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# THIOPHENE INTERCONVERSIONS IN *TAGETES PATULA* HAIRY-ROOT CULTURES

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**Key Word Index**—*Tagetes patula*; Asteraceae; French marigold; biosynthesis; naturally occurring thiophenes; root culture.

**Abstract**—On the basis of feeding experiments with $^{35}S$-labelled intermediates, we present a modified biosynthetic pathway of the bithienyls in *Tagetes*. The monothiophene 2-(but-3-en-l-ynyl)-5-(penta-1,3-diynyl)-thiophene, which is present in small amounts in *Tagetes* hairy roots, is the precursor of all bithienyls that have been described for this species but not of α-terthienyl. The current hypothesis that 5-(3-buten-l-ynyl)-2,2′-bithienyl originates from 5′-methyl-5-(3-buten-l-ynyl)-2,2′-bithienyl after oxidative decarboxylation proved untrue. The latter compound is only converted into (5′-but-3-en-l-ynyl-[2,2′]bithiophenyl-5-yl)-methyl acetate, probably via (5′-but-3-en-l-ynyl-[2,2′]bithiophenyl-5-yl)-methanol. Substitution of the butenynyl side chain of 5-(3-buten-1-ynyl)-2-2′-bithienyl results in the formation of 5-(3,4-dihydroxy-l-butynyl)-2,2′-bithienyl and 5-(4-hydroxy-l-butynyl)-2,2′-bithienyl, which are subsequently converted into respectively 5-(3,4-diacetoxy-l-butynyl)-2,2′-bithienyl and 5-(4-acetoxy-l-butynyl)-2,2′-bithienyl. The end products of this biosynthetic pathway are all bithienyl-acetate esters.

## INTRODUCTION

In the study of biosynthetic routes, plant cell cultures have been shown to be a powerful tool [1]. Major advantages are the homogeneity of both the cell cultures and the environment in which they are suspended. Radiolabelled precursors can be fed via the culture medium and their conversion by the submersed plant cells can be followed in time-course experiments. A drawback is that the cell cultures often do not synthesize the same secondary products as intact plants. Hairy-root cultures, however, usually produce the same secondary metabolites as roots on plants [2–4] although the amounts may be lower. Thus hairy-root cultures are an excellent model system to study the synthesis of secondary products in roots.

Most *Tagetes* species accumulate a range of thiophenes in their roots. These compounds are characterized by one, two or three thiophene rings linked together by their α-carbons. Formation of the thiophene rings is probably a two-step reaction [5]. The first step is the addition of a thiol to a diyne group, thus forming a thioether. The second step would be cleavage of the thioether bond and concomitant ring closure. Although the thiophenes in *Tagetes* have been the subject of several studies, the biogenetic relations of these compounds are not completely known and their biosynthesis is poorly understood [6].

The close structural relationship of the naturally occurring thiophenes in *Tagetes patula* led to the assumption that all these compounds are derivatives of the polyacetylene trideca-3,5,7,9,11-pentayn-1-ene (**1**) [7]. Labelling studies with related plant species like *Echinops sphaerocephalus* L. and *Buphthalmum salicifolium* L. confirmed this assumption [7, 8]. The biosynthetic pathway from **1** to the major thiophene derivatives comprises three types of biosynthetic steps: thiophene-ring formation, demethylation, and substitution at the side chain.

In the formation of bi- and terthienyls a monothiophene is expected as an intermediate. Addition of sulphur to **1** can yield four possible monothiophenes, of which only the diynes 2-(but-3-en-1-ynyl)-5-(penta-1,3-diynyl)-thiophene (**2**) and 2-(hex-5-en-1,3-diynyl)-5-(prop-1-ynyl)-thiophene are known to occur in plants [5]. On the basis of structural relationship only **2** can be the precursor of 5-(3-buten-1-ynyl)-2,2′-bithienyl (**3**). Addition of sulphur to **2** would yield 5′-methyl-5-(3-buten-1-ynyl)-2,2′-bithienyl (**4**) which has been proposed to be converted to **3** after oxidative decarboxylation [5, 8–12]. The presence of **2** has never been described for *Tagetes*.

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However, mutagenesis of *Tagetes* seed yielded a mutant, deficient in **3** synthesis, which accumulates large amount of **2** and **4** (Jacobs *et al.*, in preparation). This pattern supports the hypothesis that **2** and **4** are intermediates in the synthesis of **3** [7]. Probably **2** also occurs in wild-type *Tagetes*, albeit in minute amounts.

