Early Steps of Dauricine Biosynthesis in Cultured Roots of *Menispernum dauricum*

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Cultured roots of *Menispernum dauricum*, a rich source of the bisbenzylisoquinoline alkaloid dauricine (1), were fed with L-[U-14C] tyrosine, L-[3-13C] tyrosine, and [2-13C] tyramine independently, and the incorporation of possible early precursors into 1 was studied. The results demonstrated that 1 was composed of four molecules of tyrosine, and that tyramine was specifically incorporated into the isoquinoline portions of 1. The unusual chlorine-containing alkaloid acutumine (3), into which [13C]-labeled tyrosine was also incorporated, was identified as one of the main constituents in the alkaloid fraction from the roots.

**Key words:** bisbenzylisoquinoline; dauricine; biosynthesis; *Menispernum dauricum*; root culture

Bisbenzylisoquinolines are structurally constructed of two monomeric benzylisoquinoline units joined by one or more ether linkages. Dauricine (1), a bisbenzylisoquinoline, has hypotensive and anti-arrhythmic effects with little toxicity and a large safety range.\(^1\) Previously we have established dauricine-producing root cultures of *Menispernum dauricum* (Menispermaeae) which contain more than 0.5% dauricine.\(^2\) Dauricine, a simple homodimer of (R)-armepavine with all the hydroxyl groups in the isoquinoline portions methylated, is an ideal alkaloid for studying bisbenzylisoquinoline biosynthesis. The recent studies by Stadler and Zenk\(^3\) on the dimerization process of (R)- and (S)-N-methylcoclaurines to yield berberamine, a bisbenzylisoquinoline, using *Berberis stolonifera* cell cultures, showed that intramolecular phenol oxidative coupling, the most crucial step in bisbenzylisoquinoline biosynthesis, is catalyzed by a new cytochrome P-450 enzyme. However, the early steps of bisbenzylisoquinoline and benzylisoquinoline biosynthesis, especially the involvement of tyramine, are still obscure. Sugimoto and Yamada\(^4\) demonstrated that tyramine was incorporated almost exclusively into the isoquinoline portions of bisbenzylisoquinolines, aromolamine and berberamine, in *Stephania cepharanthi* root cultures. Zenk et al.\(^5\) reported that tyramine labeled both the isoquinoline and benzyl portions in a 3:1 ratio in jatrohridine, a protoberberine, in *B. canadensis* cells. In this work we have fed *M. dauricum* cultured roots with [14C]-labeled tyrosine and [13C]-labeled tyrosine and tyramine, and studied their incorporation into dauricine in an attempt to discover the exact role of these early precursors in bisbenzylisoquinoline biosynthesis.

Based on the remaining radioactivity in the medium, *M. dauricum* roots, harvested 20 days after applying L-[U-14C] tyrosine (5.92 × 10^5 dpm), accumulated 92.2% of the total radioactivity. Of the radioactivity in the roots, 5.7% (33.8 × 10^5 dpm) of which 4.2 × 10^5 dpm was present in the crude alkaloids, was recovered in the methanol extract. HPLC analysis of the crude alkaloids showed that the distribution of radioactivity closely followed UV absorption (Fig. 1). Two main peaks with t<sub>R</sub> of 7.9 and 20.0 min, attributable to highly 14C-labeled compounds, were present. One of the compounds, t<sub>R</sub> 20.0 min, was identified as dauricine (1) by comparison of its behavior on HPLC with authentic 1. The second compound, t<sub>R</sub> 7.9 min, was identified, based on physical and spectral data from a larger sample isolated in separate experiment, as acutumine (3).

These results showed, unequivocally, that tyrosine is a building block of dauricine. Incorporation and distribution of the label derived from L-[U-14C] tyrosine in the roots of *M. dauricum* were quite similar to those previously reported with cultured roots of *S. cepharantha*.\(^6\) In both cultures, more than 90% of the label was incorporated into the roots. Similar proportions of the label were recovered in the MeOH extract and crude alkaloid fraction. Moreover, each component in the crude alkaloids separated by HPLC was shown to be radiolabeled in proportion to the UV absorption. The only difference was that no labeled tyramine was detected in *M. dauricum* roots while a significant amount accumulated in *S. cepharantha* roots. 1-Tyrosine decarboxylase (TDC) activity in *S. cepharantha* was 0.462 pkat g fresh roots and that in *M. dauricum* was only 0.010 (Y. Sugimoto, unpublished data) when roots were growing exponentially. The difference of tyramine accumulation in both roots fed with L-[U-14C] tyrosine is consistent with their TDC activities.

