Involvement of Tyramine in Bisbenzylisoquinoline Biosynthesis in Cultured Roots of Stephania cepharantha

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INVOLVEMENT OF TYRAMINE IN BISBENZYLISOQUINOLINE BIOSYNTHESIS IN CULTURED ROOTS OF *STEPHANIA CEPHARANTHA*[1]

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ABSTRACT.—Cultured roots of *Stephania cepharantha*, rich sources of the bisbenzylisoquinolines aromoline [**1**] and berbamine [**2**], were fed [2-^{13}C] tyramine and the labelled alkaloids isolated. The ^{13}C-nmr spectra showed that ^{13}C was specifically incorporated into the isoquinoline portions of **1** and **2**. The results suggest that either the hydroxylation of tyramine proceeds more rapidly than its oxidation or that tyrosine conversion to the benzyl moiety proceeds via its corresponding α-keto acid.

We established bisbenzylisoquinoline-producing cultured roots of *Stephania cepharantha* Hayata (Menispermaceae) (1,2) and studied the biosynthesis of these alkaloids in those roots (3), which grow vigorously and produce more than 2% aromoline [**1**] and more than 1% berbamine [**2**] as their main metabolic constituents (4).

Bisbenzylisoquinolines have a structure of two monomeric benzylisoquinoline units joined by one or more ether linkages. Tracer experiments carried out with intact plants (5,6) and cell cultures (7) have shown that coclaurine and its *N*-methyl derivatives act as bisbenzylisoquinoline precursors. Chemical degradation of the benzylisoquinoline skeleton, obtained from tracer experiments with radio-labelled tyrosine, has shown that this amino acid is incorporated in approximately equal amounts in the upper (isoquinoline) and lower (benzyl) parts of the benzylisoquinoline molecule (8). Similar results have been shown for the bisbenzylisoquinolines, berbamunine and berbamine, from *Berberis stolonifera* callus cultures (9). The intermediacy of tyramine in benzylisoquinoline biosynthesis has been demonstrated. Roberts *et al.* (10) reported that tyramine was incorporated almost exclusively into the isoquinoline portion in the morphine and thebaine isolated from *Papaver somniferum* fed [2-^{14}C] tyramine. In contrast, in the jatrorrhizine isolated from calli of *Berberis canadense*, [2-^{14}C] tyramine labelled both the isoquinoline and benzyl portions in a 3:1 ratio (11). In all cases in which the ^{14}C tracer was used, the precise location of the labeled position was not identified.

In a previous study we fed cultured roots of *S. cepharantha* ^{14}C-labelled tyrosine and tyramine and studied the incorporation of these labels to bisbenzylisoquinolines (3). When the precursors were applied at equal radioactivities, the recovery of radioactivity in the alkaloids derived from [2-^{14}C] tyramine was 1/15 of that derived from [U-^{14}C] tyrosine. We also fed cultured roots of *S. cepharantha* [3-^{13}C] tyrosine and isolated ^{13}C-labelled aromoline [**1**]. The nmr spectrum showed labels only in the signals assigned to C-4, C-4′, C-α, and C-α′. The isotopic excess of the each carbon calculated from the peak-height analysis ranged from 9% to 15%.

Tracer molecules labelled with ^{14}C are used more frequently than are ^{13}C-labelled tracers because of their high sensitivity. Although ^{13}C-labelled tracers are less sensitive, their advantage is that the location of the labelled position can be identified by ^{13}C-nmr spectroscopy without the complicated degradation process.

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The above results indicated that sufficient ^{13}C derived from tyramine would be incorporated into alkaloids to evaluate the isotopic excess of the corresponding carbon atoms if [2-^{13}C] tyramine, the decarboxylation product of [3-^{13}C] tyrosine, was fed to the *Stephania* root cultures. We have performed such a study and examined the incorporation of label into bisbenzylisoquinolines to investigate the metabolic fate of tyrosine and to show the involvement of tyramine in bisbenzylisoquinoline biosynthesis in cultured roots of *S. cepharantha*.

