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PARTICIPATION OF C_6–C_1 UNIT IN THE BIOSYNTHESIS OF EPHEDRINE IN *EPHEDRA**

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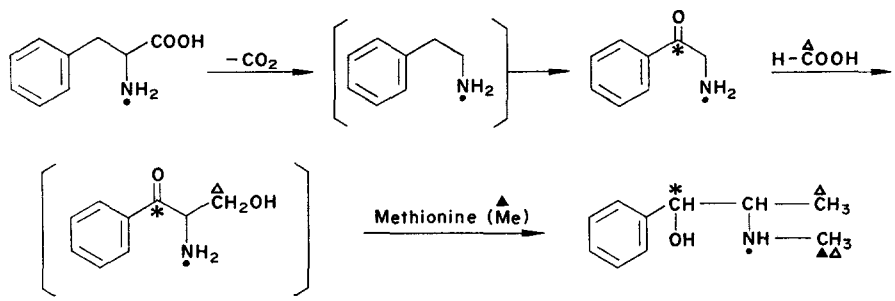
Key Word Index—*Ephedra distachya*; Ephedraceae; biosynthesis; ephedrine; C_6–C_1; benzoic acid; cinnamic acid.

Abstract—Feeding of benzoic acid-[7-^{14}C], benzaldehyde-[7-^{14}C] and cinnamic acid-[3-^{14}C] to *Ephedra distachya* resulted in efficient incorporations of ^{14}C into the α-carbon atom of the side chain of *l*-ephedrine. Thus ephedrine was shown to be biosynthesized by the condensation of a C_6–C_1 portion which is derived from phenylalanine via cinnamate and an unidentified C_2–N fragment.

INTRODUCTION

IN PREVIOUS studies on the biosynthesis of ephedrine, a biosynthetic pathway was proposed from the following evidence. Phenylalanine-[^{15}N] was incorporated into the nitrogen of ephedrine to the extent of 0·03–0·08%.[1] Methionine-[Me-^{14}C] was incorporated only into N–Me group,[2] whereas sodium formate-[^{14}C] was incorporated into both N–Me and C–Me (γ-carbon of the side chain) groups.[3] Finally an efficient incorporation of ω-aminoacetophenone-[CO–^{14}C] suggested the participation of C_6–C_2–N in the biosynthesis of ephedrine.[4]



SCHEME 1.

* Part VIII in the series of "Biosynthesis of Natural Products". A part of this study has been reported preliminarily; YAMASAKI, K., SANKAWA, U. and SHIBATA, S. (1969) *Tetrahedron Letters* 4099: For part VII see TAGUCHI, H., SANKAWA, U. and SHIBATA, S. (1969) *Chem. Pharm. Bull. (Tokyo)* **17**, 2054.

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[1] SHIBATA, S. and IMASEKI, I. (1956) *Chem. Pharm. Bull. (Tokyo)* **4**, 277.
[2] SHIBATA, S., IMASEKI, I. and YAMAZAKI, M. (1957) *Chem. Pharm. Bull. (Tokyo)* **5**, 71.
[3] SHIBATA, S., IMAZEKI, I. and YAMAZAKI, M. (1957) *Chem. Pharm. Bull. (Tokyo)* **5**, 594.
[4] SHIBATA, S., IMAZEKI, I. and YAMAZAKI, M. (1959) *Chem. Pharm. Bull. (Tokyo)* **7**, 449.

Further experiments showed that phenethylamine-[1-^{14}C] and α-amino-β-hydroxypropiophenone-[^{3}H] were incorporated nonspecifically and poorly into ephedrine. The failure of incorporation of these expected intermediates led us to examine the incorporation of phenylalanine, which was supposed to be an original precursor of ephedrine biosynthesis and reported to be an efficient precursor of *d*-norpseudoephedrine in *Catha edulis*.[5] The results obtained by the feeding experiments of phenylalanine labelled either with ^{14}C or ^{3}H suggested that it was incorporated via C_6–C_1 rather than C_6–C_2–N. In the present paper the role of C_6–C_1 in the biosynthesis of ephedrine is described and the origin of the remaining C_2–N part of ephedrine is discussed.

