Production of [^{14}C]-6-Pentyl-2-pyrone in Liquid Cultures of *Trichoderma harzianum*

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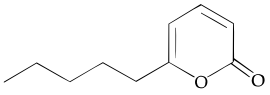
^{14}C-Radiolabeled 6-pentyl-2-pyrone (6PP), a naturally occurring compound with commercially important fragrance and antimicrobial properties, was produced by *Trichoderma harzianum* Rifai grown in liquid cultures containing D-[U-^{14}C]glucose in admixture with unlabeled D-glucose as the primary carbon source. Approximately 1.2% of the glucose consumed was converted to 6PP. Its specific incorporation rate, 5.6×10^5 Bq/mmol, was ~83% of the theoretical yield if glucose was the sole carbon source.

Keywords: *6-Pentyl-2-pyrone; Trichoderma harzianum; radiolabel; ^{14}C; fungal biosynthesis; secondary metabolite*

INTRODUCTION

The fragrance compound 6-pentyl-2-pyrone (6PP, **I**)



6PP, I

is produced naturally in ripening fruit (Engel et al., 1988) and by certain species of *Trichoderma* fungi (Collins and Halim, 1972). It is used widely as a food flavoring, while topical treatments have been shown to control *Botrytis cinerea* rots in stored kiwifruit, a recalcitrant disorder in this crop (Poole et al., 1998).

The practical uses of 6PP depend on a better understanding of its activity and fate in plant systems [e.g., Poole and Whitmore (1997)], and on more efficient biosynthesis [e.g., Cooney et al. (1997)]. Progress in such studies would be facilitated by the use of radiolabeled 6PP. Serrano-Carreon et al. (1993) showed that linoleic acid, a putative precursor of saturated lactones, was converted to 6PP only inefficiently by liquid cultures of *T. harzianum*. However, because 6PP is produced under a broad range of culture conditions, other precursors may be more suitable. We report here a convenient method for producing [^{14}C]-6-pentyl-2-pyrone in *Trichoderma harzianum* liquid cultures using D-[U-^{14}C]glucose as the primary carbon source.

MATERIALS AND METHODS

Materials. Purified water (MilliQ, Waters Corporation) was used throughout. Methanol (HPLC grade), acetic acid [analytical reagent (AR) grade], ethyl acetate (AR), diethyl ether (AR), hexanes (AR), 3,5-dinitrosalicylic acid, and NaOH were supplied by BDH (NZ) Ltd. D-Glucose, thiamine hydrochloride, potassium hydrogen tartrate, DL-tartaric acid, $NaNO_3$, deuteriochloroform, and 6PP (6-amyl-α-pyrone) were supplied by Sigma–Aldrich Chemical Co. (St. Louis, MO). Agar (PDA) was obtained from Davis (NZ) Ltd, and malt extract was from Maltexo (NZ) Ltd. D-[U-^{14}C]Glucose solution (nominal 7.40 MBq/mL 0.066 M solution in 3% ethanol) was supplied by Amersham International, Buckinghamshire, England. The measured activity was 6.34 MBq/mL glucose.

Scintillation Counting. Sample solution aliquots were mixed with a 50 times or greater volume of scintillation cocktail (Ready Safe, Beckman), and radioactive decays were counted over a 2-min period with a 1218 Rackbeta (LKB–Wallac) liquid scintillation counter.

Chromatography. High performance liquid chromatographic (HPLC) determination of 6PP followed Poole and Whitaker (1997). Thin-layer chromatography (TLC) was done on glass-backed channelled fluorescent silica gel plates (Merck 60CF254 20 × 20 × 0.1 cm), using hexanes:diethyl ether:acetic acid (40:80:1) as the mobile phase. Plates were viewed under 254 nm UV light.

Nuclear Magnetic Resonance (NMR) Spectra. ^{1}H and ^{13}C NMR spectra were obtained with a Bruker AC-300 instrument operated at 300.13 MHz for ^{1}H and 75.47 MHz for ^{13}C. Samples were dissolved in deuteriochloroform.

