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BIOSYNTHESIS OF THE BENZOYL MOIETY OF COCAINE FROM CINNAMIC ACID VIA (*R*)-(+)-3-HYDROXY-3-PHENYLPROPANOIC ACID*

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Key Word Index—*Erythroxylum coca*; Erythroxylaceae; biosynthesis; cocaine; benzoic acid; *trans*-cinnamic acid; (*R*)- and (*S*)-3-hydroxy-3-phenylpropanoic acid; β-oxidation.

Abstract—*trans*-[3-^{13}C,^{14}C]Cinnamic acid and the *N*-acetylcysteamine thioester of [3-^{13}C,^{14}C]-*trans*-cinnamic acid served as precursors of the benzoyl moiety of cocaine when fed to intact *Erythroxylum* coca plants. The specific incorporation of the thioester into the benzoyl carbonyl group of cocaine was established by means of ^{13}C NMR spectroscopy. (*R*)-(+)-[3-^{14}C]-3-hydroxy-3-phenylpropanoic acid was 11 times more effective than its (*S*)-(−)-isomer as a precursor of the benzoyl moiety of cocaine. A chemical degradation of the cocaine indicated that all the ^{14}C was located on its benzoyl moiety. Thus, the stereochemistry of the hydroxy group in 3-hydroxy-3-phenylpropanoic acid, is the same as that in the coenzyme A esters of 3-hydroxy fatty acids which are intermediates in the β-oxidation of fatty acids.

INTRODUCTION

It has been previously established that phenylalanine is a precursor of the benzoyl moiety of cocaine [1, 2]. It was later discovered that the *N*-acetylcysteamine thioester of benzoic acid was much more efficient than sodium benzoate as a precursor of this moiety of cocaine [3].

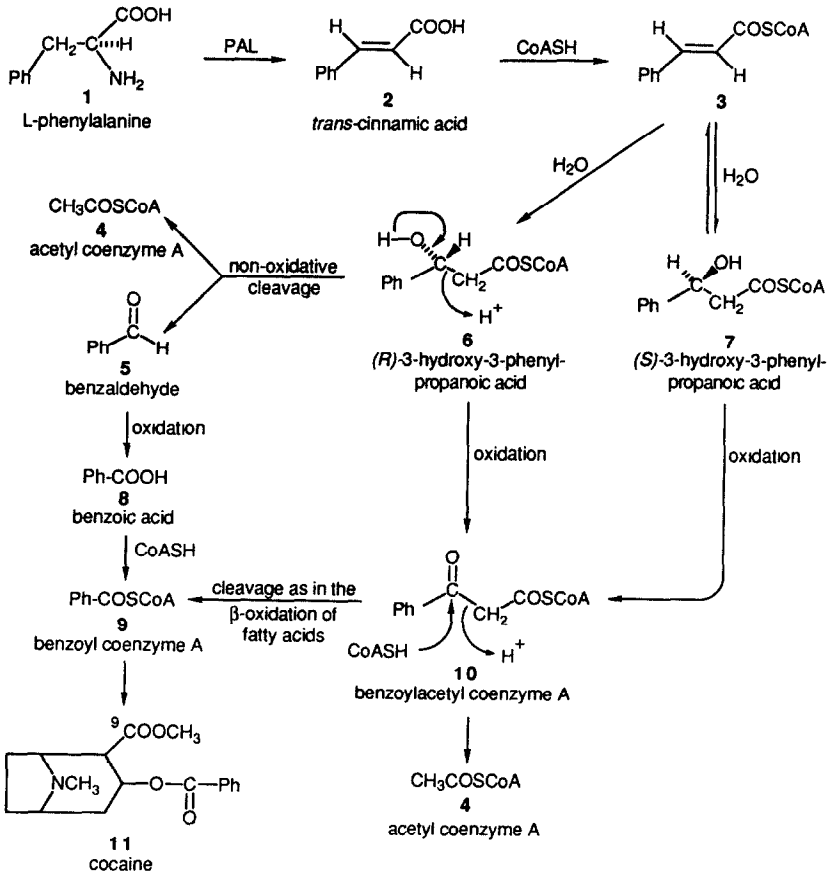
We considered that the most plausible metabolic pathway from phenylalanine to benzoic acid is the one proposed by Zenk and reviewed by him [4]. This pathway is illustrated, with some modifications, in Scheme 1. The first step is the well known conversion of L-phenylalanine (**1**) to *trans*-cinnamic acid (**2**) which is catalysed by the enzyme phenylalanine ammonia lyase which has been isolated from many higher plants. This acid is then converted to its coenzyme A thioester (**3**) which is then hydrated to yield (*R*)- and/or (*S*)-3-hydroxy-3-phenyl-propanoic acid (**6** and **7**, respectively). The intermediacy of **6** or **7** in this pathway is addressed in the present study. Two different routes to benzoyl coenzyme A are then possible. The first is a non-oxidative cleavage of 3-hydroxy-3-phenylpropanoic acid to yield benzaldehyde (**5**) and acetyl coenzyme A (**4**). Benzoic acid (**8**) is then formed by subsequent oxidation of benzaldehyde. This pathway apparently operates in the conversion of *p*-hydroxycinnamic acid (*p*-coumaric acid) to *p*-hydroxybenzoic acid in a cell-free extract of a *Lithospermum* species [5]. The more generally accepted route is by oxidation of 3-hydroxy-3-phenylpropanoic acid thioester to the benzoylacetyl coenzyme A (**10**) which is then cleaved to benzoyl coenzyme A (**9**) and acetyl coenzyme A (**4**). This latter sequence is analogous to the β-oxidation of fatty acids.

