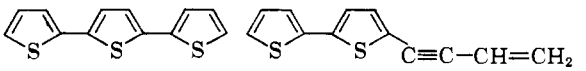
Biosynthesis of 2,2′;5′,2″-Terthienyl in the Common Marigold*

R. D. Schuetz,† T. B. Waggoner,‡ and R. U. Byerrum

ABSTRACT: Radioactive compounds suspected of being precursors to terthienyl were fed to marigold plants in nutrient solution. Terthienyl was isolated from the roots after a specified time from initial feeding. The specific activity was determined, and dilution factors were calculated. Sulfur was incorporated when fed as sodium [^{35}S]sulfate or DL-[^{35}S]methionine, but not when fed as sodium hydrogen [^{35}S]sulfide. Significant incorporation of carbon was found only when fed as DL-[2-^{14}C]methionine. A low level of incorporation was found from sodium [1-^{14}C]acetate, and no low level of incorporation was observed from DL-[2-^{14}C]glutamic acid or [2,3-^{14}C]succinic acid. Dilution factors were 294 from sodium [^{35}S]sulfate, 734 from DL-[^{35}S]methionine, and 1430 from DL-[2-^{14}C]methionine after 2 days' incubation. The "site of synthesis" was shown to be in the roots, as indicated by the results from stem and root feedings.

The initial discovery of the naturally occurring polythiophene, 2,2′;5′,2″-terthienyl, compound I (Zechmeister and Sandoval, 1945), led to its isolation and characterization from the blooms of the common marigold, *Tagetes erecta* L. (Zechmeister and Sease, 1947). Since that time an increasing number of naturally occurring compounds containing one or more thiophene rings have been reported in the literature (Katritzky, 1963). These compounds in the main are comprised of olefinic and/or acetylenic side chains attached to thiophene rings and have in the majority of cases been found in plants of the natural order *Compositae*.

Speculation on the biogenesis of terthienyl has been discussed in terms of hydrogen sulfide addition to polyacetylenes (Challenger, 1959) or to modified polyacetylenes (Sorensen and Sorensen, 1958). Terthienyl and 5-(3-buten-1-ynyl)-2,2′-bithienyl, compound II, were isolated from the same root extract of *Tagetes* (Uhlenbroek and Bijloo, 1958, 1959). These two investigators suggested that terthienyl might arise from the addition of hydrogen sulfide to 5-(3-buten-1-ynyl)-2,2′-bithienyl. With this evidence as background the present investigation was initiated, and its results are described here. This work represents the first biosynthetic[1] study of terthienyl or any of the known naturally occurring compounds containing the heterocyclic thiophene ring.



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Experimental Procedures

Materials. Sodium [^{35}S]sulfate was purchased from New England Nuclear Corp. DL-[^{35}S]Methionine, L-[^{35}S]methionine, sodium hydrogen [^{35}S]sulfide, DL-[1-^{14}C]methionine, DL-[2-^{14}C]methionine, and sodium [1-^{14}C]acetate were obtained from Volk Radiochemical Co. DL-[2-^{14}C]Glutamic acid and [2,3-^{14}C]succinic acid were secured from Tracerlab. The compounds were chromatographically pure.

Plants. The seeds of *Tagetes erecta* L. were purchased from W. Atlee Burpee Co., Fordhook Farms, Doylestown, Pa. *Tagetes* were used in all experiments except 4 and 5, where a dwarf, unknown genus was utilized. Four plants were used in each experiment unless some expired during the course of the experiment, in which case only those remaining alive were investigated. The weights of roots obtained from the plants in each experiment varied from 0.3 to 4.0 g with an average being 2.0 g. Plant age at the time of administration of the isotopically labeled compounds was 2–5 months. Each plant was transferred from the soil bed to the nutrient solution 2–4 weeks before the radioisotope was dispensed. About 80% of the roots were removed at the time the plant was removed from the soil. Each plant was placed in a separate Erlenmeyer flask containing sufficient nutrient solution to completely cover the roots. Additional solution was required during the period the new root system was developing.