RESULTS AND DISCUSSION

Phenylalanine isotopically labelled as shown in Table 1 was administered to excised shoots of *Ephedra distachya*. In each case, the plant material was extracted after an appropriate metabolic period. The method of extraction and isolation described in a previous paper[6] was modified to render it more reliable (see Experimental). Radioactive ephedrine thus obtained was oxidized by the Kuhn–Roth method to benzoic acid and acetic acid, and the latter was converted to its *p*-bromophenacyl ester for measurement of radioactivity. A sample containing ^{3}H in the aromatic ring was oxidized with permanganate instead of the Kuhn–Roth reagent which would cause the exchange of aromatic hydrogen owing to its high acidity.

TABLE 1. INCORPORATION OF ^{14}C AND ^{3}H FROM PHENYLALANINE INTO EPHEDRINE BY EXCISED SHOOTS OF *Ephedra distachya*

Expt.	(±)-phenylalanine	Month	Incorporation % × 10^5	Sp. act. dpm/mM × 10^{-3} (%)		
				Ephedrine	Benzoic acid	AcOH
1	[Arom.-^{3}H]	June	16	8·1	8·5 (106)	—
2	[Arom.-^{3}H]	June	54	5·1	—	—
	[2-^{14}C]		4·1	0·045	—	—
3	[2-^{14}C]*	June	8·2	0·27	0·25 (92)	0 (0)
	[3-^{14}C]					
4	[2-^{14}C]	Sept.	0	—	—	—
5	[3-^{14}C]	Sept.	14	0·21	—	—
6	[Arom.-^{3}H]	Sept.	12	0·13	—	—
	[2-^{14}C]		5·4	0·065	—	—
7	[Arom.-^{3}H]	Sept.	85	0·92	—	—
	[3-^{14}C]		84	1·4	1·4 (100)†	0 (0)

* [2-^{14}C]/[3-^{14}C] = 7·6/8·7.
† Only ^{14}C was counted.

The experimental conditions of feeding with phenylalanine and the results are summarized in Table 1. Although incorporation of radioactivity of phenylalanine was quite low without any remarkable seasonal difference, the degradation experiments revealed that the aromatic ring and C-3 of phenylalanine participate to form the aromatic ring and α-carbon atom of ephedrine, respectively. Contrary to this, double-labelling experiments (2, 3 and 6)

[5] LEETE, E. (1958) *Chem. Ind. (London)* 1088.
[6] SHIBATA, S. and IMAZEKI, I. (1953) *Chem. Pharm. Bull. (Tokyo)* **1**, 285.

showed that the radioactivity of phenylalanine-[2-^{14}C] was lost during the course of biosynthesis. These observations suggest that phenylalanine is cleaved between C-2 and C-3, and only the remaining C_6–C_1 part participates in the biosynthesis of ephedrine.

Phenylalanine has been known as an effective source of C_6–C_3[7] and C_6–C_1[8] compounds in higher plants, where phenylalanine ammonialyase (PAL), an enzyme which mediates the conversion of phenylalanine into cinnamate, plays an important role. The formation of benzoate and acetate by the β-oxidation of cinnamate was proved by Zenk *et al.*[8] In order to demonstrate the participation of this metabolic pathway in the biosynthesis of ephedrine, benzoic acid-[7-^{14}C], benzaldehyde-[7-^{14}C] and cinnamic acid-[3-^{14}C] were administered to the plants.

TABLE 2. INCORPORATION OF ^{14}C FROM BENZOIC ACID, BENZALDEHYDE AND CINNAMIC ACID INTO EPHEDRINE BY EXCISED SHOOTS OF *Ephedra distachya*

Expt.	Labelled compounds	Month	Incorporation %	Sp. act. dpm/mM × 10^{-3} (%)		
				Ephedrine	Benzoic acid	Acetic acid
8	Benzoic acid-[7-^{14}C]	May	0·12	333	348 (104)	0
9	Benzoic acid-[7-^{14}C]	June	0·33	9·79	—	—
10	Benzoic acid-[7-^{14}C]	May	0·025	0·964	0·947 (92·3)	—
11	Benzaldehyde-[7-^{14}C]	May	0·016	31·3	29·8 (95·5)	0
12	Cinnamic acid [3-^{14}C]	May	0·0012	0·754	0·750 (99·7)	0