*Contribution No. 225 from this laboratory, Part 49 in the series 'Biosynthesis of the Tropane Alkaloids and Related Compounds'. For part 48 see Leete, E., Bjorklund, J. A., Couladis, M. M. and Kim, S. H. (1991) *J. Am. Chem. Soc.* **113**, 9286.

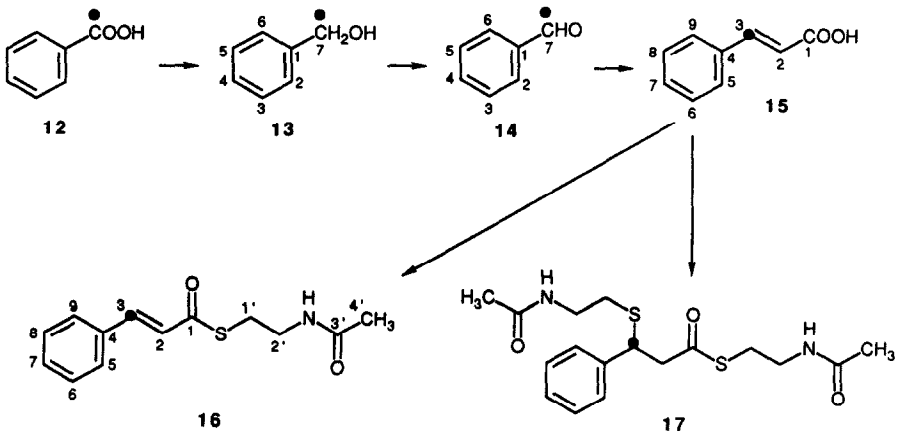
RESULTS AND DISCUSSION

Since the *N*-acetylcysteamine thioester of benzoic acid was a much more efficient precursor of cocaine than sodium benzoate [3] it was decided to feed the corresponding thioester of cinnamic acid to determine whether it was a precursor of benzoic acid in the coca plant. The synthesis is outlined in Scheme 2. [7-^{13}C]Benzoic acid (**12**) [3, 6] was reduced with lithium aluminum hydride to yield [7-^{13}C]benzyl alcohol (**13**). This alcohol was oxidized with pyridinium dichromate to [7-^{13}C]benzaldehyde (**14**). A Knovenagel condensation with malonic acid in the presence of piperidine followed by heating with pyridine yielded *trans*-[3-^{13}C]cinnamic acid (**15**). Attempts to make the thioester **16** by the usual procedure [7] which involves the use of six equivalents of *N*-acetylcysteamine resulted in the formation of compound **17** in which the *N*-acetylcysteamine has added to the α,β-unsaturated carbonyl moiety of cinnamic acid. By reducing the amount of *N*-acetylcysteamine to 1.1 equivalents and using high dilution of the reactants a satisfactory yield (60%) of the desired thioester (**16**) was obtained. In this terminal step in the synthesis a small amount of commercial [3-^{14}C]cinnamic acid was added to the [3-^{13}C]cinnamic acid so that the incorporation of the labelled thioester into cocaine could be monitored by radioactive assay as well as by ^{13}C NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra of **16** and its synthetic precursors exhibited the expected enhancements of signals and spin–spin couplings observable due to the presence of excess ^{13}C in their molecules (see Experimental).