Acutumine, an unusual chlorine-containing alkaloid, was...
reported to be isolated from *M. camadense*, *M. dauricum*, and *Sinomenium acutum*. Barton *et al.* deduced from its structure that 3 was a kind of benzylisoquinoline alkaloid and proposed a complicated biosynthetic pathway to 3 from a simple benzylisoquinoline. According to their proposal, acutamine biosynthesis branches off the pathway for dauricine just after a simple benzylisoquinoline is formed. However, there have been no experimental proofs for the proposed biosynthetic pathway for almost 30 years except for the fact that the chlorine was introduced not by a working-up process, but by a true process of biosynthesis. From our feeding experiments with L-[U-14C] tyrosine, it was verified that tyrosine was a building block of acutamine.

To obtain a more precise assessment of tyrosine incorporation into dauricine, feeding experiments of 13C-labeled tyrosine and tryptamine were done. Preliminary experiments showed that tyrosine and tryptamine had little effects on root growth. The low concentration (0.5 mM) of tyrosine promoted dauricine production while the higher concentrations (1-2 mM) were inhibitory (Table). 13C-NMR of OMD obtained by feeding 0.5 mM L-[3,13C] tyrosine showed no significant signal enhancement over that obtained from authentic OMD. In contrast, 13C-NMR of OMD from cultures fed with 1 mM L-[3,13C] tyrosine or 1 mM [2,13C] tyramine displayed significant signal increases (Fig. 2). Comparing the spectra of OMDs prepared from dauricine isolated from roots fed with L-[3,13C] tyrosine (Fig. 2A) or [2,13C] tyramine (Fig. 2B) with that of unlabeled 2 (Fig. 2C), four signals (Fig. 2A) and two signals (Fig. 2B) were specifically increased. 13C-NMR spectrum of 2 was previously assigned by Jossang *et al.* According to their assignments, enriched signals correspond to the carbons at C-4 and 4' (both δ 24.9) and C-α and α' (δ 39.5 and 39.7) of *vice versa*. The 13C-enrichments of the signals calculated from peak-height analysis of 13C-NMR spectra of 2 were as follows: for 2 from cultures fed with L-[3,13C] tyrosine, C-4 and 4' were 2.2 and 2.3% and C-α and α' were 0.8 and 0.9%. For 2 from cultures fed with [2,13C] tyramine, C-4 and 4' were both 1.3%.

These results showed that dauricine is composed of four molecules of tyrosine, and tyramine is incorporated exclusively into the isoquinoline portions. Therefore, tyrosine incorporation into the benzyl portion of the molecule proceeds not via tyramine, but via its corresponding α-keto acid (4-hydroxyphenylpyruvic). These results are consistent with the previous investigations on aromoline and berberine biosynthesis in *S. cepharanthi* root cultures. Nevertheless, 13C-enrichments of the carbons, C-4, 4', δ, α, and α', in OMD (2) were much poorer than those in aromoline and berberine. In 2 made from I isolated from *M. dauricum* roots fed with [3,13C] tyrosine or [2,13C] tyramine, 13C enrichments were less than 3% while those in aromoline and berberine from *S. cepharanthi* roots ranged from 5 to 24% when cultured in media containing similar concentrations of labeled precursors. These differences were mainly due to the contents of the studied alkaloids and their ratios to other tyrosine-derived alkaloids. In *S. cepharanthi* cultured roots, berberine and aromoline contents were about 1 and 2% of the root dry weight, respectively, and they accounted for most of the alkaloids extracted from the roots. Dauricine contents in *M. dauricum* roots was, however, about 0.5% of the root dry weight and accounted for only 10% of the alkaloid fraction. The difference of pool size of tyramine in the two cultures, shown by feeding experiments with 14C-labeled
tyrosine, could affect the incorporation of $^{13}$C, derived from $[3,13]$C tyrosine and $[2-13]$C tyramine, into the alkaloids. Moreover, uptake of labeled precursors may differ between the two root cultures when millimolar quantities of tyrosine and tyramine were fed.