Preparation of the Nutrient Solution. Stock solution of 1 N concentration was prepared; the quantities 19.2 g of ammonium dihydrogen phosphate, 50.6 g of potassium nitrate, 41.0 g of calcium nitrate tetrahydrate, and 30.0 g of magnesium sulfate were dissolved in separate volumes of 500 ml each of distilled water and

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† Present address: Institute of Biology and Medicine, Michigan State University, East Lansing, Mich.

‡ Abstracted in part from the Ph.D. dissertation, 1963, of T. B. Waggoner. Present address: M & T Chemicals, Inc., 1700 East Nine Mile Rd., Ferndale, Mich.

[1] The term "biosynthetic" signifies *in vivo* experiments. Discussions of the synthesis of terthienyl in nature have been published on speculations based upon structural similarities of terthienyl to likely precursors previously isolated. It is preferable to speak of this type of discussion, which is not based directly on actual *in vivo* experiments, as a "biogenetic" study.

stored under toluene until needed. For the preparation of a 1-liter quantity of nutrient solution, aliquots of 1, 6, 4, and 2 ml, respectively, of the above-mentioned solutions were combined and diluted to a volume of 1 liter with distilled water.

Administration of Radioisotopes. METHOD A. The plants were removed from the nutrient solution. The roots were dried by initially blotting them with absorbent paper, followed by air drying for an additional hour. Each plant was placed in a dry Erlenmeyer flask which had been wrapped in heavy black paper to prevent exposure of the roots to light during the incubation period. At this point sufficient radioisotope solution (20 μc/ml) was added to each plant, so that each one received 5 μc of activity distributed over the roots. A second such quantity of nutrient solution was added to each plant 1 hour after the initial dispensing of the radioisotope.

METHOD B. In this experimental procedure it was not necessary to remove the plants from the nutrient solution. A white cotton thread (Coats and Clark's) was inserted, with the aid of a sewing needle, through the stem of each plant approximately 3 inches above the roots. The two ends of the thread were then dipped into the radioisotopic solution making the thread function as a wick to transfer the active solution into the plant. A 5-ml beaker containing 0.25 ml (5 μc) of radioactive solution was attached at the junction of the thread and stem, and the active solution was taken up by the plant in 2 hours. At this point 0.5 ml of distilled water was added to the beaker to "wash" as completely as possible all radioisotope into the plant. The radioactivity judged to be taken up by the plant was determined by subtracting the radioactivity remaining in the thread from the amount originally fed to the plant. During the incubation period the plants were placed on the laboratory bench top under exposure to a 100-w electric light bulb.

Harvest of the Roots. The plants were removed from the nutrient solution at the end of appropriate periods. The roots were separated from the remainder of the plant, washed with 95% ethanol, and weighed after first being blotted dry with absorbent paper. The roots were then disintegrated in 100 ml of ethanol for a 5-minute period with a Waring Blendor. A sample of "cold" terthienyl was added, where required, to the ethanol mixture immediately prior to the blending operation. The blended mixture was filtered through a Soxhlet thimble, and the residue was extracted with 100 ml of ethanol for 24 hours in the Soxhlet apparatus. The combined filtrates were evaporated to dryness under reduced pressure, and the residue was redissolved in petroleum ether (bp 30–60°). The petroleum ether solution of crude terthienyl was chromatographed over activated alumina, and the specific activity was then determined. Further purification via rechromatographing or conversion of the terthienyl to its 5-acetyl derivative was carried out until a constant specific activity of terthienyl was attained.

Assay of Radioactivity. Aliquots of 0.1–1.0 ml of the desired solutions were evaporated to dryness in aluminum planchets. All counting was conducted in a windowless, gas-flow proportional counter (Baird Associates-Atomic Instrument Co., Cambridge, Mass.). No correction for self-absorption was necessary with this instrument.

Determination of Concentrations. Quantitative measurements of terthienyl were made from its absorption peak at 350 mμ (ϵ = 24,100) with a Beckman DU spectrophotometer. Absorptions of the acetyl derivative, 5-acetyl-2,2′;5′,2″-terthienyl, at 391 mμ (ϵ = 30,500) were determined. The purities of the compounds were checked by both paper and thin-layer chromatography.