(*RS*)-[3-^{14}C]-3-Hydroxy-3-phenylpropanoic acid was synthesized from *trans*-cinnamic acid and resolved into the (*R*)-(+)- and (*S*)-(−)-isomers as previously described [8]. It has been established that the (−)-isomer has the (*S*)-configuration. Attempts to make the *N*-acetylcysteamine thioester of 3-hydroxy-3-phenylpropanoic acid



Scheme 1. Hypotheses for formation of the benzoyl moiety of cocaine from L-phenylalanine.



Scheme 2. Synthesis of labelled cinnamic acid and its *N*-acetylcysteamine thioester.

were unsuccessful. All the reaction conditions resulted in an elimination reaction yielding cinnamic acid. Initial protection of the hydroxyl group, followed by thioester formation, with subsequent deprotection of the hydroxyl group was also unsuccessful. 3-Hydroxy-3-phenyl-propanoic acid was therefore fed as a solution in water with Tween 80.

As in all our previous successful feeding experiments concerned with the biosynthesis of cocaine, the potential precursors were dissolved in water or aqueous ethanol, along with Tween 80, and painted onto the leaves of *Erythroxylum coca*. Details of the feeding experiments are summarized in Table 1. One problem encountered with the *N*-acetylcysteamine thioester of cinnamic acid was its

low solubility in aqueous ethanol. In experiment 1 a 36% ethanol solution was used to maintain solution of the thioester. This high alcohol content was deleterious to the plant and many leaves fell off. The specific incorporation of the thioester into cocaine (0.15%) was not high enough to detect its incorporation into the benzoyl carbonyl group of cocaine by ^{13}C NMR spectroscopy. In experiment 2, a more dilute solution of the thioester in 10% ethanol was used along with Tween 80 and the feeding was extended over nine days. At this alcohol concentration no ill effects were observed on leaves of the plant. In this experiment the incorporation of ^{13}C into the benzoyl carbonyl group of cocaine (**11**) resulted in enhancement of this carbonyl signal (at δ166.1) relative to an internal standard (the C-9 carbonyl at δ170.7). The specific incorporation deduced from the ^{13}C NMR spectrum (1.2 %) was in good agreement with the specific incorporation determined by radioactive assay (1.34%).

A preliminary feeding with (*RS*)-[3-^{14}C]-3-hydroxy-3-phenylpropanoic acid (experiment 3) indicated that it was incorporated into cocaine. The relatively high incorporation of the (*RS*) compound compared with the subsequent incorporations of the individual (*R*) and (*S*) enantiomers (experiments 4 and 5) was due to the time of year when the feedings were performed. Experiments 4 and 5 were carried out at the same time, ensuring that the plants, growing in a greenhouse were subjected to the same environmental conditions. Two plants were used in each feeding experiment. There was a dramatic difference in the specific incorporation of the (*R*)- and the (*S*)-isomers into the benzoyl moiety of cocaine; the (*R*)-isomer being 11 times more efficiently incorporated than the (*S*)-isomer. A chemical degradation was carried out on the cocaine derived from (*R*)-(+)-[3-^{14}C]-3-hydroxy-3-phenylpropanoic acid. Hydrolysis of cocaine hydrochloride (1.07×10^6 dpm mmol^{-1}) in concentrated HCl yielded ecgonidine hydrochloride (inactive) and benzoic acid (9.9×10^5 dpm mmol^{-1}). The benzoic acid was subjected to a Schmidt reaction with sodium azide and concentrated sulphuric acid affording aniline (assayed as benzanilide, inactive) and carbon dioxide (assayed as $BaCO_3$, 9.8×10^5 dpm mmol^{-1}).

The low incorporation of (*S*)-3-hydroxy-3-phenylpropanoic acid could be due to incomplete resolution of (*RS*)-3-hydroxy-3-phenylpropanoic acid. Alternatively the (*S*)-isomer could undergo some dehydration back to cinnamic acid which is then converted to (*R*)-3-hydroxy-3-phenylpropanoic acid.

It is of interest to note that the relative stereochemistry of the hydroxyl group in (*R*)-3-hydroxy-3-phenylpropanoic acid is the same as that of the hydroxyl group in 3-hydroxy fatty acid coenzyme A esters (**18**) which are involved in the β-oxidation of fatty acids [10, 11] (Scheme 3). In this scheme the hydroxyl group has the (*S*)-configuration, but this is due to the different priorities assigned to the groups around the chiral carbon.

