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INCORPORATION OF ^{35}S INTO DITHIACYCLOHEXADIENE AND THIOPHENE POLYINES IN HAIRY ROOT CULTURES OF *CHAENACTIS DOUGLASII*

C. PETER CONSTABEL and G. H. NEIL TOWERS

Department of Botany, University of British Columbia Vancouver, British Columbia, Canada, V6T 2B1

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Key Word Index—*Chaenactis douglasii*; Asteraceae; biosynthesis; polyines; dithiacyclohexadienes; thiophenes.

Abstract—The biosynthetic relationship between dithiacyclohexadiene and related thiophene polyines was investigated in hairy root cultures of *Chaenactis douglasii*. ^{35}S was incorporated simultaneously and at the same rate into both compounds. It is concluded that these compounds are not synthesized sequentially in *C. douglasii*.

INTRODUCTION

Root and root culture extracts of *Chaenactis douglasii* have been found to contain, in addition to the antibiotic dithiacyclohexadiene polyines thiarubrine A (**1**) and thiarubrine B (**2**), the related thiophenes **3** and **4** as minor constituents [1]. These thiophenes are known from almost 100 members of the Asteraceae, usually occurring in the absence of the thiarubrines [2]. The biosynthesis of thiarubrines and thiophenes is poorly understood; however both are presumably derived from tridecapentaynene [2]. Since the thiophenes **3** and **4** are involved in the synthesis of more complex thiophenes and are structurally very similar to thiarubrines, a possible biosynthetic relation between thiarubrines and the corresponding thiophenes in *C. douglasii* was postulated and investigated. A tissue culture system using *Agrobacterium rhizogenes*-transformed hairy roots of *C. douglasii* [3] provided a well-defined environment within which labelling studies were conducted.

RESULTS

Polyine pattern of C. douglasii *hairy root cultures*

The thiarubrines and thiophenes isolated from intact plant roots and root cultures of *C. douglasii* are each found as two isomers, A and B. Since these occur in a constant ratio, they are analysed together. Preliminary experiments showed the thiophenes to be present in *C. douglasii* root extracts at concentrations of 2–10% of the more abundant thiarubrines (Table 1). Thiophenes were present in greater amounts relative to thiarubrines early in the culture period. There was a large difference in polyine content of intact plant roots from two populations examined. *Chaenactis douglasii* is known to be a variable species complex [4].

^{35}S incorporation in relation to SO_4^{2-} availability

Roots are able to regulate rates of uptake of specific ions dependent on their availability [5]. Such regulation has been observed with SO_4^{2-} in cultured tobacco cells [6] and seedling roots of pea [7]. In order to optimize the uptake and incorporation efficiency of ^{35}S into the compounds of interest, cultures of *C. douglasii* roots were grown in SH medium with a range of reduced SO_4^{2-} concentrations. Appropriate amounts of $MgCl_2$ were added to maintain the Mg^{2+} concentration constant. After a three-day labelling period, thiarubrines were extracted and assayed for radioactivity. The results are shown in Fig. 1. Cultures that had been grown in SH medium with one half the normal concentration of SO_4^{2-} showed the greatest total activity of thiarubrines. The specific activity was also at its maximum at this SO_4^{2-} concentration. The increase in specific and total activity was greater than expected from a reduced dilution of the label; the rate of SO_4^{2-} uptake had been significantly increased by the S-limiting conditions. Thiarubrine synthesis was not inhibited by SO_4^{2-} limitation except at 0.36 and 0.12 mM SO_4^{2-}, and growth was not affected except at the lowest SO_4^{2-} concentration (data not shown). For further labelling experiments cultures were grown in SH with 0.86 mM $MgSO_4$ + 0.86 mM $MgCl_2$

Rate of incorporation of ^{35}S into thiarubrines and thiophenes

Experiments were carried out to determine how rapidly ^{35}S, supplied as $^{35}SO_4^{2-}$, is incorporated into the polyines of interest. Cultures in the logarithmic growth phase were fed 5 μCi of $^{35}SO_4^{2-}$ and four flasks were extracted and analysed at intervals between 1 and 120 hr. The results are presented in Table 2. No activity could be detected with a labelling period of 1 hr or less, and the rate of incorporation decreased after 24 hr. Based on the above results, a pulse of 24 hr was chosen for pulse-chase experiments.