Chromatographic Methods. A. COLUMN. Terthienyl was separated from the petroleum ether solution of the root extract by chromatography of the crude sample over 7.5 g of alumina (Alcoa, F-20) in a 25 × 1.5-cm glass column fitted with a stopcock. The sample size used was about 15 ml and fractions of 10 ml were collected. Terthienyl was eluted with petroleum ether (bp 30–60°) after 40–60 ml of eluate had been collected. A fraction containing compound II was always collected prior to terthienyl. Both compounds displayed a positive reaction to a 0.1% isatin solution in concentrated sulfuric acid. Terthienyl changed color from violet to purple and compound II showed a wine-red color. The 5-acetyl-2,2′; 5′,2″-terthienyl was purified in the manner described except the solvent used was carbon tetrachloride, and the eluent was 10% ether in carbon tetrachloride.

B. PAPER. The ascending method was employed using Whatman No. 1 paper. The solvent used was methanol–water (66:34, v/v). The compounds were located on the paper from their fluorescence in ultraviolet light. The R_F values determined were: terthienyl, 0.71; compound II, 0.80; 5-acetylterthienyl, 0.46; solvent front, 13.5 cm. Quantities of these compounds as small as 0.1 μg could be detected.

C. THIN LAYER. Alumina (Alcoa, F-20) and plaster of paris (commercial) were thoroughly mixed dry in a weight ratio of 7:3. A thick slurry was produced from 10 g of dry mix and 8 ml of water. For increased hardness of the surface, the water was replaced by an equal volume of 2% sodium hydroxide solution. Eight chromatostrips (microscope slides) were obtained from 10 g of dry mix. The strips were dried 2–5 hours at 75° and stored over potassium hydroxide pellets in a vacuum desiccator until used. Samples of 0.01–1.0 ml were applied as a spot or line at the origin; precautions were taken to minimize spreading of the spot. The development solvent was *n*-hexane containing 1–10% of a polar solvent, usually ether. The strip was placed in a 3 × 12-cm test tube containing 1–2 ml of solvent. Development time was less than 5 minutes. The strip was removed from the solvent and air-dried, and the spots were located by observing their fluorescence in ultraviolet light. Terthienyl and compound II were separated by this method, as well as terthienyl and 5-acetylterthienyl. Compound II traveled faster than terthienyl on the chromatostrips, paper chromatograms, and through alumina columns, while terthienyl always traveled faster than its 5-acetyl derivative under the same chromatographic conditions.

TABLE I: Purification of 2,2′;5′,2″-[^{35}S]Terthienyl from Marigold Roots.

	Total (cpm × 10^4)	Specific Activity (cpm/μmole)	Total (cpm × 10^4)	Specific Activity (cpm/μmole)
Ethanol extract	37.2	1350	65.4	2740
Petroleum ether solution	6.56	289	17.1	674
First chromatogram	1.27	47	1.84	97
Second chromatogram			1.40	90
5-Acetyl derivative	0.109	47		

TABLE II: Incorporation of Sulfur-35 into 2,2′;5′,2″-Terthienyl in Marigold Roots.

Expt	Days of Incubation	Fed via	Specific Activity, Fed × 10^9	cpm/mmole Isolated × 10^7	Dilution Factor[a]
		Sodium [^{35}S]sulfate			
1[b]	2	Stem	1.49	[c]	
2	2	Root	5.42	1.84	294
3	15	Root	4.83	1.86	260
4[d]	5	Root	4.46	2.77	161
5	10	Root	4.46	5.80	77
		Sodium hydrogen [^{35}S]sulfide			
6[e]	1.2	Root	1.51	[c]	
7	1.7	Root	3.26	[c]	
		L-[^{35}S]Methionine			
8[f]	2	Stem	37.6	[c]	
9	2	Stem	37.6	[c]	
10	6	Stem	37.6	[c]	
11	12	Stem	37.6	[c]	
12	17	Stem	37.6	0.063	59,600
		DL-[^{35}S]Methionine			
13[g]	2	Root	4.38	0.596	734

[a] Dilution factors were calculated by dividing the specific activity of the administered radioisotope by the specific activity of the isolated terthienyl. [b] *Tagetes*, aged 21 weeks, expts 1–3. [c] No radioactivity was detected in the isolated terthienyl. [d] Dwarf, unknown genus, aged 7 weeks, expts 4–5. [e] *Tagetes*, aged 7 weeks, expts 6–7. [f] *Tagetes*, aged 21 weeks, expt 8; aged 6 weeks, expts 9–12. [g] *Tagetes*, aged 7 weeks.

