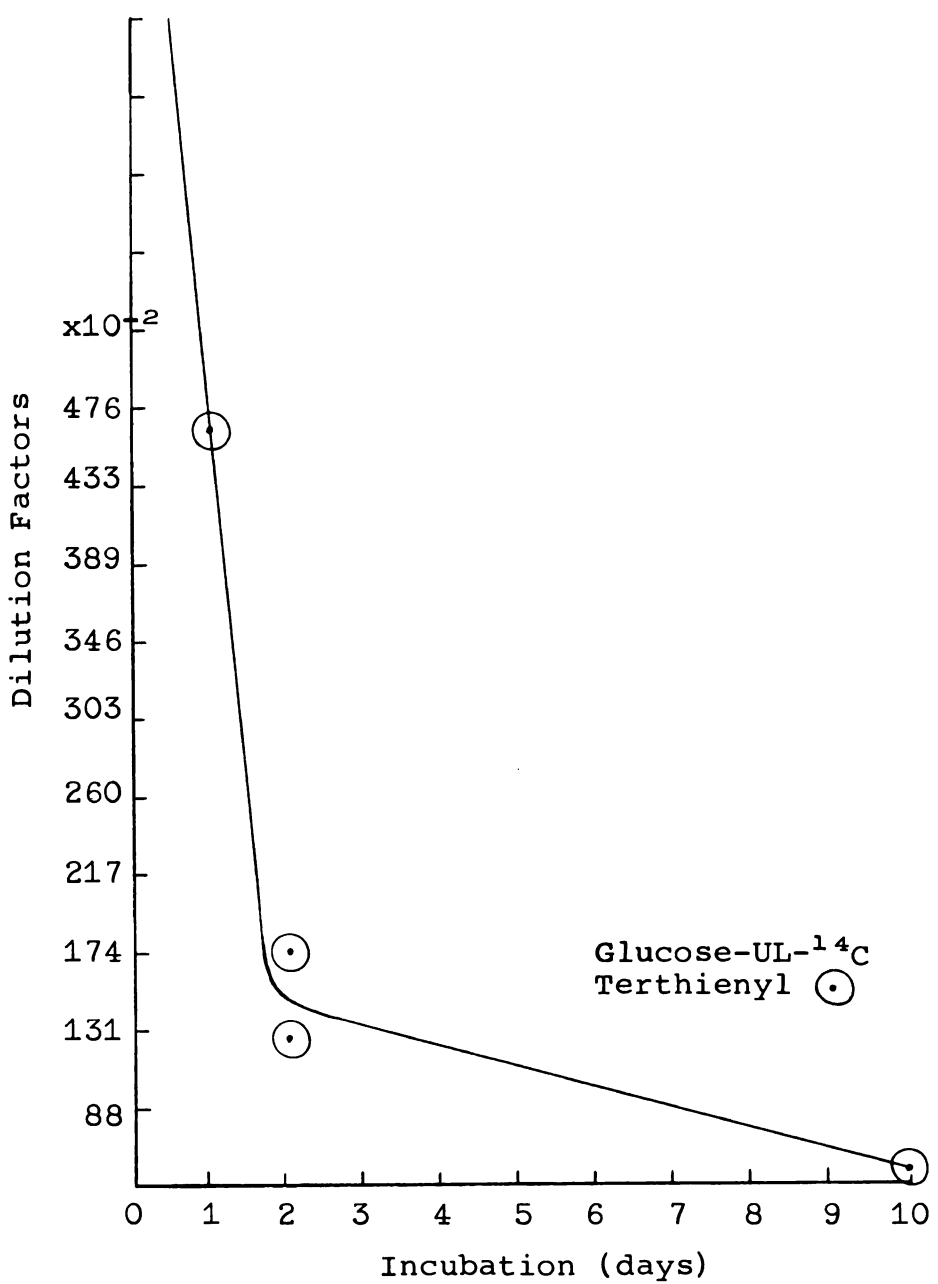


Fig. 12. Time Dependent Study on ^{14}C

reactive unit in the acetate hypothesis is acetyl CoA and not acetate, the investigation of the physiological precursors of acetyl CoA, glucose and pyruvate, is required before the hypothesis can be rejected.