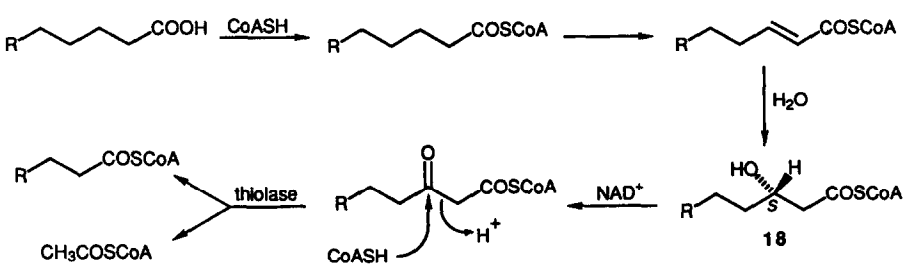
In summary, we have established that the benzoyl moiety of cocaine is formed from cinnamic acid via (*R*)-3-hydroxy-3-phenylpropanoic acid. The problem of whether the benzoic acid is formed via benzoylacetyl coenzyme A or benzaldehyde will probably not be solved until this pathway is examined in cell-free systems at the enzyme level.

EXPERIMENTAL

General. Mps: corr. Radioactive materials were assayed by liquid scintillation counting using dioxane–EtOH as solvent with the usual scintillators [12]. NMR spectra were determined at 300 and 75.5 MHz for ^1H and ^{13}C, respectively. All recorded spectra are ppm from TMS. MS were determined by Dr E. Larka at the University of Minnesota. GC was carried out on a 25 m glass capillary coated with a cross-linked methyl silicone (0.52 μm thick), i.d. 0.31 mm, using the following instrument parameters: He flow rate 1 ml min^{-1}, inj. temp. 250°, init. oven temp. 50°, equilibration time 4 min, rate of temp. increase 30° min^{-1}. Elemental analyses were carried out by M.H.W. Laboratories, Phoenix, Arizona.

[7-^{13}C]*Benzyl alcohol* (**13**). [*carboxyl*-^{13}C]Benzoic acid, >99% ^{13}C at the carboxyl group, [6], (1.035 g, 8.41 mmol), was dissolved in dry THF (15 ml) and slowly added to a stirred soln of $LiAlH_4$ (1.2 g, 32.mmol) in dry THF (80 ml) in a N_2 atm. After the addition, the mixt. was refluxed for 4 days. Wet THF was then added to the cooled reaction mixt. to decompose excess $LiAlH_4$. A few drops of 2 M HCl were then added. The reaction mixt. was then filtered through Celite, dried (Na_2SO_4), filtered and evapd to yield [7-^{13}C]benzyl alcohol (881 mg, 8.16 mmol, 97%). ^{13}C NMR ($CDCl_3$): δ140.7 (*d*, $^1J_{1-7}$ = 46.4 Hz, C-1), 128.4 (*d*, $^2J_{2,6-7}$ = 3.6 Hz, C-2, C-6), 127.4 (*s*, C-4), 126.8 (*d*, $^3J_{3,5-7}$ = 3.6 Hz, C-3, C-5), 64.9 (C-7, enhanced signal). ^1H NMR ($CDCl_3$): δ7.30 (*m*, 5H, aromatic), 4.48 (*d*, $^1J_{H\,^{13}C}$ = 142.5 Hz, H_2-7), 2.8 (*br s*, 1H, OH). GC R_t 8.09 min. EIMS *m/z* (rel. int.) 109([M]$^+$, 12), 77([C_6H_5]$^+$, 100).

trans-[3-^{13}C]*Cinnamic acid* (**15**). [7-^{13}C]Benzyl alcohol (881 mg, 8.16 mmol) dissolved in CH_2Cl_2 (10 ml) was added to a stirred soln of pyridinium dichromate (7.03 g, 18.7 mmol) in CH_2Cl_2 (20 ml) in a N_2 atm. After stirring for 16 hr, the reaction mixt. was dil. with Et_2O (75 ml) and filtered through a small amount of silica gel. The filtrate was evapd and residual benzaldehyde dissolved in a small amount of pyridine and added to a soln of malonic acid (2.23 g, 21.4 mmol) in dry pyridine (15 ml) along with piperidine (0.3 ml). The reaction mixt. was



Scheme 3. β-Oxidation of fatty acids.

