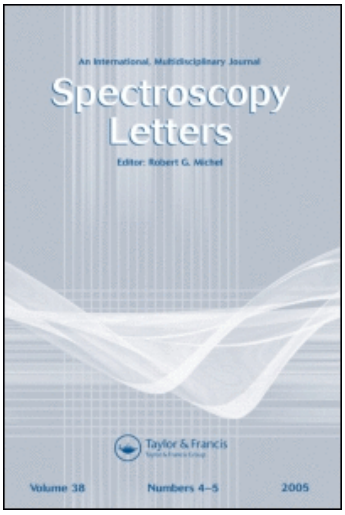

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Biosynthetic Studies of Bithiophenes in Hairy Root Cultures of Tagetes Patula Using ^{13}C-labeled Acetates

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BIOSYNTHETIC STUDIES OF BITHIOPHENES IN HAIRY ROOT CULTURES OF TAGETES PATULA USING ^{13}C-LABELED ACETATES

Key Words: *Tagetes patula*, root cultures, bithiophenes, biosynthesis

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IN MEMORY OF ANNE F. MORRISON

ABSTRACT - Biosynthetic studies of two bithiophenes, [5-(3-buten-1-ynyl)-2,2'-bithiophene (BBT) and 5-(4-acetoxy-1-butynyl)-2,2'-bithiophene (BBTOAc)], were performed in hairy root cultures of *Tagetes patula* using [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-labeled sodium acetate as precursors. The data support previous biogenetic proposals and demonstrate that hairy root cultures provide a highly suitable medium for biosynthetic studies of root constituents.

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INTRODUCTION

A wide range of structural types of acetate-derived compounds (acetogenins) with multiple triple bonds (polyacetylenes) are found as natural products and they are very common constituents in many members of the large sunflower family (Asteraceae) [1]. Recently, these relatively unstable, often highly conjugated compounds have attracted considerable attention due to their broad spectrum of biological activities [2]. Previous biosynthetic studies of polyacetylenes and the derived thiophenes were performed with radioactive ^{14}C- and tritium-labeled precursors [3,4]. Based on these results and previous biogenetic proposals it is now generally accepted that polyacetylenes are formed by linear combination of acetate units [4]. The thiophenes are presumably derived from pentaynene by addition of sulfide to the triple bonds, a process that can be mimicked *in vitro* by the addition of H_2S to diynes [1,5] and was also supported by biosynthetic incorporations of ^{35}S-labeled sulfate into thiophenes [6].

The introduction of pulsed Fourier transform NMR techniques in the early 1970s permitted the use of ^{13}C NMR labeling experiments in biosynthetic studies [7]. The advantage of the use of ^{13}C-labeled precursors is the gain of a wealth of biosynthetic information without requiring specific degradation experiments which is necessary when ^{14}C- and tritium-labeled precursors are used. Its disadvantage is the low sensitivity, necessitating relatively high incorporations of the ^{13}C-labeled precursors. Generally, biosynthetic ^{13}C-experiments with live plants are limited due to the low yield of incorporation of precursors, thus excluding the insensitive ^{13}C methodology for these studies [8]. Since to the best of our knowledge no detailed biosynthetic studies of the carbon framework of bithiophenes had been previously performed and the use of hairy

root cultures in biosynthetic studies has not been exploited, we attempted the incorporation of [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-labeled acetates into BBT (**1**) and BBTOAc (**2**) using hairy root cultures of *Tagetes patula*. It was of particular interest to learn whether biosynthetic incorporations of ^{13}C-labeled percursors into constituents of hairy root cultures are effective.

In this paper we report the results of our biosynthetic experiments with [1-^{13}C]-, [2-^{13}C]- and [1,2-$^{13}C_2$]-acetates and their incorporations into BBT and BBTOAc in hairy root cultures of *T. patula*. The data clearly support previous biogenetic proposals [9] and demonstrate that hairy root cultures can be highly suitable media for biosynthetic studies of root constituents.

RESULTS AND DISCUSSION

Hairy root clones of *Tagetes patula* were grown in bioreactors and the labeled acetates were added aseptically to the reactors at the end of the growth period, that is, one week before harvesting the root culture. Complete assignments of the carbon signals were achieved by the use of DEPT 135° and DEPT 90° experiments as well as carbon-hydrogen correlations [10], INAPT experiments [11,12] and by spectral comparison with published results for BBT [9]. DEPT 135, DEPT 90 (Fig. 1) and ^{13}C-^{1}H correlation experiments (Fig. 3) were used for the assignment of the methylene, methyl and methine carbons. Unambiguous assignments of the proton signals were achieved by 2D ^{1}H-^{1}H correlation experiment (COSY-45) shown in Figure 2.