The extent of incorporation of glucose-UL-^{14}C into terthienyl reported in experiments 3, 4, 6, and 7 of Table 19 is small. The high dilution of carbon-14 in terthienyl shows glucose to be of little significance as a precursor to terthienyl. Carbon labeling could have arisen from randomization of the carbon-14 in the entire organism. Alternatively, the high dilution factors may be caused by storage or discrimination among catabolic routes open to glucose. The glucose carbon which is directed to polyacetylene synthesis in the Basidiomycete strain, B.841, is known to diminish during periods of active growth (51), the same period used in these experiments. The shape of the glucose dilution curve, Figure 12, can be interpreted as the result of two mechanisms of incorporation. The first can be assumed to be due to the catabolism of glucose-UL-^{14}C and the second is the consequence of metabolism of glucose-UL-^{14}C to starch, a process known to occur in plants (97). The carbon-14 labelled starch can be thought of as a reservoir for acetyl-1,2-^{14}C CoA which in turn is a source of carbon-14 in fatty acids (Chart 1) and polyacetylenes.

The dilution factors in experiments 16 and 17, pyruvate-3-^{14}C, are too high to be significant. No activity was

observed in experiment 18. An experiment of shorter duration, for instance 0.5 to 0.8 day, ought to be performed before placing any interpretation on these results. A shorter duration experiment is suggested because pyruvate is readily converted to acetyl CoA and is not stored in the plants.

The examination of malonate-2-^{14}C and malonic-2-^{14}C acid in experiments 8 to 12 and 33 confirms a role for malonyl CoA in the biosynthesis of terthienyl. Malonyl CoA functions as a chain elongation unit in plant and animal tissue. No ready explanation is available for the high dilution factor observed in experiment 9 compared with experiments 8 and 33, Table 19. Citrate which was added to the nutrient solution in experiment 10 would be expected to cause a higher dilution factor if, as in mammalian tissue, citrate inhibits the metabolism of malonic acid (98). Data for the chromatographic examination of aliquots of the ethanolic root extracts from experiments 8, 9, and 11 are listed in Table 27. The fluorescent areas corresponding to terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl and the assay of the radioactivity present in these areas reveal the decrease in activity which accounts for the dilution factors.

The amino acid serine functions as a precursor of cysteine and cystine by way of cystathionine. The dilution factors observed for DL-serine-3-^{14}C and DL-cystine-1-^{14}C, experiments 24, 25, and 27, are comparable in magnitude. This would suggest a comparable mode of incorporation into terthienyl.

Table 27. Fluorescent Components of Ethanol Extracts

Experiment[a] Number	Rf[b]	Incubation (days)	Total CPM x 10^{-3}
8	0.63	2.0	10.1
	0.50[c]		8.11
9	0.75	2.0	10.4
	0.63[c]		4.38
11	0.65	8.7	4.80
	0.53[c]		4.15

[a]Sodium malonate-2-^{14}C.

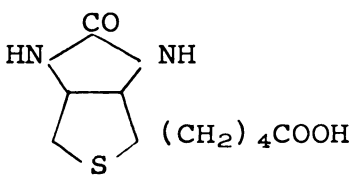
[b]Paper chromatography. Developing solvent: methanol-water (60:40).

[c]This corresponds to the Rf of co-chromatographed terthienyl.

DL-Methionine-2-^{14}C is diluted about one-third the amount of the dilution factors for the three-carbon amino acids. One catabolic product of methionine is α-ketobutyric acid. This fact and the observation that pimelate-7-^{14}C, experiment 30, is incorporated to the greatest extent of all the likely carbon precursors to the polythienyls examined in this work suggest that straight chain carboxylic acids function as the source of the carbon atoms in terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl. A preformed polyacetylene is, therefore, not a requirement for the synthesis of polythienyls in Tagetes. Since a polyacetylene has been shown to be incorporated into the polythienyls of Echinops sphaerocephalus (5), the incorporation of pimelate-7-^{14}C into the polythienyls of Tagetes erecta L. may be construed as

evidence for the synthesis of polyacetylenes from organic acids rather than other routes postulated in Chart 1. Data from a systematic carbon-by-carbon degradation are required before there can be assurance that there was no multiple entry of the acid into the polythienyls.