Feeding experiments with radiolabelled **3** indicated that this compound is readily converted into 5-(4-hydroxy-1-butynyl)-2,2′-bithienyl (**5**) [13] and 5-(4-acetoxy-1-butynyl)-2,2′-bithienyl (**6**) [7, 14–16]. The diol 5-(3,4-dihydroxy-1-butynyl)-2,2′-bithienyl (**7**) may also be a **3** derivative [7]. The mechanism of **3** derivatization is not really established, but it seems probable that all derivatives are formed via an epoxidation of the vinyl group [5]. Reduction of the epoxide moiety would yield the alcohol **5**, whereas hydrolysis would give **7**. The alcohols, in turn, may be converted into **6** and 5-(3,4-diacetoxy-1-butynyl)-2,2′-bithienyl (**8**) respectively.

In this paper the presence of **2**, the 'missing link' in the biogenesis of bithienyls, will be described in isolated *Tagetes* roots. Furthermore, the hypothetical, but still unresolved, steps in the biosynthetic pathway, i.e. oxidative decarboxylation of **4** to **3** and the conversion of **3** into **6** and **8** via **5** and **7**, will be investigated in experiments in which $^{35}$S-labelled intermediates were fed to *Tagetes* hairy roots.

## RESULTS

All thiophenes that have been described in *T. patula* roots could be identified in the HPLC chromatogram of an extract from the *T. patula* hairy-root line Tp9402. Moreover, after culturing the hairy roots in the presence of $^{35}$S-labelled sulphate or cysteine, radioactive label could be traced in two minor peaks that had not yet been identified as thiophenes. One peak was identified as **2** on the basis of HPLC retention time and GC-MS pattern that were compared with those of authentic samples. The other compound has not yet been identified.

The presence of **2** in the root extract makes it plausible that this compound is a precursor in polythienyl biosynthesis. To test this idea we fed [$^{35}$S]**2** to Tp9402 hairy roots. After 48 hr the roots were extracted and the extracts fractionated by HPLC. Radioactive label was found in all bithienyl-containing fractions but not in 2,2′:5′,2″-terthienyl (**9**). This indicates that the bithienyls, but not terthienyl, are derived from **2**.

Feeding [$^{35}$S]**4** to the roots led to the formation of labelled (5′-but-3-en-1-ynyl-[2,2′]bithiophenyl-5-yl)-methyl acetate (**10**) whereas no radioactive **3** or other bithienyls were detected (Table 1). This result sheds doubt on the hypothesis of **3** formation by oxidative decarboxylation of **4**. A considerable amount of radioactivity (20%) was present in a fraction that did not contain any of the known thiophenes.

To clarify the point whether **4** is converted into **3** via oxidative decarboxylation, we synthesized the presumed intermediates in this decarboxylation reaction, (5′-but-3-en-1-ynyl-[2,2′]bithiophenyl-5-yl)-methanol (**11**) and 5′-formyl-5-(3-buten-l-ynyl)-2,2′-bithienyl (**12**). Feeding the radiolabelled aldehyde or the alcohol both led to the formation of [$^{35}$S]**10**, whereas no [$^{35}$S]**3** could be detected. When [$^{35}$S]**10** was fed, it was taken up by the roots but not converted.

Table 1. Conversion of thiophenes in transformed roots of *Tagetes patula*

| | Isolated compound | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **11** |
| Precursor | Distribution of activity in the root extract (%) | | | | | | | | | |
| **2** | 4 | 8 | 22 | 21 | 10 | 3 | 2 | — | 18 | 6 |
| **3** | — | 61 | — | 1 | 20 | — | 16 | — | — | — |
| **4** | — | — | 46 | 1 | — | 1 | 1 | — | 31 | 1 |
| **5** | — | 1 | — | 2 | 87 | — | 3 | — | — | — |
| **6** | — | — | — | 5 | 85 | — | 4 | — | — | 1 |
| **10** | — | — | — | — | 1 | — | 3 | — | 70 | 5 |
| **11** | — | — | — | — | — | — | — | — | 81 | 3 |

Eight-day-old root cultures were placed in an emulsion containing $^{35}$S-labelled thiophenes, 2 kBq ml$^{-1}$. After 40 hr of incubation the roots were rinsed and thiophenes were extracted. The radioactivities of the individual thiophenes were measured after separation by HPLC and expressed as percentages of the total activity in the extract. The values are means of at least three determinations, the standard error was 5% or less throughout.