Table 1. Precursors fed to *Erythroxylum coca* and their incorporation into cocaine

Expt No.	Precursor wt, mmol, total activity (^{14}C)	Feeding details	Fresh weight of leaves	Cocaine hydrochloride isolated		
				Weight (mg)	Activity (^{14}C) (dpm mmol^{-1})	Specific inc.*
1	[3-^{13}C, ^{14}C]Cinnamic acid, *N*-acetylcysteamine thioester, 70 mg, 0.28 mmol, 1.25×10^7 dpm	Dissolved in 36% aqueous EtOH + 3% Tween 80. Leaves painted with 3% Tween in H_2O for a total of 21 days.	83 g + 14 g of air-dried fallen leaves	136	6.5×10^4	0.15%
2	As in expt 1, 74 mg, 0.295 mmol, 1.32×10^7 dpm	Thioester dissolved in 10% aq. EtOH (45 ml) + 3% Tween 80 and the feeding spread over 9 days for a total time of 21 days.	106 g	100	5.97×10^5	1.34% [1.2%]†
3	(*RS*)-[3-^{14}C]-3-Hydroxy-3-phenylpropanoic acid, 79 mg, 0.48 mmol, 1.67×10^6 dpm	Dissolved in H_2O + 3% Tween 80. Fed over 6 days for a total time of 21 days.	164 g	178	5.4×10^4	1.56%
4	(*R*)-(+)-[3-^{14}C]-3-Hydroxy-3-phenylpropanoic acid (**6**), 12 mg, 0.072 mmol, 2.32×10^7 dpm	Dissolved in H_2O + 3% Tween 80. Leaves painted over 2 days for a total time of 14 days.	62 g	120	1.07×10^6	0.31%
5	(*S*)-(−)-[3-^{14}C]-3-Hydroxy-3- phenylpropanoic acid (**7**), 12 mg, 0.072 mmol, 2.32×10^7 dpm	Dissolved in H_2O + 3% Tween 80. Leaves painted over 2 days for a total time of 14 days.	71 g	94	0.089×10^6	0.028%

*Specific incorporation: specific activity of cocaine hydrochloride (dpm mmol^{-1}) divided by specific activity of administered precursor.
†Deduced from enhancement of signal (at 166.1 ppm from TMS) emanating from benzoyl carbonyl group.

refluxed for 4 hr and then cooled to 0° and added to cold 6 M HCl (80 ml). The ppt. was extracted with CH_2Cl_2 (2 × 20 ml) which was then washed with brine and dried (Na_2SO_4). The residue obtained on evapn of CH_2Cl_2 was sublimed (80–90°, 0.1 mm Hg) to yield *trans*-[3-^{13}C]cinnamic acid (963 mg, 6.46 mmol, 79%), mp 132–133°. ^{13}C NMR ($CDCl_3$): δ172.6 (*d*, $^2J_{1,3}$ = 2.5 Hz, C-1), 147.2 (greatly enhanced signal plus a quartet of two satellites: $^1J_{3-2}$ = 70.7 Hz, $^1J_{3-4}$ = 55.3 Hz, C-3), 134.1 (*d*, $^1J_{4-3}$ = 55.2 Hz, C-4), 130.8 (*s*, C-7), 129.0 (*d*, $^3J_{6,8-3}$ = 4.5 Hz, C-6, C-8), 128.4 (*d*, $^2J_{5,9-3}$ = 2.0 Hz, C-5, C-9), 117.3 (*d*, $^1J_{2-3}$ = 70.8 Hz, C-2). ^{1}H NMR ($CDCl_3$): δ7.80 [*dd*, *J* = 16.0 Hz (coupling with *trans*-H at C-2), $^1J_{H-^{13}C}$ = 159.3 Hz, H-3, 7.55 (*m*, 2H, aromatic), 7.41 (*m*, 3H, aromatic), 6.47 [*d*, *J* = 16.0 Hz (coupling with *trans*-H at C-3, H-2)]. GC R_t 10.86 min. EIMS *m/z* (rel. int.) 149([M]$^+$, 84), 132 ([$C_6H_5{}^{13}CH{=}CH{-}CO$]$^+$, 34), 104 ([$C_6H_5{}^{13}CH{=}CH$]$^+$, 57), 77 ([$C_6H_5$]$^+$, 71).