The incorporation of benzoic acid-[7-^{14}C] was very high as shown in Table 2. Incorporation of benzaldehyde-[7-^{14}C] and cinnamic acid-[3-^{14}C] were approx. one-tenth and one-hundredth of that of benzoic acid-[7-^{14}C], respectively. The radioactivity of the ephedrine was shown by degradation to be localized in its C_6–C_1 part. The Schmidt reaction of benzoic acid obtained by the degradation gave inactive aniline (see Experimental). Radioactivity derived from these precursors is located in the α-carbon atom of ephedrine, indicating the direct participation of C_6–C_1. Benzoic acid is a more efficient precursor than benzaldehyde, which may be due to its high permeability and mobility in the living plants. Our present data are not incompatible with the efficient incorporation of phenylalanine-[3-^{14}C] into *d*-nor-pseudoephedrine in *Catha edulis*,[5] if the radioactivity is introduced via C_6–C_1. The incorporation of phenylalanine-[^{15}N] was interpreted as indirect, possibly by transamination or via free ammonia liberated by the action of PAL. The occurrence of benzylmethylamine in *Ephedra*[9] also supports the presence of the intermediate C_6–C_1 compound. The relatively low incorporation of phenylalanine and cinnamic acid may suggest an alternative route to give benzoate derived directly from shikimate. This possibility has been pointed out for a fungus producing benzofuran derivatives, the benzene ring and

[7] KOUKOL, J. and CONN, E. E. (1961) *J. Biol. Chem.* **236**, 2692.
[8] ZENK, H. M. (1971) *Metabolism of Prearomatic and Aromatic Compound in Plants in Pharmacognosy and Phytochemistry* (WAGNER, H. and HÖRHAMMER, L., eds.), pp. 330–339, Springer, Berlin.
[9] RETI, L. (1953) *Ephedra Bases in The Alkaloids* (MANSKE, R. H. F. and HOLMES, H. L., eds.), Vol. III, p. 349, Academic Press, New York.

one carbon of which were derived from C_6–C_1.[10] We must retain this possibility until the problem is fully resolved.

TABLE 3. INCORPORATION OF ^{14}C FROM ALANINE, SERINE AND GLYCINE INTO EPHEDRINE BY EXCISED SHOOTS OF *Ephedra distachya*

Expt.	Labelled compounds	Month	Incorporation (%)	Spec. act. dpm/mM × 10^{-3} (%)			
				Ephedrine	Benzoic acid	Acetic acid	N–Me
13	L-Alanine-[U-^{14}C]	May	0·0017	1·7	0·76 (45·1)	0·30 (17·5)	(37·8)
14	L-Serine-[U-^{14}C]	July	0·00046	6·6	1·1 (17·4)	0·44 (6·7)	(75·9)
15	Glycine-[2-^{14}C]	June	0·001	1·5	0·29 (18·5)	0·051 (3·3)	(78·2)

The N–Me is unequivocally derived from active methionine, as confirmed in the previous study.[4] The remaining C_2–N part of ephedrine demands discussion. Since nor-pseudoephedrine was synthesized chemically by Akabori and Momotani,[11] it seemed worthwhile to test the incorporation of alanine and related amino acids into ephedrine. As shown in Table 3, ^{14}C-labelled L-alanine, L-serine and glycine showed similar incorporation to that of phenylalanine. Labelling from alanine-[U-^{14}C] was higher in the C_6–C_1 part than in the C_2–N part. High incorporation of formate-[^{14}C] into the C_2–N part observed in the previous study[3] suggested serine as a possible origin of the C_2–N moiety. More than 70% of the radioactivity from serine-[U-^{14}C] was found in N–Me as had been expected, while only one-third of radioactivity of the C_6–C_1 part was found in the C_2–N part. The efficient and specific incorporation of ω-aminoacetophenone-[CO–^{14}C] reported in the previous paper[4] might suggest an intermediate formed by the condensation of C_6–C_1 and glycine. However, this possibility was ruled out by the lack of incorporation of serine-[U-^{14}C] into C_2–N, since these amino acids are known to be biosynthetically equivalent. The direct feeding of glycine-[2-^{14}C] showed an almost identical distribution pattern of radioactivity to that of serine-[U-^{14}C]. As the feeding experiments of C_2 and C_3 amino acids did not give any conclusive evidence for the origin of C_2–N, compounds related to the TCA cycle were tested as precursors in the next series of experiments using glucose-[6-^{14}C] as a control (Table 4).