The preceding experiments indicated that the thiophene biosynthetic pathway branches at **2**. The end product of one branch is **10**, which is derived from **4**. The other branch leads to the five demethylated bithienyls.

[$^{35}$S]**3** is readily converted into [$^{35}$S]**5**, [$^{35}$S]**6**, [$^{35}$S]**7** and [$^{35}$S]**8**. After feeding of [$^{35}$S]**5**, incorporation of label into **6** was high. When [$^{35}$S]**6** was fed to the roots, it was taken up but not converted. The results indicate that **6** is produced from **3** via **5**. During root growth, **6** hardly underwent any turnover and thus can be considered as a metabolically inactive end product.

To obtain additional information on the order in which the individual bithienyls are synthesized *in vivo*, carrier-free [$^{35}$S]sulphate was fed to a growing root culture and the specific activity of the thiophenes that were formed, was determined at intervals. The bithienyls appeared in the order **3**, **5/8**, and **6/10** (Table 2). The specific activities at 15–60 min. decreased in the same order. The amounts of **2**, **4**, **11** and **7** were too small to accurately determine their specific activities.

These results show that the sequence of thiophene formation is **3**, **5/8**, **6/10**. Three of these thiophenes, i.e. **3**, **5** and **6**, are supposed to be part of the same branch in the biosynthetic pathway. The sequence of formation and the differences in specific activities of these three compounds confirmed that **3** is converted into **5**, which is subsequently converted into **6**.

## DISCUSSION

The only monothiophene in *Tagetes* (**2**) is the precursor of all the bithienyls in this species (Scheme 1). This implies that the formation of the first thiophene ring in bithienyl

Table 2. Synthesis of individual thiophenes in transformed roots of *Tagetes patula*

| Time after $Na_2{}^{35}SO_4$ addition (min) | Specific activity in thiophenes ($GBq\,mol^{-1}$) | | | | |
|---|---|---|---|---|---|
| | **3** | **5** | **6** | **8** | **10** |
| 15 | 0.7 | 0.1 | 0 | 0.3 | 0 |
| 30 | 2.4 | 0.8 | 0 | 1.2 | 0 |
| 60 | 35.4 | 16.2 | 8.9 | 16.2 | 10.2 |

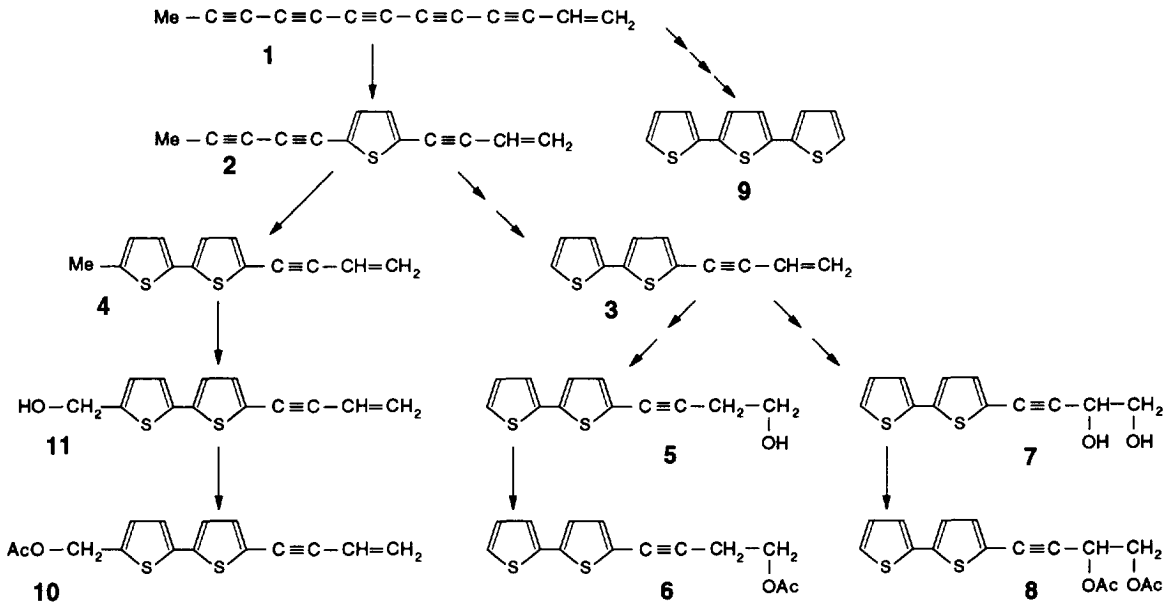
Tips were cut from exponentially (8 day) growing roots and preincubated in sulphate-free medium for 2 hr. Carrier-free [$^{35}$S]sulphate, 0.75 $MBq\,ml^{-1}$, was then added and the roots were extracted after various periods. The radioactivities and the chemical concentrations of individual thiophenes were measured after separation by HPLC. Data are means of at least three determinations, the standard errors were 5% or less.