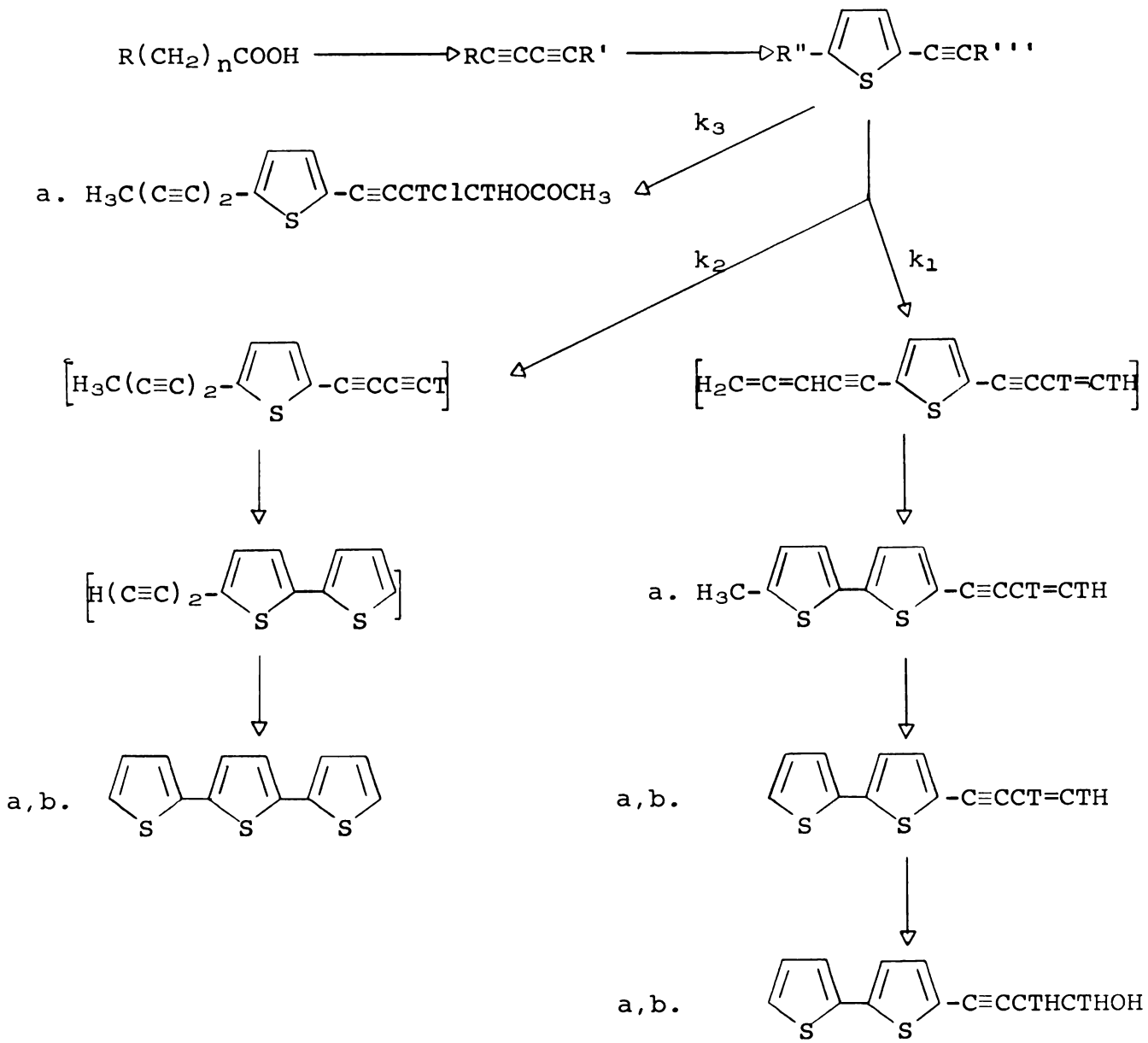
Pimelic-1,7-^{14}C acid has been identified as the precursor of biotin, **XXXIII**, in a microorganism and fungus (99,100). No degradation of biotin labeled from pimelic-1,7-^{14}C acid has been reported. It would be of great significance to compare the labeling pattern of biotin-^{14}C with that of terthienyl-^{14}C generated <u>in vivo</u> from pimelic-1,7-^{14}C.