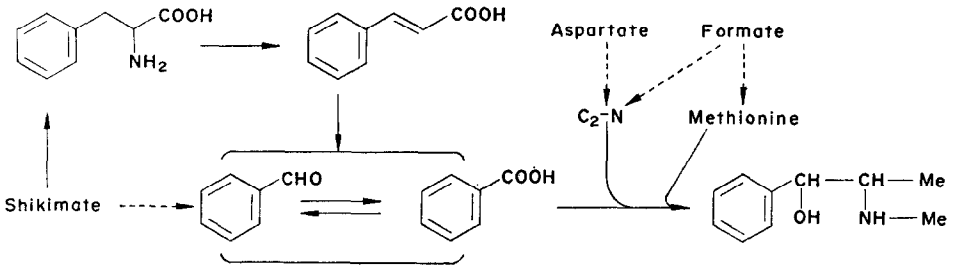
TABLE 4. INCORPORATION OF ^{14}C FROM SEVERAL COMPOUNDS RELATED TO THE TCA CYCLE INTO EPHEDRINE BY EXCISED SHOOTS OF *Ephedra distachya*

Expt.	Labelled compounds	Month	Incorporation (%)	Spec. act. dpm/mM × 10^{-3} (%)			
				Ephedrine	Benzoic acid	Acetic acid	N–Me
16	Succinic acid-[2,3-^{14}C]	June	0·00005	0·28	—	—	—
17	Propionic acid-[2-^{14}C]	June	0·0013	3·3	0·14 (4·3)	0·27 (8·12)	(87·6)
18	L-Aspartic acid-[U-^{14}C]	July	0·0011	5·3	1·40 (26·3)	1·45 (27·3)	(46·4)
19	D-Glucose-[6-^{14}C]	July	0·00023	0·67	0·14 (21·0)	0·14 (21·0)	(58·0)
20	Na-formate-[^{14}C]	June	0·00062	2·96	0·53 (17·8)	0·93 (31·4)	(50·8)

Propionic acid-[2-^{14}C] and succinic acid-[2,3-^{14}C] were chosen on the assumption that C_2 and N may react simultaneously with C_6–C_1 to build up ephedrine. Relatively high incorporation into the C_2–N moiety was observed in the feeding of L-aspartic acid-[U-^{14}C]

[10] BU'LOCK, J. D., KAYE, B. and HUDSON, A. T. (1971) *Phytochemistry* **10**, 1037.
[11] AKABORI, S. and MOMOTANI, K. (1941) *Proc. Imperial Acad. (Tokyo)* **17**, 506.
[12] GURIN, S. and DELLVA, A. M. (1947) *J. Biol. Chem.* **170**, 545.

and glucose-[6-^{14}C], suggesting that the C_2–N might be derived from aspartate or some closely related compound, but no firm conclusion could be drawn, since formate-[^{14}C] was also incorporated well into the C_2–N portion of ephedrine, as reported previously.[3]



SCHEME 2.

Although the origin of the C_2–N part of ephedrine has not yet been clarified, the present study has indicated a biosynthetic scheme for ephedrine, where C_6–C_1 compounds such as benzoic acid or benzaldehyde react with C_2–N compounds or their equivalent to give ephedrine. No tenable evidence to support the direct participation of ω-aminoacetophenone was obtained in the present study. Good incorporation of ω-aminoacetophenone-[CO–^{14}C] probably occurs indirectly, via C_6–C_1.