biosynthesis is strictly regulated with respect to its position. The formation of the second ring occurs in two ways. First, addition of a thiol compound to the diyne moiety in **2** results in the formation of **4**. This reaction scheme is the same as for the formation of the first ring. Therefore, both processes may be catalysed by the same enzyme. In the alternative pathway, that leads to **3**, the methyl group of the monothiophene is removed prior to the closure of the second thiophene ring. The oxidative removal of a methyl group directly linked to a triple bond is a reaction described for several subtribes of the Heliantheae and in the tribe Anthemideae [12]. Demethylation of **2** would lead to the $C_{12}$ compound 2-(but-en-l-ynyl)-5-(but-1,3-diynyl)-thiophene, which has been found to accumulate in *Eclipta* species (Heliantheae) [17]. If the enzyme that catalyses the formation of the thiophene ring in *Eclipta* is able to form a second ring after demethylation, then **3** would be expected as the main accumulation product in this species. However, the occurrence of **3** in *Eclipta* has not been described. Therefore, it is unlikely that the enzyme involved in the formation of the second ring of **3** is the same as in the other thiophene ring formations.

Compound **4** is an intermediate in the formation of **10** in *Tagetes*. We propose a reaction scheme involving first oxidation of the methyl group to form **11**, then acetylation of the alcohol. Compound **4** is detected in wild-type *Tagetes* only in small amounts. This indicates that the oxidation step occurs fast. Compound **11** is present in wild-type roots, again in very small amounts compared to its derivative **10**.

The precursor for **5** and **6** is **3**. We found no evidence for the conversion of **4** into **5** and **6** as proposed by Jente *et al.* [16]. This incongruity can be explained by a reinterpretation of their original results. In the column and TLC steps used [16], the alcohols **5** and **11** are not separated since their retardation factors differ too little in these separation systems. The same is true for the corresponding acetate esters **6** and **10**. However, with reversed-phase HPLC the four compounds are easily separated. Since the introduction of this technique several authors have described a major absorption peak attributable to **10** [18–21]. Though this compound was already known from *Flaveria* [9] and *Buphthalmum* [8], its presence has only recently been reported for *Tagetes* [22].

It is unlikely that **5** and **6** are biosynthesized separately, as was suggested by Jente *et al.* [13]. Two lines of evidence support this conclusion. After feeding radiolabelled sulphate, the label is incorporated into **5** first and then into **6**, which results in a higher specific activity of **5**. Moreover, when $^{35}$S-labelled **5** is fed to roots, it is almost




Scheme 1. Biogenetic relations of the thiophenes in *Tagetes*.

completely converted into [$^{35}$S]**6** within 40 hr. The conclusion that **5** is the direct precursor of **6** is in agreement with results of Metschulat and Sütfeld [23] who showed the activity of a specific acetyl-transferase catalysing this conversion. The bithienyl acetate esters **6**, **8** and **10** are not converted *in vivo* (Table 1) and thus are metabolically inactive end products of the bithienyl biosynthetic pathway.

## EXPERIMENTAL

*Root cultures.* Hairy-root line Tp9402 was obtained by transformation of *Tagetes patula* L. cv. 'Nana' with *Agrobacterium rhizogenes* LBA 9402 (pRil855). Root line A-II-5 was obtained by transformation of a *Tagetes erecta* mutant which accumulated **2** and **4**, with the same *Agrobacterium* strain (Jacobs *et al.*, in preparation).

*Growth of isolated roots.* Roots were maintained in liquid Gamborg's B5 medium [24], supplemented with 3% (w/v) sucrose and 100 $\mu g l^{-1}$ biotin. The cultures grew in 50 ml flasks filled with 20 ml medium, on a rotary shaker (100 rpm) at 25° in the dark and were subcultured every two weeks.

*Kinetics of thiophene biosynthesis.* Tips of 1 cm were cut from roots precultured for 8 days and incubated in sulphate-free medium for 2 hr. Carrier-free [$^{35}$S]sulphate (0.8 MBq $ml^{-1}$) was then added to the culture. The thiophenes were extracted at intervals and separated by HPLC. The specific activity of each of the compounds was calculated from the radioactivity and the chemical amount present in the peaks.