Pulse-chase experiments

A 24 hr $^{35}SO_4^{2-}$ (20 μCi) pulse was followed by addition of unlabelled $MgSO_4$. Four culture flasks each were harvested 0, 2, 4, 8, and 12 days after the end of the labelling period and analysed for thiarubrine and thio-

Table 1. Polyines in *C. douglasii* hairy root culture and intact plant root extracts (mg/g EDW)‡

Source	Thiarubrines	Thiophenes
Root cultures*		
12-day-old	6.35 (2.63)†	0.66 (0.05)
24-day-old	5.00 (0.22)	0.21 (0.03)
Intact plant roots		
Site 1	5.11 (2.12)	0.36 (0.01)
Site 2	1.80 (0.78)	0.13 (0.04)

*Hairy root culture line CD-HR211.

†Means (±1s.d.), $n=10$ (intact plants), or $n=4$ (cultures).

‡EDW = Extracted dry wt.

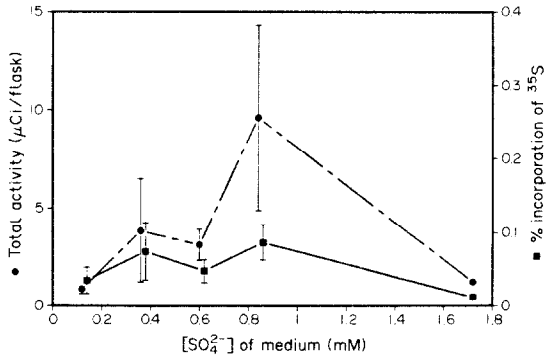


Fig. 1. Incorporation of ^{35}S into thiarubrines under various conditions of SO_4^{2-} limitation. Each point is the mean of four independent determinations. Bars represent ±1 s.d. 1.72 mM is the standard concentration of $MgSO_4$ in SH medium.

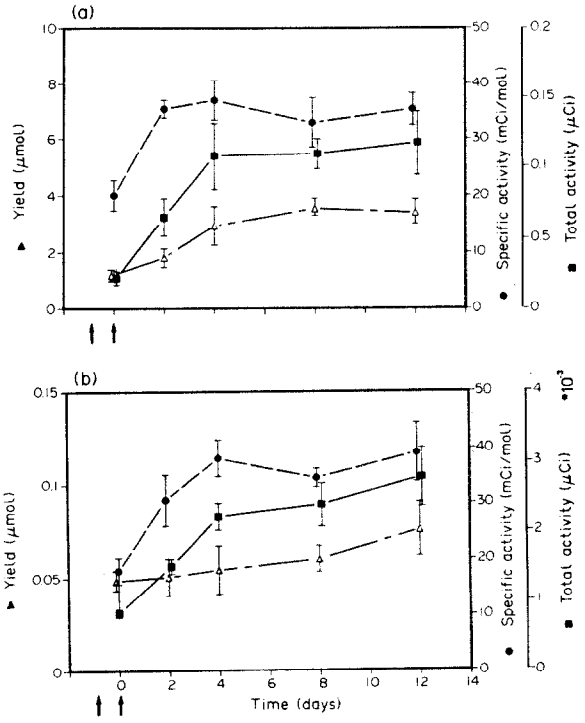


Fig. 2. Specific and total activity of thiarubrines (A) and thiophenes (B) versus time after pulse-chase labelling with $^{35}SO_4^{2-}$. Each point is the mean of four independent determinations. Bars represent ±1 s.d. The arrows indicate beginning and end of the 24 hr pulse.

phene content and activity. The results are summarized in Fig. 2. Thiarubrine yield followed a sigmoidal curve while thiophene increases were less dramatic and more linear. Earlier time-course experiments with hairy root cultures of *C. douglasii* had shown that thiarubrine synthesis parallels the typically sigmoidal pattern of biomass accumulation. In both thiophenes and thiarubrines the specific activity increased simultaneously up to two days after labelling. The total activities of both compounds increased steadily to day four and then remained constant. Although the $^{35}SO_4^{2-}$ in the medium was diluted by the unlabelled $MgSO_4$ at time 0, ^{35}S continued to be incorporated into the polyines for several days. Presumably sufficient ^{35}S was taken up by the roots in 24 hr to feed into the biosynthetic pathway for the following four days. As a result, the pulse actually received by the thiarubrine pathway spanned more than 24 hr.

DISCUSSION

The pattern of ^{35}S incorporation into thiarubrine and thiophene indicates that the label appeared in both compounds at the same time, and once incorporated, remained at a constant level in both cases. This is inconsistent with the hypothesis that these compounds are synthesized sequentially as part of the same pathway, with either thiarubrine being converted to thiophene or vice-versa. Several alternative possibilities could account for the observed pattern: both compounds could be synthesized by separate pathways, or in separate reactions from a common precursor. Biosynthetically, the latter is more likely. However, previous observations indicate that thiarubrine is an unstable molecule and decomposes to the thiophene upon exposure to light, heat, and other agents [8]. It is therefore possible that at least some of the thiophenes detected are not synthesized by the plant but result from degradation in culture or during the extraction procedure. The similar specific

Table 2. Rate of incorporation of ^{35}S into thiarubrines (mCi/mol) in hairy root cultures of *C. douglasii*

Time (hr)	1	3	8	24	120
Sp. act.	0	0.64 (0.35)*	0.89 (0.15)	11.8 (2.89)	27.6 (2.70)

*Mean of four independent determinations (±1 s.d.)