XXXIII

Biosynthetic Scheme B, Chart 6, is proposed for <u>Tagetes erecta</u> L. to account for (a) the efficiency of pimelate as a precursor to both terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl, (b) the relationship observed between the polythienyls of <u>Tagetes</u> revealed by experiments 21 to 23, (c) the small differences in dilution factors observed for terthienyl compared with the bithienyl derivative in experiments 24, 25, and 30 and in Bohlman's work (5), and (d) the biogenetic evidence which has been presented here.

Chart 6. Biosynthetic Scheme B.



[a]Natural product from Echinops sphaerocephalus L.
[b]Natural product from Tagetes erecta and/or minuta.

The groups R and R' are introduced into Scheme B because the order of the steps in the sequence leading to acetylenic bonds is not confirmed. Rates k_1 and k_2 are assumed to be comparable although as long as both polythienyl pools are saturated with radioactivity from the hydrocarbon skeleton within 12 hours (Bohlman's experiment with tritium labeling in reference 5), this is not essential. Rate k_3 is very different from k_1 and k_2. There is no direct evidence of the biological mechanism for the formation of a triple bond. Since acetylenic bonds adjacent to a thiophene ring are apparently stable towards addition of H_2S, relative to other triple bonds (Table 5), it is not unreasonable to postulate desaturation to a terminal conjugated acetylenic system and the isomerization to the less stable allenic form before incorporation of sulfur and cyclization to a thiophene ring. Independent pathways for the polythienyls allow routes designated by rates k_1 and k_2 to be about equally saturated with the first pulse of tritium, carbon-14 and sulfur-35 radioactivity. The incorporation of further sulfur-35 along an independent pathway and the catabolism of terthienyl will account for the dilution factor of tritium labeled as well as sulfur-35 labeled terthienyl (^{3}H:4140; ^{35}S:60.8) compared to the bithienyl derivative (^{3}H:2000; ^{35}S:6.94).

The biological conversion, if any, of carbon-14 labeled 3-(2,2'-bithenoyl)propionic acid to naturally occurring thiophenic compounds in Tagetes would contribute to the

clarification of desaturation, chain elongation, and cyclization on sulfur which can be effected enzymically. The synthesis of this bithienyl derivative has been reported for the first time in this work.

The investigation of precursors to terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl in Tagetes erecta L. is limited to the identification of compounds efficiently incorporated into the biosynthetic pathway. Elucidation of the mode of incorporation requires systematic degradation of the radioactive biosynthetic material and identification of the distribution of the radioisotope in the molecule. The relative stability of terthienyl with respect to the other naturally occurring thiophenic compounds is the determinant for its selection in a degradative investigation. The stability allows purification to constant specific activity with minimal loss of terthienyl radioactivity and contamination by decomposition products. Any study of a reaction sequence to degrade terthienyl is necessarily concerned with the conversion of the symmetrical molecule to a form which may undergo further and controlled degradation, and secondly, with the selection of the route for the further degradation.

The control of mono- and disubstitution in terthienyl can be accomplished reasonably well by control of reagent quantities and dilution. The acetylation procedure reported here has produced a 37-40% yield of the monoacetylated product with recovery of 25-31% terthienyl. This enables a yield of

at least 50% to be realized by recycling the recovered terthienyl. The maximum yield of diacetylated terthienyl reported is 51% (26).

The chemistry of bithienyl which has been studied has established that substitution occurs in the 5,5'-positions when these positions are not substituted. The unique pattern in the aromatic region of the nuclear magnetic resonance spectrum, namely, the low field doublet and the sequence of apparent pairs of signals, provides a rapid and easily discerned characterization of the mono- and disubstituted polythienyl. The coupling constants are those reported for thiophene itself (38). The chemistry of terthienyl and higher polythienyls, on the other hand, has not been extensively studied.

Direct metalation of terthienyl has precedence with the homologs, thiophene (31) and bithienyl (26). Two synthetic routes to the carboxylic acid are available from the thienyllithium. Carbonation with dry ice has been known to produce thiophenecarboxylic acids directly and in better yields (31, 33) than the alternate procedure of formylation followed by oxidation. 2,2'-Bithienyl-5-carboxylic acid synthesized by the latter route in this work is obtained in 52.7% in contrast to the 74% yield reported by the direct carbonation (26). Formylation with dimethylformamide carbonyl-^{14}C followed by oxidation, however, is a practical route to the carboxyl-^{14}C acid, a derivative which is useful in the evaluation of the efficiency of subsequent degradative procedures.