*Preparation of radiolabelled precursors.* Roots that had been grown in B5 medium with $Na_2{}^{35}SO_4$ (specific activity 40 GBq $mol^{-1}$), synthesized radiolabelled thiophenes. Root line Tp9402 was used for the production of $^{35}$S-labelled **3**, **6** and **10**. The amount of **8** in these roots was too small to allow isolation for precursor-feeding experiments. The mutant root line A-II-5 was used for the production of labelled **2** and **4**. After fractionation of the root extracts [25], thiophenes were recovered from the petrol phase. This phase was fractioned on a column of 4 g silica gel G-60 (Merck) by stepwise elution with 30-ml fractions of 0, 10 and 40% $Et_2O$ in petrol. The fractions were evapd to dryness, dissolved in EtOH and purified by reversed-phase HPLC. Purity of the isolated compounds was confirmed by analytical HPLC and TLC. The alcohols **5** and **11** which are minor metabolites in the root, were made by saponification of the corresponding acetate esters [16]. Formylation of **3** via the Vilsmeier–Haack reaction gave **12**. In this reaction 5 mg **3** was mixed with 5 mg $POCl_3$ and 5 mg *N*-methylformanilide for 20 min at 50°. After cooling, 1 ml of a 1 M NaOAc solution and 2 ml EtOAc were added. After mixing for 30 min, the mixture was shaken with 6 ml hexane–t-butylmethyl ether (1:1). The hexane layer was evapd under $N_2$ at ambient temp. and the residue taken up in 200 $\mu l$ ethylene–glycol monomethyl ether. The structure of the chemically synthesized compound was confirmed by GC–MS and $^1$H NMR. The procedure was repeated using [$^{35}$S]**3** as starting material. The identity of [$^{35}$S]**12** was confirmed by HPLC analysis, using the non-labelled compound as reference.

*Precursor feeding.* In the feeding experiments the labelled thiophene precursors dissolved in 100 $\mu l$ EtOH were added to 10 ml 0.1% Tween-20 in Gamborg's B5 medium supplemented with 3% (w/v) sucrose and 100 $\mu g l^{-1}$ biotin. The radioactivity of the incubation medium was 2 kBq $ml^{-1}$. Eight-day-old root cultures were placed in these emulsions and incubated for 40 hr. Then roots were rinsed with unlabelled B5 medium and thiophenes extracted as described above. The organic phase was fractionated with reverse-phase HPLC, thiophenes were monitored by UV absorption. Fractions of 0.5 ml were collected, mixed with scintillation fluid and radioactivity was measured.

*Identification of thiophenes.* GC–MS: a capillary column (fused silica WCOT, coated with CP-Sil 5CB, 25 m × 0.32 mm ID) was used to fractionate the samples. Carrier gas was He, and the flow rate 1.5 ml $min^{-1}$. Samples of 1 $\mu l$ were injected with a splitting ratio of 1:10, at an injection port temp. of 250°. The initial oven temp. was 100°, the temp. was raised by 15° $min^{-1}$ to 280°, this temp. was maintained for 8 min. The electron impact method (EI) was used to ionize the fractions. Recorded spectra were compared with spectra known from literature [5, 22, 26–28]. Since **5** and **11** are not stable at the oven temp. used, these compounds were acetylated prior to GC–MS identification. This was done by dissolving the alcohols in $Ac_2O$ and subsequent heating at 70°, for 1 hr. The anhydride was removed by evapn.

Fourier-transformed $^1$H NMR spectra were recorded on a spectrometer operating at 400 MHz. Samples were measured in $CDCl_3$ with TMS as an internal standard. NMR spectra were compared with spectra known from literature [9–11, 26, 29, 30]. UV spectra were recorded in EtOH. Absorption was measured between 210 and 600 nm. Spectra were compared with known spectra from the literature [9–11, 29, 31, 32]. HPLC was performed on a Lichrosorb RP-18 column (particle size 7 $\mu m$). Column dimensions were 25 × 0.4 cm, the eluent was MeCN–$H_2O$ (318:7). The flow rate was 1.5 ml $min^{-1}$, thiophenes were detected by their UV absorption at 340 nm. TLC: strongly lipophilic thiophenes were separated on $Al_2O_3$ by elution with petrol, thiophenes of moderate polarity on silica gel 60 $F_{254}$ by elution with $C_6H_6$–$CHCl_3$ 9:1 [33]. Alternatively, thiophenes of moderate polarity were separated on silica gel 60 $F_{254}$ by elution with hexane–EtOAc (9:1), which resulted in similar retardation factors.