EXPERIMENTAL

Preparation of DL-*phenylalanine-[Arom.-*^{3}H]. Phenylalanine-[Arom.-^{3}H] was prepared by the method of Gurin and Delluva.[12] The location of labelled ^{3}H was confirmed by the conversion of phenylalanine-[Arom.-^{3}H] to benzoic acid. Permanganate oxidation of phenylalanine-[Arom.-^{3}H] (sp. act. 6·2 × 10^7 dpm/mM) gave benzoic acid (sp. act. 6·1 × 10^7 dpm/mM). This indicates almost all the radioactivity (98%) is in the aromatic ring.

Administration of labelled compounds. Ephedra distachya L., which was originally cultivated in Chiba Experimental Station of Tokyo University Forest was transplanted and cultivated in Kemigawa Experimental Station for Medicinal Plants of Tokyo University for 1–2 yr before use. The roots of the plants were removed to leave 2–3 cm root tips at the end of aerial parts. Water was supplied occasionally to maintain a suitable water level. After 6 days more than 90% of the radioactivity in the solution was absorbed by the plants. The plants were harvested for extraction after 7–8 days.

Extraction and isolation of ephedrine. The plant material was dried at 60° before extraction. The extraction method used in the previous study[6] was modified to make it more reliable. Dried *Ephedra* plants (50 g) were exhaustively extracted with MeOH (×3). The solvent was removed, and the residue was dissolved in 1% H_2SO_4. After removing acidic and neutral substances by filtration and extraction with Et_2O, the acidic aqueous solution was made alkaline with K_2CO_3 (pH 11). The basic fraction obtained by the extraction of the alkaline solution with Et_2O was washed with H_2O and dried ($MgSO_4$). Oily basic compounds obtained on evaporation of Et_2O were dissolved in a small vol. of H_2O and neutralized with dil. HCl. Crude ephedrine. HCl obtained on evaporation was recrystallized from EtOH–Et_2O to give pure ephedrine–HCl (*ca.* 50 mg) of m.p. 220°. The purity was examined by TLC or scanning radiochromatography.

The Kuhn–Roth oxidation of ephedrine. Ephedrine–HCl (50 mg) in oxidizing soln (10 ml, prepared by dissolving CrO_3 (168 g) and H_2SO_4 (250 ml) in H_2O (1 l.)) was heated at 140° in a flask equipped with a condenser. Water was added occasionally to maintain a suitable vol. After 1 hr, the temp. was raised to 165–170°, and distillation was continued until 150 ml distillate was collected. The distillate was extracted with Et_2O (×3) and the Et_2O soln was dried. Removal of solvent left a solid which was sublimed to yield benzoic acid (6·3 mg). The remaining aqueous phase of distillate was neutralized with 0·1 N NaOH, and H_2O was removed *in vacuo* to give a solid residue, which was taken up in 60% aq. EtOH. A calculated quantity of *p*-bromophenacyl bromide was added to the soln and the mixture was refluxed for 1 hr. Crystals obtained on standing the reaction mixture were submitted to preparative TLC. *p*-Bromophenacyl acetate recovered from the plate was recrystallized from aq. EtOH (18 mg, m.p. 85°).

Permanganate oxidation of ephedrine. To a soln of ephedrine–HCl (26 mg) in 1% KOH (1 ml) 4% $KMnO_4$ was added dropwise with stirring. After 2 hr of stirring, brown precipitates were removed by filtration. The filtrate was made alkaline with NaOH, and extracted with Et_2O to remove basic and neutral

compounds. The aqueous layer was acidified with dil. H_2SO_4 and extracted with Et_2O. After drying, Et_2O was removed to obtain crude benzoic acid which was purified further by sublimation (6 mg).

The Schmidt reaction of benzoic acid. Ephedrine–HCl isolated from the *Ephedra* fed with benzoic acid-[7-^{14}C] was degraded by the Kuhn–Roth oxidation to obtain benzoic acid. The benzoic acid (51 mg, sp. act. $6{\cdot}90 \times 10^4$ dpm/mM) was subjected to the Schmidt reaction to afford aniline–HCl, which was converted into benzoate (23 mg, m.p. 162°, sp. act. $2{\cdot}57 \times 10^2$ dpm/mM).

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