EXPERIMENTAL

PLANT MATERIALS.—Cultured roots of *S. cepharantha* were established as described elsewhere (2). Cultures were maintained in the dark at 26° in flasks containing SB5 medium (modified B5 medium) supplemented with 3% (w/v) sucrose, 10 μM indolebutyric acid, and 1 μM gibberellic acid agitated on a gyratory shaker at 80 rpm. Roots were subcultured at three-week intervals.

CHEMICALS.—L-[3-^{13}C] Tyrosine (99.8 atom %) was purchased from Isotec, Inc., and L-tyrosine decarboxylase (EC 4.1.1.25) from Sigma.

PREPARATION OF [2-^{13}C] TYRAMINE.—[2-^{13}C] Tyramine was prepared according to Gunsalus and Smith (12). The mixture of 380 mg L-[3-^{13}C] tyrosine, 50 mg L-tyrosine decarboxylase (70 units), 100 ml 0.2 M acetate buffer (pH 5.5), and 200 ml H_2O was incubated for 4 h at 37°. The incubation mixture was made alkaline (pH 10) with NH_4OH and treated repeatedly with EtOAc. The organic phase was dried overnight with Na_2SO_4 and concentrated to dryness. The residue was dissolved in MeOH and filtered. The filtrate, concentrated to dryness, yielded 0.29 g crude [2-^{13}C] tyramine as a yellow powder. HCl aqueous (25 ml) (0.4 N) was added to the powder, and the solution was filtered. The dried filtrate yielded 0.33 g of crude tyramine HCl as a pale yellow powder. This was crystallized from a mixture of EtOH and MeOH (10:1), yielding 241 mg of [2-^{13}C] tyramine hydrochloride. Eims *m/z* $[M]^+$ 138 (19.1%), 109 (100), 108 (64.7), 78 (25.3).

The decarboxylation process was monitored by hplc (Shimadzu LC-6A) at room temperature. The stationary phase was Develosil ODS-3 (150×4.6 mm) and the solvent 70% MeOH containing 0.2% NH_4OH. The flow rate was 0.35 ml/min. A short pre-column (30×4.6 mm) was placed between the injector and separation column. Tyrosine and tyramine were detected by uv absorption at 277 nm. The respective Rt's of tyrosine and tyramine were 4.2 min and 8.4 min.

Mass spectra were recorded with a gc-ms spectrometer (HP 5890 II). A di mode was used, with an ionizing energy of 70 eV and an ion source temperature of 250°.

FEEDING PROCEDURE AND ISOLATION OF ALKALOIDS.—Roots were cultured for 25 days in SB5 medium containing 200 ppm [2-^{13}C] tyramine, then harvested and freeze-dried. A 6-g root sample was treated with MeOH (200 ml×3) at room temperature to obtain 3.0 g of MeOH-soluble extract. The residue was suspended in 250 ml of 3% aqueous citric acid and filtered. The filtrate was made alkaline to pH 9–10 with NH_4OH and treated with $CHCl_3$ (150 ml×4). Removal of the $CHCl_3$ gave 245 mg of basic residue which, when a small amount of MeOH was added, produced 74 mg of aromoline [**1**] as pale yellow crystals.

After the removal of **1**, CH_2N_2 was introduced to the residual MeOH solution to convert berbamine [**2**] to its methyl ether, isotetrandrine [**3**]. Compound **3** was separated by tlc on a 0.25 mm Si gel plate (Merck Art. 5715) with a solvent system of $CHCl_3$-MeOH-NH_4OH (500:25:1). It was located by uv illumination and by spraying the plate with modified Dragendorff's reagent. The R_f value was 0.65. The band was scraped off, and the alkaloid was eluted with MeOH and dried. A small amount of EtOH added to the residue (33 mg) gave **3** (7.7 mg) as pale yellow crystals.