In Figure 4 shows the ^{1}H, ^{13}C and the INAPT spectra of compound **1** are given. Polarization transfer of H-1b enhanced the acetylenic carbon C-3, while polarization of H-2 was transferred to the other acetylenic carbon C-4. The INAPT spectrum obtained from polarization transfer of H-6 affected carbons 8

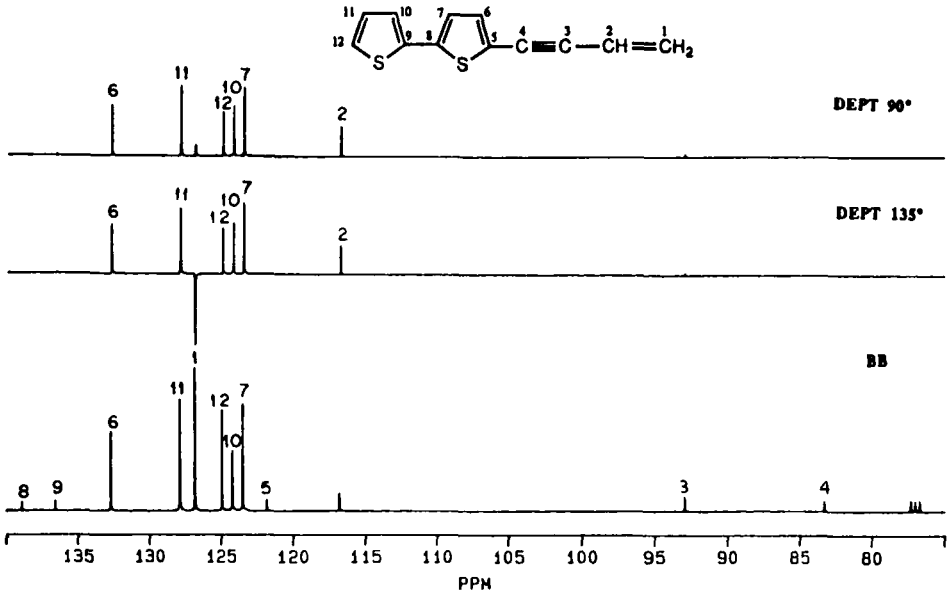

Figure 1. Broad Band ^{13}C NMR Spectrum and Dept 135 and Dept 90 Subspectra ($CDCl_3$) of Bithiophene **1**.

and 4. Since the proton H-7 absorbs very close to H-11 it could not be selectively polarized. The INAPT spectrum of H-7 and H-11 enhanced the signals for carbons 9,5,12,11 and 6 of which the last three were caused by a 2-bond carbon-hydrogen polarization transfer.

The ^{13}C NMR spectra of the unlabeled and the singly labeled experiments were recorded on the same scale to allow for easy comparison of the relative intensities of the peaks thus deriving the magnitude of ^{13}C-incorporations. The ^{13}C NMR spectra of unlabeled acetate, acetate-[1-^{13}C]- and acetate-[2-^{13}C]-enriched bithiophenes **1** and **2** are shown in Figures 5 and 7. [1-^{13}C]-Acetate enriched bithiophenes **1** and **2** exhibit enhanced signals for carbons 2,4,6,8,10 and 12 while acetate-[2-^{13}C] enriched BBT and BBTOAc

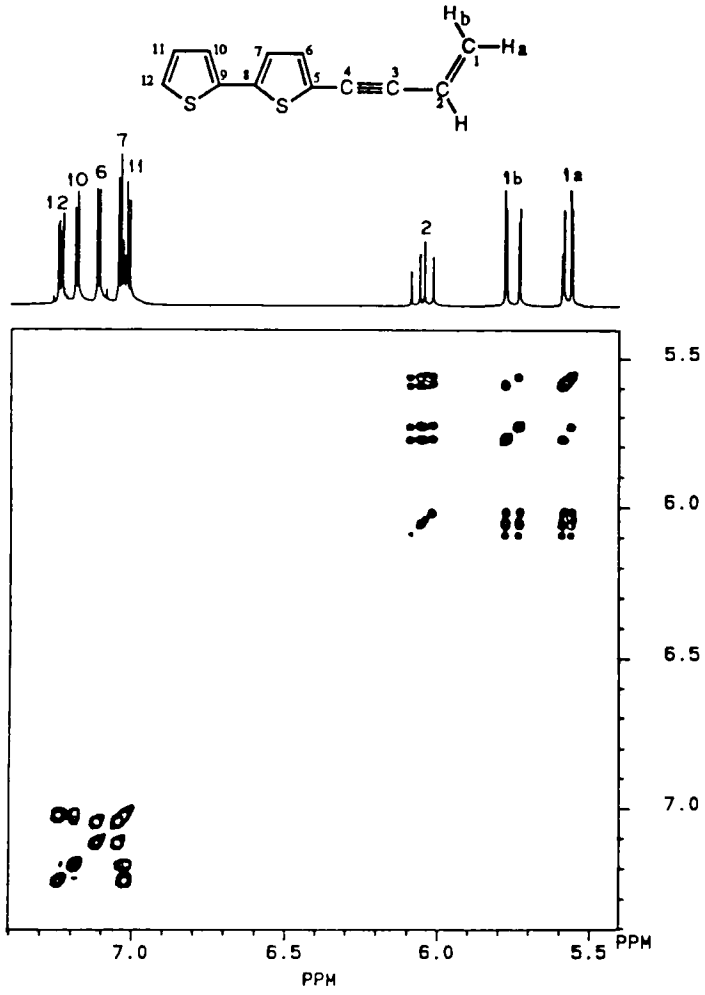

Figure 2. The 2D ^{1}H - ^{1}H Shift Correlation of Bithiophene **1** by COSY - 45 at 400 MHz in $CDCl_3$.