**Experimental**

**Plant materials.** Roots of *M. dauricum* were obtained from established cultures. The excised roots were cultured on a rotary shaker (70 rpm) in the dark at 27 °C in a B5 medium supplemented with 7.5 μM 1-naphthaleneacetic acid (NAA) and 3% sucrose. The medium was selected as it has been shown to be an optimum medium for dauricine production.


**Feeding experiments with $[1-13]$C tyrosine and $[2-13]$C tyramine.** $[1-13]$C Tyrosine (5.92 x 10$^{-3}$ dpm) was fed to vigorously growing 20-day-old cultured roots of *M. dauricum*. The roots were then allowed to grow for an additional period of 20 days before harvest. Harvested roots were freeze-dried and soaked overnight in MeOH. The suspension was centrifuged for 5 min at 3000 rpm and the supernatant decanted and saved. The pellets were re-extracted with MeOH and centrifuged. The supernatants were combined and evaporated to dryness at 40 °C. The residue was dissolved in 2.0 ml of 3% citric acid. The acidic aqueous solution was filtered through a filter paper into a glass tube and made alkaline (pH 10) with NH$_4$OH. A 1.0-ml portion of this alkaline solution was put onto an Extrelut column (Merck Art. 11738). The column was left for 10 min and then eluted with CHCl$_3$ (3.5 ml x 2). The combined CHCl$_3$ eluates were evaporated to dryness at 30 °C. The residue was dissolved in MeOH and analyzed by HPLC. The stationary phase was Devosil ODS-3 (50 x 4.6 mm) and the mobile phase was 75% MeOH containing 0.2% NH$_4$OH. The flow rate was 0.3 ml min$^{-1}$. A short pre-column (30 x 4.6 mm) was placed between the injector and the separation column. Dauricine, t$_R$ 20.0 min, was monitored by UV absorption at 283 nm. Peak areas were calculated with an electronic integrator. Radioactivity of the fraction separated by HPLC was measured in a toluene-based scintillator.

**Isolation and identification of acetamin (3).** Roots were cultured for 50 days in a B5 medium with 3% sucrose and then harvested and freeze-dried. A 30-g sample of the roots was extracted with MeOH and then treated as described above. The basic residue (0.73 g) was chromatographed over silica gel (120 g) with CHCl$_3$-MeOH. The proportion of MeOH in the solvent system was increased stepwise (50:1; 30:1; 20:1; 10:1). Fractions eluted with CHCl$_3$-MeOH (20:1) yielded 81 mg white powder. Recrystallization of the white powder from hot MeOH gave 3 as colorless crystals, mp 215°C (dec) [lit.]$^{13}$ 238-240°C (dec)]. $^1$H-NMR, IR, UV, and mass spectra matched literature values.

**Feeding experiments with $[1-13]$C tyrosine and $[2-13]$C tyramine.** $[1-13]$C Tyrosine (0.5 mm and 1 mm) or $[2-13]$C tyramine (1 mm) were added to the medium. Excised *M. dauricum* roots were cultured, allowed to grow for 40 days, harvested, and freeze-dried. The basic fraction, obtained from the roots as described in the previous experiments, was treated with CH$_3$N$_2$ to convert dauricine to its O-methyl derivative. Thereafter O-methyldauricine (OMD; 2) was separated by TLC on a 0.25-mm silica gel plate (Merck Art. 5715) with a solvent system of CHCl$_3$-MeOH-NH$_4$OH (200: 50: 1). OMD was located by UV illumination and by spraying the plate with a modified Dragendorff’s reagent. The band containing 2, R$_f$ 0.72, was scraped off, and the alkaloid was eluted with MeOH. OMD was further purified by semi-preparative HPLC. The column was Capcell Pak C$_{18}$ (250 x 20 mm) and the solvent 75% MeOH with 0.2% NH$_4$OH. The flow rate was 8 ml min$^{-1}$. A short pre-column (10 x 4.6 mm) was placed between the injector and the separation column. OMD (2), t$_R$ 26.8 min, was detected by UV absorption at 283 nm. Analytical HPLC showed that purity of OMDs, thus obtained, was more than 98%. $^1$C-NMR spectra of 2 were measured at 100 MHz using JEOL JNM GX-400 spectrometer. Sample concentration was 7 mg ml$^{-1}$ in CDCl$_3$. Measuring parameters were: pulse width 5 μs (flip angle), number of scans 18,000, pulse delay 1.362 s. $^1$C-Enrichments of NMR signals were calculated from peak-height analysis of the spectra.

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**References**