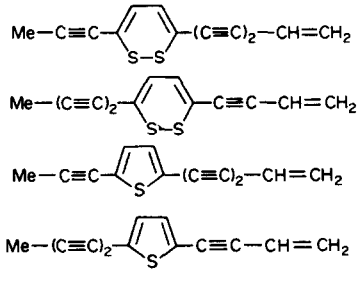
activities of both compounds at all times despite differing pool sizes (yield) support this hypothesis; it remains to be explained, however, why the different number of sulphur atoms per molecule of the compound is not reflected in their specific activities. All procedures were performed in dim light to minimize possible decomposition; nevertheless, duplicate extractions with a known quantity of HPLC-purified thiarubrine gave decomposition rates of between 2 and 5%, depending on the extraction procedure used. These rates would give concentrations in the range observed for thiophenes. Greater conversion occurred when fresh roots were first extracted into methanol and then partitioned into petrol, rather than being freeze-dried and extracted into petrol directly, suggesting that *in vitro* degradation could account for the presence of the thiophenes. The higher ratio of thiophene yield to thiarubrine yield during the early culture period is probably the result of increased degradation during culture inoculation. However, our data cannot exclude other possibilities.

Whatever the origin of the thiophenes, our data give no evidence for a direct biosynthetic relation to thiarubrines. Based on synthesis experiments, Bohlmann and Bresinsky [9] had already suggested that *in planta* thiophene synthesis via a dithiacyclohexadiene was unlikely. Our conclusion that the thiophenes may not be actively synthesized in *C. douglasii* hairy root cultures is surprising in light of the wide distribution of the thiophenes **3** and **4** in the Asteraceae. Their role as precursors for numerous mono-, bi- and terthienyl polyines has been documented [2, 8].

The data reported here give no indication of any turnover of thiarubrine in hairy root cultures of *C. douglasii* during the late logarithmic and stationary growth phases. As thiarubrine A has been shown to be very toxic to fungi, bacteria, and nematodes [10], the absence of a turnover of thiarubrine suggests that, in the plant, thiarubrine has a protective rather than a physiological function.

EXPERIMENTAL

Root cultures. Hairy root cultures were induced on *C. douglasii* with *Agrobacterium rhizogenes* strain TR7 as described previously [3]. The cultures were routinely maintained in liquid SH medium [11] on a rotary shaker (100 rpm) at 25° in the dark and subcultured every 4 weeks. For some experiments, 1.72 mM $MgSO_4$ was replaced by 0.86 mM $MgSO_4$ + 0.86 mM $MgCl_2$ in the medium (see Results).



Radiochemicals. $Na_2{}^{35}SO_4$ (40 μCi/μmol) was obtained from Amersham Radiochemicals. It was administered to cultures as a filter-sterilized aq soln.

Extraction of thiarubrines and thiophenes. Root cultures were rinsed (3x) in dist. H_2O, blotted dry, and frozen to −80°. After lyophilizing for 48 hr, samples were extracted with petrol for 24 hr. The extract was filtered, the filtrate evaporated and the compounds resuspended in HPLC-grade MeOH. The polyine components were separated by HPLC on a 0.4 × 30 cm Varian Micro Pak MCH-10 reverse-phase column using 72% aq. MeCN (1 ml/min), quantified by absorption at 340 nm, and collected for liquid scintillation counting. An int. standard of known concn was used to correct for variations in injection vol. The molar absorptivities used for concentration determinations were 10 300 for the thiarubrines and 31 500 for the related thiophenes [1]. All manipulations involving thiarubrines were carried out in dim light.

Liquid scintillation counting. Samples (2 ml) were mixed with 10 ml of a scintillation mixture consisting of 10 mg PPO-BisMSB (ICN Radiochemicals) and 50 g naphthalene in 1 l-*p*-dioxane. Counts were obtained on a Searle Isocap/300 liquid scintillation counter, and corrected for quenching using the channels method.

Tracer studies. 11- to 16-day-old root cultures (logarithmic growth phase) were labelled with a filter-sterilized aq soln of $Na_2{}^{35}SO_4$ (5–20 μCi). In pulse-chase experiments, the label was diluted out by adding unlabelled $MgSO_4$ (0.080 mmol/flask).

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