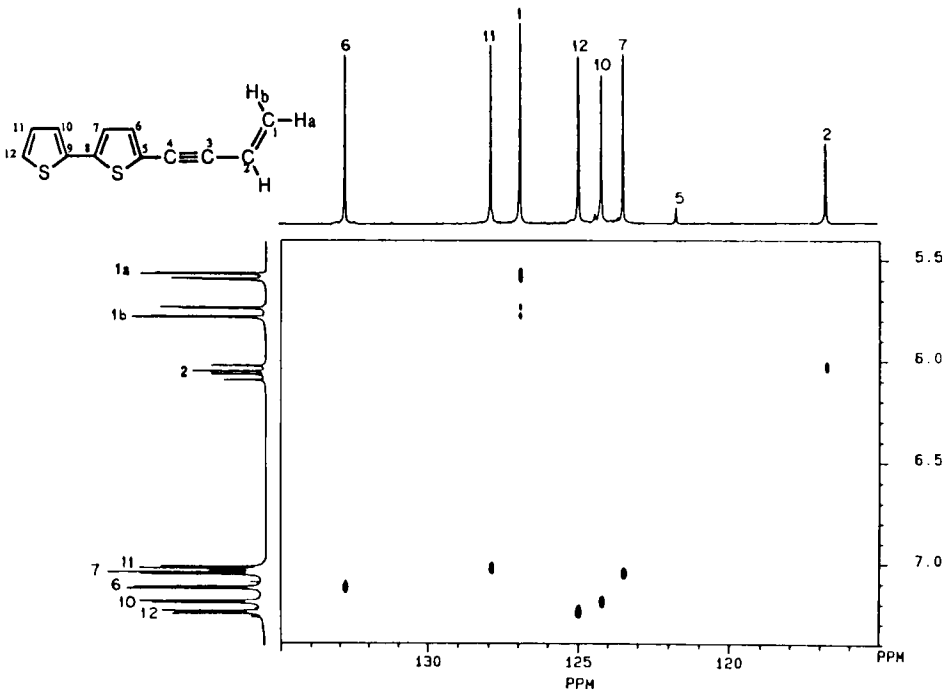

Figure 3. The 2D ^{13}C - ^{1}H Correlation Spectrum of Bithiophene 1.

show enhancements of carbon signals 1,3,5,7,9 and 11. In acetate-[1-^{13}C]-enriched bithiophene **2** the carbonyl carbon is enhanced, while acetate-[2-^{13}C] enriched bithiophene **2** shows the acetate methyl signal as an enhanced singlet. These results are in agreement with the biogenetic proposal which suggests that the twelve-carbon chain polyacetylenes and their thiophene derivatives **1** and **2** must be derived from a fourteen carbon chain precursor followed by loss of a carbon moiety at both ends of the carbon chain [1]. The most probable biosynthetic intermediate for bithiophenes **1** and **2** is the common tridecapentaynene which represents a degradation product of eighteen-carbon