The direct metalation of terthienyl with n-butyllithium, formylation of the polythienyllithium with dimethylformamide, and the silver oxide oxidation of the crude reaction product have been found to give a mixture of products. The difficult isolation of a pure sample of the carboxylic acid causes this reaction to be an impractical step in the synthesis of a terthienyl derivative suitable for systematic degradation. This reaction and the direct carbonation of terthienyllithium may be a profitable study. The reaction mechanism (38) and Wynberg's metalation study on 2,3'-bithienyl (26) suggest the basicity of sulfur to be a factor in the reaction.

The desulfurization of 5,5'' -diacetylterthienyl and many other thiophene compounds have been reported in literature (26). The activity of Raney nickel which is effective for a given thiophenic compound apparently varies. W-7 Raney nickel refluxed with 2,5-diphenylthiophene for six hours is reported to yield 45.9% of diphenylbutane and two other products (101). Raney nickel prepared rapidly at $0^{o}C$, slowly brought to room temperature, and washed to remove the concentrated base is effective in the desulfurization of a crude sample of terthienyldicarboxylic acid. The infrared spectrum of the esterified product confirms the degradation to a long chain fatty acid.

Two procedures for the degradation of fatty acids have been examined. Four criteria define an optimal procedure. It is desired (a) to degrade one carbon at a time, (b) to

obtain a good yield on a small scale, (c) to obtain the product in a form which is easily further degraded, and (d) to isolate in a convenient manner the carbon lost. The reason for failure of the Beckman monooxime degradation scheme is not readily apparent. Failure to form the tosylated intermediate may be one explanation. The Barbier-Wieland sequence, on the other hand, has given a satisfactory yield of the degradation products, the fatty acid ester (53.8%), and benzophenone (52.2%).

This complete examination of possible precursors to terthienyl and 5-(3-buten-1-ynyl)-2,2'-bithienyl in Tagetes erecta L. decidedly indicates that an organic acid of high specific activity (pimelic acid) and accurately known labeling pattern ought to be the precursor used in a degradation study of terthienyl. The preferred degradative scheme suggested by this work consists of the conversion of 5-acetylterthienyl to 5-terthienylcarboxylic acid by the already known haloform reaction (102), desulfurization with slightly basic and active Raney nickel, and Barbier-Wieland degradation of the fatty acid.

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APPENDICES

APPENDIX 1

SOURCE OF LABELED COMPOUNDS

1. Volk Radiochemical Company Skokie, Illinois	DL-Glucose-UL-^{14}C Malonic-2-^{14}C acid DL-Methionine-2-^{14}C Sodium pyruvate-3-^{13}C DL-Cysteine-^{35}S DL-Ornithine-2-^{14}C
2. Nichem Inc. Bethesda, Maryland	DL-Serine-3-^{14}C DL-Cystine-1-^{14}C
3. Baird Atomic Inc. Cambridge, Massachusetts	Pimelic-7-^{14}C acid
4. New England Nuclear Corp. Boston, Massachusetts	Sodium sulfate-^{35}S

APPENDIX 2

FORMULAE FOR COUNTING STATISTICS AND ERRORS (64,65)

1. Standard Error of an Observed Activity, n.

$$\sigma = (n)^{\frac{1}{2}}$$

2. Standard Deviation of a Net Rate, R_s.

$$\sigma_R = \left(\frac{R_{s+b}}{t_s} + \frac{R_b}{t_b} \right)^{\frac{1}{2}}$$

R = rate
s = sample
b = backround
t = time

3. Optimal Counting Time Distribution.

$$\frac{t_s}{t_b} = \left(\frac{R_{s+b}}{R_b} \right)^{\frac{1}{2}}$$

4. Probable Standard Error of the Specific Activity, A.

$$\sigma_A = \left(\frac{\sigma^2_R}{R^2_s} + \frac{\sigma^2_c}{C^2} \right)^{\frac{1}{2}}$$

A C = concentration

$$P = 0.6745\ \sigma_A$$