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## REFERENCES

1. Hahlbrock, K. and Scheel, D. (1989) *Annu. Rev. Plant Mol. Biol.* **12**, 347.
2. Flores, H.E. (1987) *Proc. Am. Chem. Soc.* **190**, 66.
3. Berlin, J., Mollenschott, C., Greidziak, N., Erdogan,

S. and Kuzovkina, T. (1990) *Current Plant Science and Biotechnology in Agriculture*, Vol. 9, p. 763. Kluwer Academic, Dordrecht.
4. Mukundan, U. and Hjortso, M. A. (1990) *J. Exp. Botany* **41**, 1497.
5. Bohlmann, F., Burkhardt, T. and Zdero, C. (1973) *Naturally Occurring Acetylenes*. Academic Press, London.
6. Constabel, C. P. and Towers, G. H. N. (1989) *Phytochemistry* **28**, 93.
7. Bohlmann, F. and Hinz, U. (1965) *Chem. Ber.* **98**, 876.
8. Bohlmann, F., Wotschokowsky, M., Hinz, U. and Lucas, W. (1966) *Chem. Ber.* **99**, 984.
9. Bohlmann, F. and Kleine, K.-M. (1963) *Chem. Ber.* **96**, 1229.
10. Bohlmann, F., Arndt, C., Kleine, K.-M. and Bornowski, H. (1965) *Chem. Ber.* **98**, 155.
11. Bohlmann, F. and Berger, E. (1965) *Chem. Ber.* **98**, 883.
12. Christensen, L. P. and Lam, J. (1991) *Phytochemistry* **30**, 11.
13. Jente, R., Richter, E., Bosold, F. and Olatunji, G. A. (1988) *Bioactive Molecules*, Vol. **7**, p. 187. Elsevier, Amsterdam.
14. Schulte, K. E. and Foerster, S. (1966) *Tetrahedron Lett.* 773.
15. Schulte, K. E., Henke, G., Rücker, G. and Foerster, S. (1968) *Tetrahedron* **24**, 1899.
16. Jente, R., Olatunji, G. A. and Bosold, F. (1981) *Phytochemistry* **20**, 2169.
17. Christensen, L. P. and Lam, J. (1990) *Phytochemistry* **29**, 2753.
18. Downum, K. R. and Towers, G. H. N. (1983) *J. Nat. Prod.* **46**, 98.
19. Pensl, R. and Sütfeld, R. (1985) *Z. Naturforsch.* **40c**, 3.
20. Sütfeld, R. (1987) *Modern Methods of Plant Analysis*, Vol. 5, p. 104. Springer, Berlin.
21. Ketel, D. H. (1986) *Physiol. Plant.* **66**, 392.
22. Caniato, R., Filippini, R. and Cappelletti, E. M. (1990) *Anal. Chim. Acta* **232**, 253.
23. Metschulat, G. and Sütfeld, R. (1987) *Z. Naturforsch.* **42c**, 885.
24. Gamborg, O. L. (1970) *Plant Physiol.* **45**, 372.
25. Norton, R. A., Finlayson, A. J. and Towers, G. H. N. (1985) *Phytochemistry* **24**, 719.
26. Bohlmann, F., Kleine, K.-M. and Arndt, C. (1964) *Chem. Ber.* **97**, 2125.
27. Groneman, A. F., Posthumus, M. A., Tuinstra, L. G. M. Th. and Traag, W. A. (1984) *Anal. Chim. Acta* **163**, 43.
28. Bicchi, C., Frattini, C., Pellegrino, G., Rubiolo, P., Raverdino, V. and Tsoupras, G. (1992) *J. Chromatog.* **609**, 305.
29. Atkinson, R. E., Curtis, R. F. and Phillips, G. T. (1964) *Tetrahedron Letters* 3159.
30. Bohlmann, F. and Zdero, C. (1985) *The Chemistry of Heterocyclic Compounds*, Vol. **44**, p. 261. John Wiley and Sons, New York.
31. Uhlenbroek, J. H. and Bijloo, J. D. (1959) *Rec. Trav. Chim.* **78**, 382.
32. Bohlmann, F. and Herbst, P. (1962) *Chem. Ber.* **95**, 2945.
33. Curtis, R. F. and Phillips, G. T. (1962) *J. Chromatog.* **9**, 366.