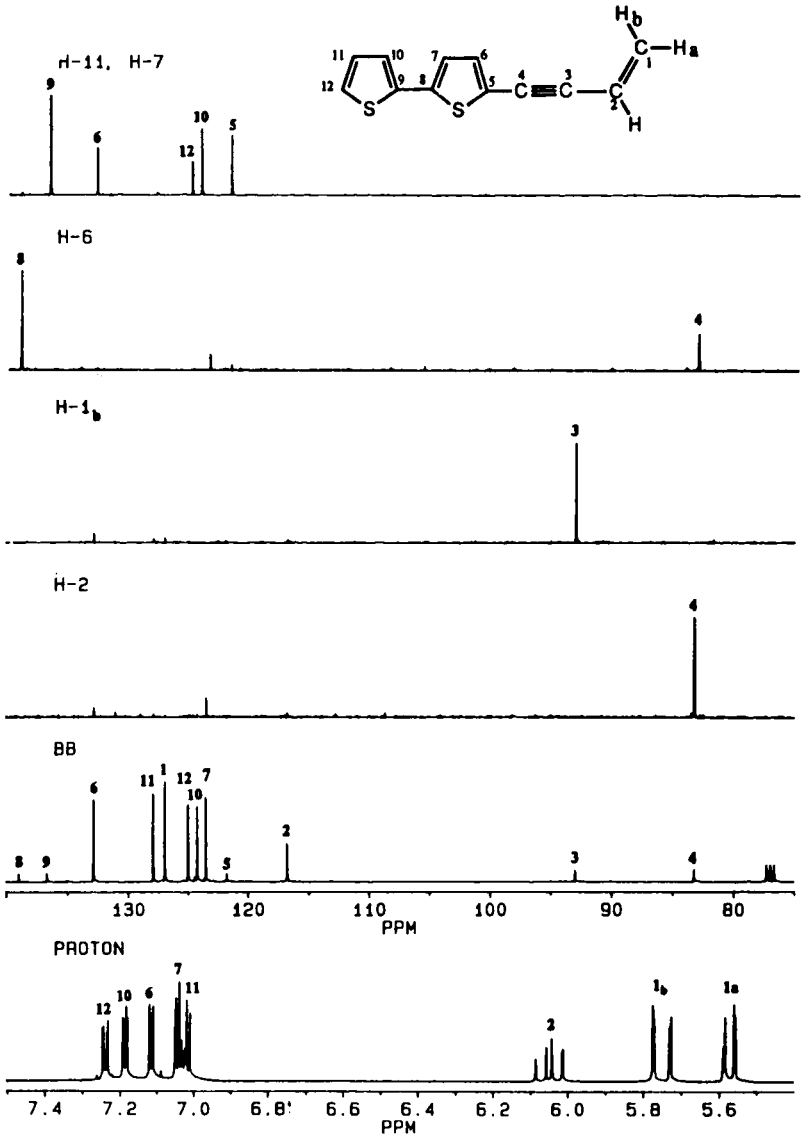

Figure 4. INAPT Spectra of Bithiophene 1.

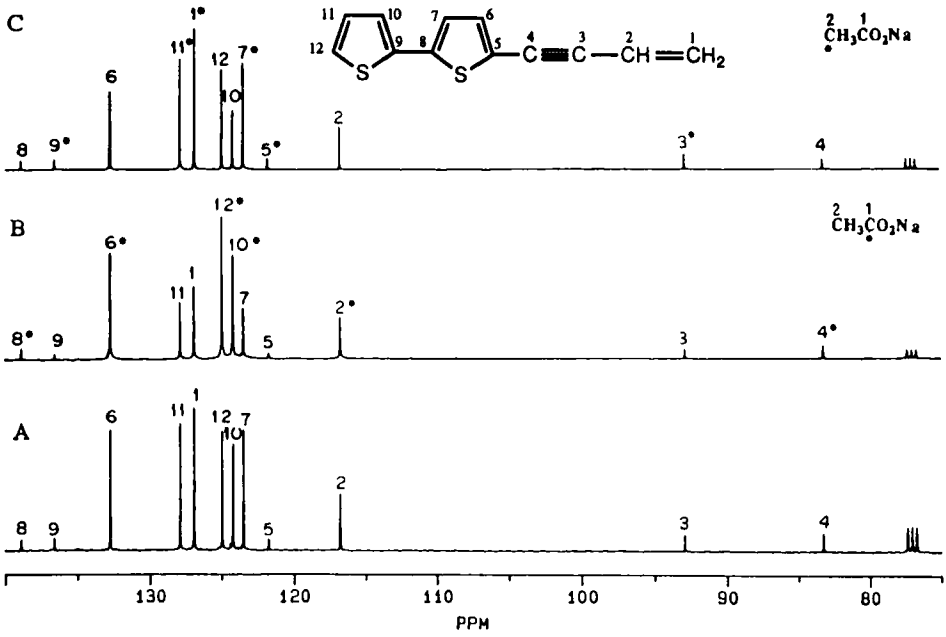

Figure 5. Broad Band ^{13}C NMR Spectra ($CDCl_3$) of Bithiophene **1**. (A): Natural Abundance, (B): Acetate-[1-^{13}C]-Enriched and (C): Acetate-[2-^{13}C]-Enriched Bithiophene **1**.

fatty acids [1]. Earlier feeding experiments had shown that ^{14}C-labeled oleic acid was transformed to shorter chain acetylenes [4].

In both compounds a higher enrichment was observed for acetate-[1-^{13}C] incorporations. Also, within a set of data, some carbons showed higher enrichments indicating higher incorporation of acetate into these positions. In bithiophene **2** the acetate carbonyl carbon signal was enhanced approximately five times when compared to the other carbons in the spectrum of the acetate-[1-^{13}C] enriched compound, and the acetate methyl enrichment was about nine times higher in the acetate-[2-^{13}C]-enriched spectrum. The ^{13}C-enrichments were calculated using a method reported previously [13].