Preparation of 5-Acetyl-2,2′;5′,2″-terthienyl. A typical procedure consisted of heating to its reflux temperature a solution of 8 mg of terthienyl dissolved in 20 ml of dry benzene, and adding to it two drops each of stannic chloride and acetyl chloride, followed by heating the reaction mixture at its reflux temperature for 15 hours. The reaction mixture was then poured into 50 ml of 1 N HCl and an equal volume of crushed ice, neutralized with sodium bicarbonate, and extracted with ether. The average yield was 40% of the 5-acetyl derivative and 25% of the 5,5″-diacetyl derivative; the remainder of the material balance was unreacted terthienyl. The mono- and diacetyl derivatives were separated by alumina chromatography and checked for purity by both paper and thin-layer chromatography. 5-Acetyl-2,2′;5′,2″-terthienyl, mp 168–169°; 391 mμ (ε = 30,500) max in ethanol.

Typical Purification of Terthienyl. Two typical isolations of terthienyl are shown in Table I comparing purifications by rechromatography and preparation of its 5-acetylterthienyl.

Results

The results of sulfur-35 incorporation into terthienyl are summarized in Table II. The per cent of radioisotope incorporated ranged from 0.04 to 0.08 from sodium [^{35}S]sulfate and was 0.03 from DL-[^{35}S]methionine fed via roots and 0.003 fed via stems.

The results of the carbon-14 experiments are sum-

TABLE III: Incorporation of Carbon-14 into 2,2′;5′,2″-Terthienyl in Marigold Roots.

Expt	Days of Incubation	Fed via	Specific Activity, Fed × 10^9	cpm/mmole Isolated × 10^7	Dilution Factor
		DL-[2-^{14}C]Glutamic acid			
14[a]	1.8	Root	0.33	[b]	
		[2,3-^{14}C]Succinic acid			
15	2	Root	2.96	[b]	
		DL-[1-^{14}C]Methionine			
16[c]	10	Stem	3.02	[b]	
17	15	Stem	3.02	[b]	
18	19	Stem	3.02	[b]	
		DL-[2-^{14}C]Methionine			
19	2	Root	1.13	0.79	1,430
20	10	Root	1.13	[b]	
21	20	Root	1.13	[b]	
		Sodium [1-^{14}C]acetate			
22	2	Root	6.96	[b]	
23	10	Root	6.96	0.0198	35,100
24	20	Root	6.96	0.0680	10,200
25	2	Stem	6.96	[b]	
26	9	Stem	6.96	[b]	
27	17	Stem	6.96	[b]	

[a] *Tagetes*, aged 6 weeks, expts 14–15. [b] No radioactivity was detected in the isolated terthienyl. [c] *Tagetes*, aged 8 weeks, expts 16–27.

TABLE IV: Summary of Sulfur-35 Taken Up by the Roots of Marigold Plants.

Expt	Radioisotopic Form	Total cpm Dispensed	Per Cent cpm Nutrient Solution	Remaining in EtOH Extracted
2	Sodium sulfate	1.99×10^7	Unknown[a]	1.9[b]
6	Sodium bisulfide	3.33×10^7	73	0.90
7	Sodium bisulfide	5.96×10^7	50	9.0
13	DL-Methionine	3.80×10^7	13	30

[a] In expt 5, 15 days' incubation, the activity remaining in the nutrient solution was 16% of the amount dispensed. [b] In all sodium [^{35}S]sulfate experiments the activity in the ethanol extract was 1–1.9% of the amount dispensed.

marized in Table III. The per cent of carbon-14 incorporated from DL-[2-^{14}C]methionine was 0.01, and 0.002–0.004 from sodium [1-^{14}C]acetate after 10 and 20 days, respectively.