***Trichoderma harzianum* Cultures.** A *T. harzianum* Rifai strain known to produce high yields of 6PP (strain A: Cooney et al., 1997) was recultured on gel media containing 30 g/L agar, 10 g/L malt extract, and 2 g/L potassium hydrogen tartrate. Conidia were harvested from ~20-day-old gels by rubbing the surfaces gently with ~1 mL of water, filtering through glass wool, centrifuging to a pellet, rinsing, and resuspending in water. The density (conidia/mL) was counted with a hemocytometer. This suspension was then used for inoculating the liquid cultures for production of 6PP.

The base liquid culture medium comprised Czapek's minerals (1 mL/L; without nitrate or ammonium salts), thiamine hydrochloride (0.1 g/L), DL-tartaric acid (2 g/L), sodium nitrate (40 mg/L), and D-glucose (10 g/L). For the cultures, 50 mL was placed in each of four 125-mL conical flasks. For production of labeled 6PP, 0.16 mL of D-[U-^{14}C]glucose solution was also added. Flasks were inoculated with freshly harvested *T. harzianum* conidia to give a concentration of 3.3×10^7/mL.

Flasks were agitated gently (100 orbits/min) at 22 °C in a 12-h lighting cycle. Incubation was terminated by placing the cultures in a −80 °C freezer, for the base medium, after 11, 13, 15, and 19 days (without radiolabel) or after 15 days only (with radiolabel). The effects of varying the concentrations of DL-tartaric acid were assessed by inoculating media containing 1, 0.5, 0.25, and 0 g/L DL-tartaric acid with a different batch

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of *T. harzianum* conidia and incubating for 8 days under similar conditions.

Quantification of 6PP and D-Glucose Content in Cultures. Subsamples (0.5 mL) of the supernatant were withdrawn, diluted with methanol (0.5 mL), and filtered (0.45 μm syringe tip, Phenomenex). 6PP concentrations were determined in the filtrates by HPLC.

Glucose contents were measured only for cultures in the base medium sampled at 15 days. The filtrates (0.2 mL) were first diluted to 2 mL with 0.1 M, pH 6.4, phosphate buffer. Aliquots (0.5 mL) were then mixed with 0.5 mL of a solution containing 1.0 g of 3,5-dinitrosalicylic acid, 30 g of sodium potassium tartrate, and 1.6 g of sodium hydroxide per 100 mL, heated at 80 °C for 5.0 min, and then quenched in ice. The solutions were diluted with water to 3 mL, and the absorbance at 540 nm was measured. Readings for D-glucose standard solutions, prepared similarly, were linear ($R^2 = 0.991$) in the range 0−1 mg/mL.

Purification of ^{14}C-Labeled 6PP. The cultures were thawed, extracted twice with an equal volume of ethyl acetate, which was evaporated at 30 °C or lower, and redissolved in ethyl acetate (1.0 mL). A 50-μL subsample was withdrawn to determine its ^{14}C content.

The remaining material was loaded uniformly onto channelled TLC plates. Reagent 6PP (2 mg in 50 μL of ethyl acetate solution) was placed in one channel as an elution standard. One of the sample channels was scraped off in bands at 0.1 R_f intervals over the entire eluted distance and extracted in ethyl acetate (0.2 mL) and then in 0.2 mL of methanol:water (90:10 by volume). These two extracts were combined, and ^{14}C activity was determined for each R_f interval.

Authentic 6PP was observed as an intense absorption band at $R_f = 0.80-0.90$. This band was scraped off the remaining sample channels and extracted with ethyl acetate (3 × 2 mL). The purity of the separated product was verified by HPLC and by ^{1}H and ^{13}C NMR.

RESULTS

6PP Production and the Effect of Tartaric Acid Concentration. 6PP concentrations in the 2 g/L DL-tartaric acid base media were 57, 73, 75, and 99 μg/mL after 11, 13, 15, and 19 days incubation, respectively. In the cultures with varying amounts of tartaric acid, 6PP concentrations after 8 days were 126, 110, 173, and 144 μg/mL for 1, 0.5, 0.25, and 0 g/L DL-tartaric acid concentrations, respectively. Residual glucose in the base medium was 2.7 g/L at 15 days, the time when the radiolabeled cultures were terminated.