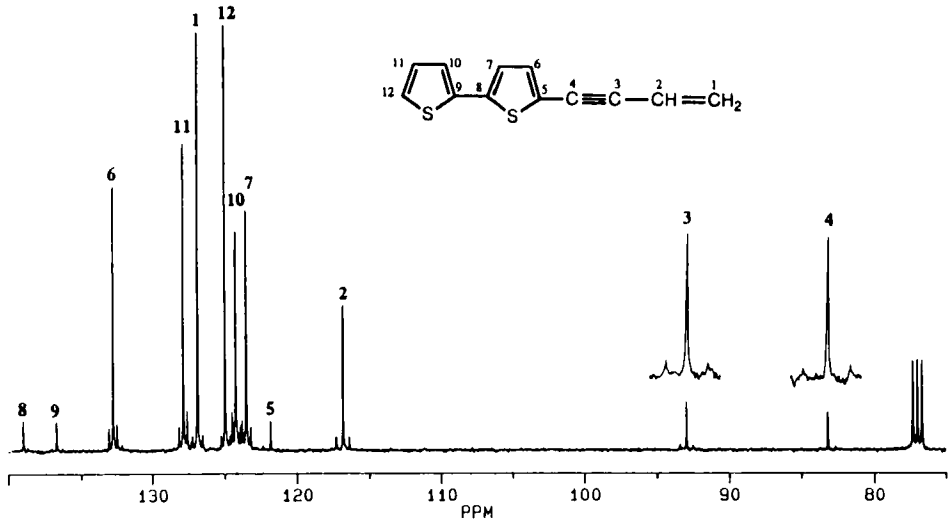

Figure 6. Broad Band ^{13}C NMR Spectrum ($CDCl_3$) of Acetate-[1,2-$^{13}C_2$]-Enriched Bithiophene 1.

In the experiments with doubly labeled acetate the [1,2-$^{13}C_2$]-acetate was > 97% enriched with ^{13}C-isotopes. The ^{13}C NMR spectrum of acetate-[1,2-$^{13}C_2$] enriched bithiophene **1** is shown in Figure 6. The carbons derived from acetate units which are incorporated intact into the molecule appear as triplets, the center peak being due to the natural abundance signal and the two satellite signals are due to the ^{13}C-^{13}C coupling from incorporations of intact acetate units [8]. Couplings of 88.5 (C-2/C-3), 106.8 (C-4/C-5), 58.0 (C-6/C-7), 71.7 (C-8/C-9) and 58.0 Hz (C-10/C-11) were observed, which indicated that these carbon pairs were derived from intact acetate units. C-1 and C-12 appeared as enhanced signals when compared to those of the natural abundance ^{13}C NMR spectrum. They also showed small satellite signals with couplings of 71.7 Hz for C-1 and 62.6 Hz for C-12. This can only be accounted for by substantial ^{13}C-^{13}C

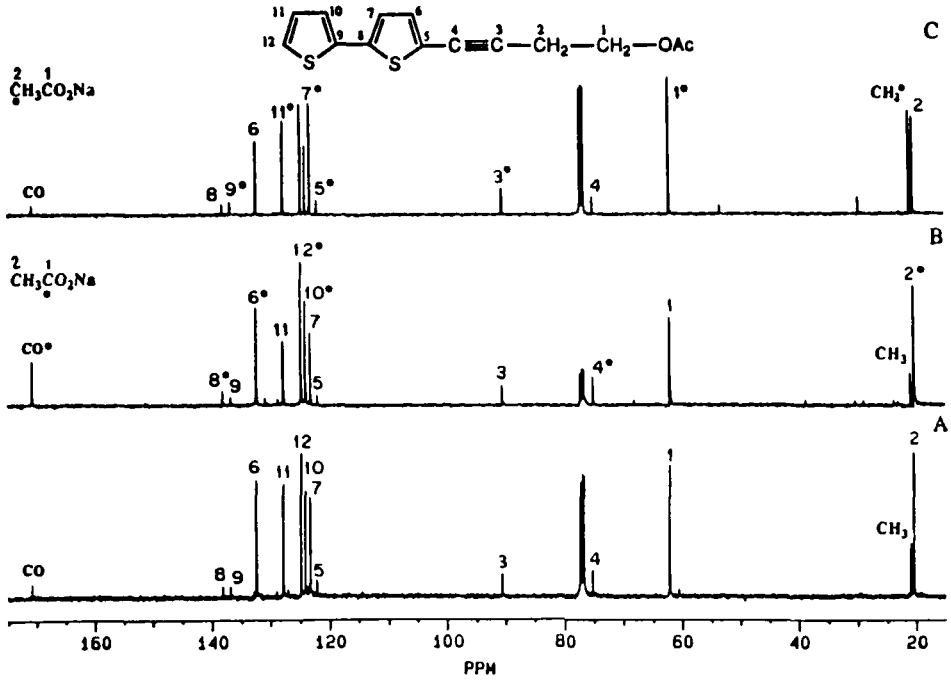

Figure 7. Broad Band ^{13}C NMR Spectra ($CDCl_3$) of Bithiophene 2. (A): Natural Abundance, (B): Acetate-[1-^{13}C]-Enriched and (C): Acetate-[2-^{13}C]-Enriched Bithiophene 2 .

coupling between adjacent carbons due to higher incorporation of ^{13}C-enriched doubly labeled acetate in these positions [14].