Discussion

It was assumed that all substrates were transported into the plant equally well through the roots. This was important when the negative results of the sodium hydrogen [^{35}S]sulfide experiments were considered.[2] In Table IV a summary of the activity remaining in the nutrient solution and extracted from the roots is shown. It was concluded from the data given in Table IV that sulfur-35 was taken up in the sulfide form, since 9% of the activity dispensed was extracted from the roots as compared to 1.9% extracted when sulfate was fed. The sulfate experiment in Table IV showed incorporation into terthienyl as presented in Table II. It was also

[2] One referee pointed out that if the sulfide was administered in near neutral or slightly acid media, loss of sulfide as gaseous H_2S would probably be considerable. The sulfide was administered in distilled water and remained in contact with the roots for 1 hour prior to immersion in nutrient solution. It was hoped that most of the sulfide would be taken up during this 1-hour period. As shown in Table IV, the sulfide was taken up the least amount of the three compounds tried.

assumed that the substrate entered the root in the same form as it was administered; however, no experimental evidence was obtained to support this assumption.

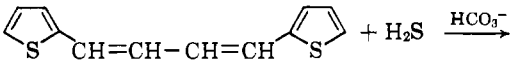
It was believed that the "site of synthesis" was located in the roots, since no incorporation was observed from [^{35}S]sulfate and L-[^{35}S]methionine when fed via stems (Table II,).[3] The dilution factor of 59,600 from [^{35}S]-methionine after 17 days of incubation was not considered to be of significant value. Further evidence was obtained from the [^{14}C]acetate experiments, although the incorporation was not considered to be of significance because of the high dilutions (Table III).

The results of [2-^{14}C]methionine require some comment, since incorporation occurred only during incubation periods of 2 days.[4] The first thought was that radioactive terthienyl was further metabolized, thus suggesting that terthienyl was not a "storage product."[5] Unfortunately, further experimental evidence was lacking, and one could only speculate.[6]

In addition, sulfur-35 incorporation during 2–15 days of incubation was possible, since sulfur-35 was always present in the nutrient solution (footnote *a*, Table IV). It has also been established previously that excess sulfate not required for carbon compounds remains unchanged in the plant (Wiame, 1958). Therefore, a continual supply of sulfur-35 was possible, and incorporation from incubations longer than 2 days could be expected. Again, this argument remains somewhat speculative.

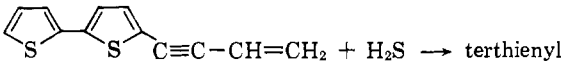
The discovery and identification of a carbon-sulfur compound as a precursor to terthienyl remains to be shown. Future work in locating the position of carbon-14 incorporated from [2-^{14}C]methionine is needed by degradation of terthienyl.

Terthienyl has been prepared in the laboratory under weakly alkaline conditions at 20–60° (Horner, 1962):



terthienyl

A similar reaction is possible in the roots of the marigold (Uhlenbroek and Bijloo, 1959):[7]



The incorporation of sulfur-35 from sulfate and methionine and the lack of incorporation from the sulfide suggest the possibility that H_2S is not involved in the biosynthesis of terthienyl. It would be of interest to investigate the incorporation of cysteine-sulfur into terthienyl. The identification of the pathway of sulfur to terthienyl is, as yet, not definitely established.

Acknowledgment

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[3] During experiments 2 and 3, the stems and leaves were extracted with ethanol. All attempts to locate terthienyl in these extracts failed to detect at least 0.1 μg as shown by both paper and thin-layer chromatography.

[4] This crucial 2-day experiment has been repeated twice in our laboratory by Sister Mary de Paul (unpublished work) with dilution factors of the same magnitude being obtained in both experiments.

[5] In experiment 20, 10 days' incubation, the activity remaining in the nutrient solution was 0.2% of that dispensed. The nature of the activity was not investigated.

[6] The presence of terthienyl obtained from the roots was conclusively shown in each experiment by paper chromatography even though it may not have been radioactive.

[7] Phenylheptatriyne, Ph—(C≡C)$_3$—Me, was isolated from a *Compositae, Coreopsis* (Sorensen, 1961). 2-Phenyl-5-(α-propynyl)thiophene was also isolated from a species of *Coreopsis* (Sorensen and Sorensen, 1958).