DETERMINATION OF THE ISOTOPIC EXCESS OF ^{13}C-ENRICHED AROMOLINE [**1**] AND ISOTETRANDRINE [**3**].—^{13}C-enrichment was calculated from the peak-height analysis of ^{13}C-nmr spectra of **1** and **3**. ^{13}C-nmr spectra were measured at 50.32 MHz with a Bruker AC-200P spectrometer. The sample concentration of **1** was 37.5 mg/ml and of **3** 19.3 mg/ml in $CDCl_3$ with TMS as the internal standard. The measurement parameters were: pulse width 3.6 μsec (45° flip angle), number of scans 12,000, pulse delay 2 sec.

RESULTS AND DISCUSSION

Preliminary experiments showed that less than 200 ppm of tyramine did not significantly reduce cell growth or alkaloid formation (Table 1). Roots were cultured for 25 days in SB5 medium containing 200 ppm [2-^{13}C] tyramine and harvested. [2-^{13}C] Tyramine was prepared by decarboxylation of [3-^{13}C] tyrosine as described in the

TABLE 1. Effects of Tyramine on Growth and Alkaloid Formation in *Stephania cepharantha* Root Cultures.[a]

Tyramine concentration (ppm)	Growth Index (n-fold)	Alkaloid Content (% dry wt)	
		Aromoline	Berbamine
0	22.4	2.83	1.29
40	18.0	2.88	1.26
200	21.6	2.64	1.21
400	15.8	2.21	1.08

[a]Roots (0.1 g fresh wt) were cultured for 29 days in 100-ml flasks containing 25 ml of SB5 medium with various concentrations of tyramine. Each value is the mean of three replicates.

Experimental section. Dry roots (6 g) were subjected to the isolation procedure, yielding 74 mg of aromoline as pale yellow crystals. After removal of aromoline [**1**], CH_2N_2 was introduced to the residual solution. This converted berbamine [**2**] to its methyl ether, isotetrandrine [**3**], which was isolated as pale yellow crystals (7.7 mg). The ^{13}C-nmr spectra of ^{13}C-enriched **1** and **3** are shown in Figures 1 and 2, respectively. In each alkaloid only two signals were specifically enhanced as compared with the spectra of the unlabelled compounds [not shown, see Sugimoto and co-workers (3,13)].

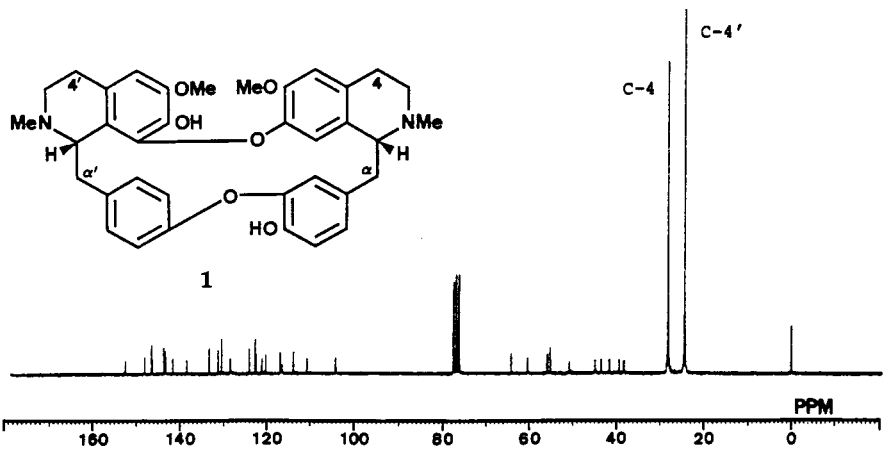


FIGURE 1. ^{13}C-nmr spectrum of aromoline isolated from cultured roots of *Stephania cepharantha* administered [2-^{13}C] tyramine.

The ^{13}C-nmr spectrum of **1** was assigned by Koike *et al.* (14), who reported that enhanced signals correspond to the carbons at C-4 (δ 28.5) and C-4′ (δ 24.6) of **1**. The ^{13}C enrichments of these two signals were calculated from the peak-height analysis of the ^{13}C nmr as C-4 (23.98%) and C-4′ (21.40%).