The ^{13}C NMR spectrum of acetate-[1,2-$^{13}C_2$] enriched bithiophene **2** showed triplets for carbon signals 2,6,7,10,11, as well as the acetate carbonyl and its methyl absorptions. Carbons 3,4,5,8, and 9 represent quaternary carbons and their ^{13}C-absorptions are of such low intensity that no satellites were detected above the spectral noise level [14]. Carbons 1 and 12 appeared as enhanced singlets, which must be derived from two acetate units that have not been incorporated intact into the molecule (Fig. 8 and Table 2). The results

Table 1. ^{13}C-chemical shifts (100.6 MHz, $CDCl_3$, TMS as internal standard), enrichments in acetate-[1-^{13}C] and acetate-[2-^{13}C] labeled bithiophene **1** and ^{13}C-^{13}C coupling constants observed in acetate-[1,2-$^{13}C_2$]-labeled bithiophene **1**.

Carbon	d(ppm)	Enrichment		J(^{13}C-^{13}C) (Hz)
		acetate-[1-13C]	acetate [2-^{13}C]	
1	71.75		0.3	71.7
2	116.78	0.6		88.5
3	92.95			88.5
4	83.23	0.8		106.8
5	121.76		0.5	106.8
6	132.75	1.1		58.0
7	123.45		0.3	58.0
8	138.95	1.1		71.7
9	136.63		0.4	71.7
10	124.19	1.9		58.0
11	127.85		0.5	58.0
12	124.94	1.3		62.6

obtained from the acetate-[1,2-$^{13}C_2$]-enriched bithiophenes **1** and **2** corroborated the results from the singly labeled experiments and support the biogenetic proposal on the formation of twelve-carbon thiophenes from their 14-carbon polyacetylene precursors [1]. The ^{13}C-^{13}C coupling constants, especially those of bithiophene **1**, showed that carbons 2 and 3, 4 and 5, 6 and 7, 8 and 9, and 10 and 11 were incorporated intact into the molecule while carbons 1 and 12 must be derived from two acetate units each of which lost their adjacent terminal carbon.

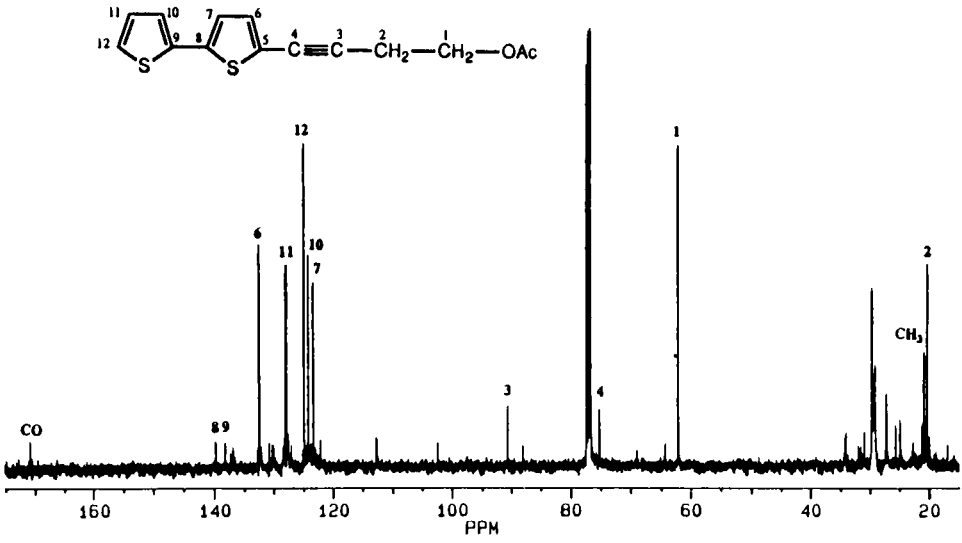

Figure 8. Broad Band ^{13}C NMR Spectrum ($CDCl_3$) of Acetate-[1,2-$^{13}C_2$]-Enriched Bithiophene 2.

This indicates that the twelve-carbon bithiophenes **1** and **2** are most likely formed from fourteen-carbon precursors followed by the loss of the two terminal carbons. Alternatively, direct carbon cleavages of longer chain precursors could occur.

Comparison of the mass spectral data of unlabeled and ^{13}C-labeled bithiophenes very clearly supported the incorporation of ^{13}C-labeled acetates by the appearance of peaks of up to [M+7] in acetate-[1,2-$^{13}C_2$]-enriched bithiophene **1**. The results are summarized in Table 3.

The data presented above clearly demonstrate that hairy root cultures represent a suitable medium for biosynthetic studies involving ^{13}C-labeled precursors. Hairy root cultures grow relatively fast and produce secondary metabolites in quantities sufficiently large to be used for ^{13}C NMR labelling experiments. In the present case, this permitted non-destructive biosynthesic

Table 2. ^{13}C-chemical shifts (100.6 MHz, $CDCl_3$, TMS as internal standard), enrichments in acetate-[1-^{13}C] and acetate-[2-^{13}C]-labeled bithiophene **2** and ^{13}C-^{13}C coupling constants observed in acetate-[1,2-$^{13}C_2$]-labeled bithiophene **2**.