Separation of Labeled 6-Pentyl-2-pyrone. The crude ethyl acetate culture extracts contained 2.0% of the added ^{14}C label. Approximately 65% of this activity was recovered after small-scale fractionation on TLC; most of the recovered material occurred in the low polarity (R_f 0.8−0.9) fraction corresponding to 6PP (Figure 1). A further UV-absorbing band was observed near the origin, but it is likely that it was not fully extracted from the silica. A total of 10.3 mg of 6PP and 0.86% of the added label were recovered from the R_f 0.8−0.9 band for the combined cultures with D-[U-^{14}C]-glucose, giving a specific incorporation rate of 5.6×10^5 Bq/mmol. It was calculated that 1.16% of the carbon atoms in the consumed D-glucose was incorporated into the 6PP.

The HPLC of this material contained a single peak absorbing at 301 nm, which eluted at the same retention time as authentic 6PP. The ^{1}H and ^{13}C NMR spectra were consistent with published spectra (Claydon et al., 1987). Peaks not attributable to 6PP had negligible intensity.

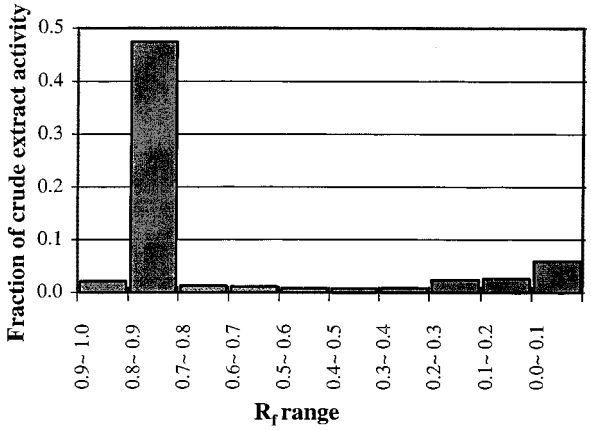

Figure 1. Distribution of ^{14}C in TLC fractions of a crude ethyl acetate extract of *Trichoderma harzianum* liquid cultures containing D-[U-^{14}C]glucose.

DISCUSSION

The results show that ^{14}C was readily incorporated in reasonable yields into the 6PP produced by *T. harzianum* cultures growing on glucose as the primary carbon source. The activity of the purified 6PP was 83% of that expected if 6PP had been produced solely from the labeled glucose. This suggests that a small amount of a carbon source other than glucose was incorporated into the product, presumably from the DL-tartaric acid, which was included in the medium to limit bacterial growth. It is probable that carbon atoms from either source would be incorporated randomly. The results for the lower amounts of tartaric acid show that it is not needed for 6PP production and could be omitted if it was necessary to produce 6PP in which the labeling was unequivocally uniform. The differences in yield for the two inoculum batches may reflect normal batch to batch variations resulting from minor differences in inoculum age, vigor, or culture conditions rather than any suppressive effect of DL-tartaric acid.

The proportion of ^{14}C incorporated into 6PP (~1.2% of the glucose consumed) was considerably higher than the 0.17% reported for linoleic acid, a suggested precursor of 6PP (Serrano-Carreon et al., 1993). They attributed the low yields to the slow rates of early steps in the proposed pathway in which the linoleic acid was reduced to a 12 carbon chain. The higher yields in our work, using glucose as the sole carbon source, suggest that alternative biosynthetic pathways function for unsaturated lactones. The lower yield from radiolabeled linoleic acid would occur if the bulk of the molecule was consumed in a nonspecific manner, but might alternatively result from differences in 6PP biosynthesis or intracellular transport for the different *Trichoderma* species used.

Greater quantities could be produced by scaling the production, and higher specific activity could be achieved by increasing the proportion of labeled to carrier glucose. Nevertheless the production and cleanup is straightforward, and the product is being used in continuing studies of 6PP in biocontrol and crop protection systems.

ABBREVIATIONS USED

6PP, 6-pentyl-2-pyrone; HPLC, high performance liquid chromatography; UV, ultraviolet; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

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