The ^{13}C-nmr spectrum of **3** has been assigned by our group (13). Enhanced signals were the carbons at C-4 (δ 23.6) and C-4′ (δ 25.8) of **3**, the ^{13}C enrichment being C-4 (14.02%) and C-4′ (15.10%).

Our experiments unequivocally show that tyramine is specifically incorporated into the isoquinoline portion of the bisbenzylisoquinolines aromoline [**1**] and berbamine [**2**] in cultured roots of *S. cepharantha*, without scrambling of label in other carbons.

It is well established (15) that coclaurine, the monomeric precursor of

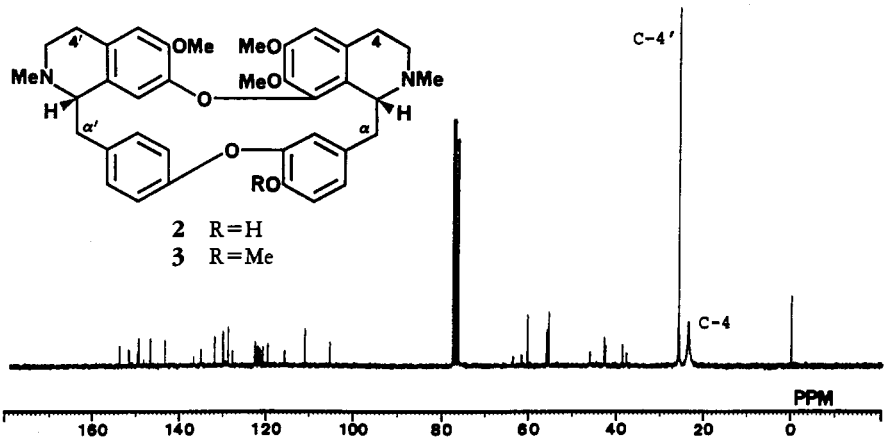
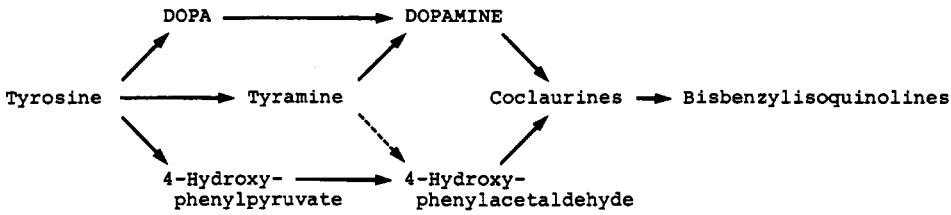


FIGURE 2. ^{13}C-nmr spectrum of isotetrandrine isolated from cultured roots of *Stephania cepharantha* [2-^{13}C] tyramine.

bisbenzylisoquinoline, is formed by the condensation of dopamine with 4-hydroxyphenylacetaldehyde (Scheme 1). The specific incorporation of tyramine into the upper dopamine-derived portion of bisbenzylisoquinolines as reported here clearly demonstrates the hydroxylation of tyramine to dopamine. Theoretically, 4-hydroxyphenylacetaldehyde could be formed from tyramine by way of an amineoxidase or via the decarboxylation of 4-hydroxyphenylpyruvate.



SCHEME 1. Biosynthetic sequence leading from tyrosine to the bisbenzylisoquinolines.

In a previous investigation (3,13), [3-^{13}C] tyrosine was incorporated in approximately equal amounts into the isoquinoline and benzyl portions of aromoline [**1**] and berbamine [**2**]. When 200 ppm of tyrosine or tyramine was fed to *Stephania* root cultures and allowed to be metabolized for one month, millimolar quantities of tyramine were detected in the roots, approximately 4 mM for tyrosine and 6 mM for tyramine. However, none of [2-^{13}C] tyramine administered exogeneously was incorporated into the benzyl moiety of **1** and **2** as shown in Figures 1 and 2, respectively. These results suggest that the hydroxylation of tyramine proceeds more rapidly than its oxidation or that tyrosine conversion to the benzyl moiety proceeds via its corresponding α-keto acid in *Stephania* root cultures.

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