Carbon	d(ppm)	Enrichment		$J(^{13}C-^{13}C)$ (Hz)
		acetate-[1-^{13}C]	acetate-[2-^{13}C]	
1	62.09		0.2	
2	20.27	0.8		68.7
3	90.58		0.4	
4	75.17	1.4		
5	122.09		0.03	
6	132.39	0.8		59.5
7	123.27		0.2	59.5
8	138.10	1.1		
9	136.77		0.2	
10	124.11	0.9		56.5
11	127.87		0.1	51.9
12	124.85	0.6		
CO(Ac)	170.82	4.7		59.5
CH_3(Ac)	20.87		1.7	59.5

studies of bithiophenes. In future experiments, the general scope of the application of hairy root clones as a medium for biosynthetic experiments will be further tested. This will include root cultures of various plant species and biosynthetic studies of structurally and biosynthetically different types of secondary metabolites.

Table 3. Ion intensities* in the molecular region of the mass spectra of natural abundance and acetate-[1-^{13}C], acetate-[2-^{13}C] and acetate-[1,2-$^{13}C_2$]-enriched bithiophenes **1** and **2**.

Compound	Sample	M^+	M+1	M+2	M+3	M+4	M+5	M+6	M+7
Bithiophene **1**									
	Natural abundance	100	14.6	6.8	-	-	-	-	-
	Acetate-[1-^{13}C] enriched	100	20.7	12.3	2.7	0.3	-	-	-
	Acetate-[2-^{13}C] enriched	100	36.6	12.1	2.1	1.5	0.02	-	-
	Acetate-[1,2-^{13}C] enriched	100	17.0	8.4	1.5	0.7	0.04	0.04	0.01
Bithiophene **2**									
	Natural abundance	100	25.4	-	-	-	-	-	-
	Acetate-[1-^{13}C] enriched	100	25.8	9.2	4.2	1.1	0.07	-	-
	Acetate-[2-^{13}C] enriched	100	20.3	10.9	1.1	-	-	-	-
	Acetate-[1,2-^{13}C] enriched	100	19.5	13.9	2.5	0.03	-	-	-

*Molecular ion $[M]^+$ was arbitrarily set to 100.

EXPERIMENTAL

^1H and ^{13}C NMR Data.

The NMR spectra were acquired at 25° C in $CDCl_3$ on a Bruker AM 400 spectrometer using a 5 mm dual tuned $^1H/^{13}C$ probe with observation frequencies of 400.13 and 100.62 MHz, respectively.

INAPT Experiments of BBT (**1**).

The INAPT experiments were performed using the following pulse sequence [11,12]:

^1H: D1 - 90° - D2 - 180° - D2 - 90° - D3 - 180° - D3 - BB
^{13}C: - 180° - 90° - FID

Proton relaxation delay (D1) was set to 1.38 sec. Length of delay D2 and D3 were optimized for three-bond coupling constants of the various carbon hydrogen bonds. The delay D2 was set to 37.5 msec (J = 5 Hz) (H-2), 18.75 msec (J = 8 Hz) (H-1b), 12.5 msec (J = 10 Hz) (H-11, H-7) and 2 msec (H-6), while D3 was set to 13.75 msec (H-8), 6.25 msec (H-1b), 3.75 msec (H-11, H-7), and 3.5 msec (H-6). The ^1H pulse was set at 12.5 msec for H-2, H-1b, H-7 and H-11 and 27 msec for H-6.

The INAPT spectra were processed with line broadening LB = 2 and with magnitude calculation after Fourier transformation giving positive signals.

Hairy Root Cultures.

Transformed roots, popularly called hairy roots, were used in these experiments due to their rapid growth rate. These roots were obtained by

infection of the plant with the bacterium *Agrobacterium rhizogenes* and they generally synthesize the metabolites characteristic of the normal root [15].

The hairy root clone of *Tagetes patula* TS was a gift from Dr. Hector Flores, Pennsylvania State University. Roots for analysis were grown in bioreactors in 9 liters of MS medium without phytohormones [16]. All reactors were inoculated with 50 tips, approximately 1 cm long. Sodium acetate was added aseptically to the reactors on day 37 and fermentation continued for one week before the roots were harvested. Several acetate concentrations were tried to determine the optimal amount. For the results reported in this paper, 0.3735g and 0.2950g were added of the singly labeled acetate and the doubly labeled acetate, respectively. The roots used for control were grown for 40 days before harvest.

*Extraction and Isolation of Unlabeled Bithiophenes **1** and **2**.*

Root cultures of *Tagetes patula* (48.4g of dry wt) were soaked in 800 ml of CH_2Cl_2 for 24 hrs. After suction filtration and evaporation of the solvent in vacuo, the crude extract (1.17g) was subjected to Vacuum Liquid Chromatography (VLC) [17] using hexane, CH_2Cl_2 and ethyl acetate (EtOAc) mixtures of increasing polarity, yielding twelve 50 ml fractions. The first fraction (90 mg) was further chromatographed by preparative thin layer chromatography (prep. TLC) using hexane as solvent yielding 46 mg of pure bithiophene **1** and 20 mg of β-farnesene. Fraction 6 (30 mg), when chromatographed by prep. TLC using 10:1 hexane:EtOAc (2X), yielded 15 mg of bithiophene **2**.

*Extraction and Isolation of Labeled Bithiophenes **1** and **2**.*

*[1-^{13}C]-acetate labeled bithiophenes **1** and **2**.*

112.8g of root cultures fed with [1-^{13}C]-acetate were soaked in 800 ml of CH_2Cl_2 (2X) for 24 hr, yielding 522 mg of crude extract after evaporation of the

solvent in vacuo. VLC of this extract using hexane, CH_2Cl_2 and EtOAc mixtures of increasing polarity yielded twelve 50 ml fractions. The first fraction (106 mg) was chromatographed on prep. TLC using hexane giving 31 mg of bithiophene **1** and 49 mg of β-farnesene. Fraction 6 gave 50 mg of bithiophene **2** after prep. TLC separation using 1:1 hexane:CH_2Cl_2 (3X) as solvent.

*[2-^{13}C]-acetate labeled bithiophenes **1** and **2**.*

94.0 g of root cultures fed with [2-^{13}C] acetate were soaked in 1600 ml of CH_2Cl_2 for 24 hr yielding 733 mg of crude extract. VLC separation with hexane, CH_2Cl_2 and EtOAc mixtures of increasing polarity gave sixteen, 50 ml fractions. Fraction 1 gave after prep. TLC separation 62 mg of bithiophene **1** and 55 mg of β-farnesene. Bithiophene **2** (36 mg) was obtained from fraction 8 after prep. TLC using hexane: CH_2Cl_2 (2:1, 3x).

*[1,2-$^{13}C_2$]-acetate labeled bithiophenes **1** and **2**.*

104.6 g of root cultures fed with [1,2-$^{13}C_2$] acetate were soaked in 1600 ml of CH_2Cl_2 (2X) for 24 hr, yielding 1.13 g of crude extract. Chromatographic separation using VLC and hexane CH_2Cl_2, EtOAc mixtures of increasing polarity gave 12, 50 ml fractions. Fraction 1 was chromatographed by prep. TLC using hexane yielding 29 mg of bithiophene **1** and 23 mg of β-farnesene. Fractions 4 and 5 were combined (70.7 mg) and chromatographed on prep. TLC using hexane:CH_2Cl_2(3X) giving 5 fractions of which the third one contained bithiophene **2** (22 mg), which was rechromatographed by prep. TLC giving 15.8 mg of pure bithiophene **2**.

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REFERENCES

1. Bohlmann, F., T. Burkhardt and C. Zdero, *Naturally Occurring Acetylenes.* Academic Press, London and New York, 1973.
2. Towers, G.H.N., *Plant. Physiol.* (Life Sci. Adv.) **1987**, *6*, 85.
3. Jente, R., G. A. Olatunji and F. Bojold, *Phytochemistry* **1981**, *20*, 2169.
4. Bu' Lock, J. D. and G. N. Smith, *J. Chem. Soc.* (C). **1967**, 332.
5. Bohlmann, F. and U. Hinz, *Chem. Ber.* **1965**, *98*, 876.
6. Constabel, C. P. and G.H.N. Towers, *Phytochemistry* **1989**, *28*, 93.
7. Tanabe, M., H. Seto and L. Johnson, *J. Amer. Chem. Soc.* **1970**, *92*, 2157.
8. Simpson, T. J., In: *Modern Methods of Plant Analysis*, Vol. **2**, (H. F. Linskens and J. F. Jackson, eds.) pp. 1-41, Springer, New York, 1986.
9. Bohlmann, F. and C. Zdero, In: *Heterocyclic Compounds*, Vol. **44**, Part 1 (A. Weissberger and E. C. Taylor, Eds.) John Wiley and Sons, Gronowitz, 1985.
10. Parodi, F. J., N. H. Fischer and H. E. Flores, *J. Nat. Prod.* **1988**, *51*, *594*.
11. Bax, A., *J. Magn. Res.* **1984**, *57*, 314.
12. Bax, A., J. A. Ferreti, N. Nashed and D. M. Jerina, *J. Org. Chem.* **1985**, *50*, 3029.
13. Holker, J. S. E., R. D. Lapper and T. J. Simpson (1974) *J. Chem. Soc. Perkin I*, 2135.
14. Simpson, T. J. and S. E. Hoeker, *Phytochemistry* **1977**, *16*, 229.
15. Flores, H. E., In: *Biotechnology in Agricultural Chemistry* (H. M. Lebaron, R. O. Mumma, R. C. Honeycutt, J. H. Duesing, J. F. Phillips and M. J. Haas, Eds.) pp. 66-86, ACS Symposium Series *334*, Washington, DC., 1987.
16. Murashige, T. and F. Skoog, *Physiol. Plant* **1962**, *14*, 473.
17. Coll, J. C. and B. T. Bowden, *J. Nat. Prod.* **1986**, *49*, 934.

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