This *trans*-[3-^{13}C]cinnamic acid (500 mg) was dil. with *trans*-[3-^{14}C]cinnamic acid (0.18 mg, nominal activity 54 μCi, Research Products International Corp.) by soln in C_6H_6 and then evapn to afford *trans*-[3-^{13}C, ^{14}C]cinnamic acid with a sp. act. of 4.47×10^7 dpm mmol^{-1} (26% higher than the activity expected from information on the label of the undil. [3-^{14}C]cinnamic acid).

trans-[3-^{13}C,^{14}C]*Cinnamic acid, N-acetylcysteamine thioester* (**16**). Triethylamine (0.95 ml) and diphenylphosphoryl azide (1.5 ml, 9 mmol) were added to a stirred soln of *trans*-[3-^{13}C, ^{14}C]cinnamic acid (500 mg, 3.36 mmol, 4.47×10^7 dpm mmol^{-1}) in CH_2Cl_2 (20 ml) at 25°. After 1 hr, *N*-acetylcysteamine [7] (481 mg, 3.67 mmol) dissolved in CH_2Cl_2 (2 ml) was added and the mixt. stirred at 25° for 16 hr. The solvent was then removed under red. pres. and the residual crude thioester purified by medium pressure LC on a 2.5 × 30 cm column of silica gel with a flow rate of 8.5 ml min^{-1}. The solvent gradient varied from EtOAc (100%), frs 1–11; with 3% EtOH, frs 11–25; with 50% EtOH, frs 26–40; with 75% EtOH, frs 41–50. Frs were collected every min. The thioester was detected in frs 10–20 (by TLC). Evapn of these frs yielded *trans*-[3-^{13}C, ^{14}C]cinnamic acid, *N*-acetylcysteamine thioester (503 mg, 2.01 mmol, 60%). The thioester was dissolved in EtOAc containing a small amount of CH_2Cl_2. Hexane was then added until the soln became cloudy and on standing needles of the thioester (495 mg) sepd, mp 100–102°, sp. act. 4.46×10^7 dpm mmol^{-1}. TLC on silica gel, R_f 0.32 with 3% EtOH in EtOAc as developing solvent. ^{13}C NMR ($CDCl_3$): δ190.03 (*s*, C-1), 170.4 (*s*, C-3'), 141.11 (highly enhanced signal, C-3, $^1J_{3-2}$ = 70.8 Hz, $^1J_{3-4}$ = 56.2 Hz), 130.8 (*s*, C-7), 128.9 (*d*, $^3J_{6,8-3}$ = 4.9 Hz, C-6, C-8), 128.4 (*d*, $^2J_{5,9-3}$ = 2.4 Hz, C-5, C-9), 124.4 (*d*, $^1J_{2-3}$ = 70.8 Hz, C-2), 39.7 (*s*, C-2'), 28.5 (*s*, C-1'), 23.1 (*s*, C-4'). ^{1}H NMR ($CDCl_3$): 7.62 (*dd*, 1H at C-3, *J* = 15.8 Hz (coupling with the *trans*-H at C-2), $^1J_{H-^{13}C}$ = 150.1 Hz), 7.53 (*m*, 2H, H-5 and H-9), 7.37 (*m*, 3H, other aromatic H), 6.72 (*d*, *J* = 15.8 Hz, H-2), 3.49 (*q*, *J* = 6.2 Hz, Hz-2'), 3.18 (*t*, *J* = 6.6 Hz, H_2-1'), 2.0 (*s*, H_3-4'). EIMS (30 eV), *m/z* (rel. int.) 250 ([M]$^+$, 0.59), 132 ([$C_6H_5{}^{13}CH{=}CH{-}CO$]$^+$, 100), 104 ([$C_6H_5{}^{13}CH{=}CH$]$^+$, 28), 77 ([$C_6H_5$]$^+$, 10). Elemental analysis (on unlabelled sample): Calc. for $C_{14}H_{15}NO_2S$: C, 62.63; H, 6.06; N, 5.62; S, 12.86%. Found: C, 62.61; H, 6.17; N, 5.85; S, 12.73%.

(RS)-[3-^{14}C]-3-*Hydroxy-3-phenylpropanoic acid.* This was synthesized from [3-^{14}C]cinnamic acid and resolved into its (*R*)-(+) and (*S*)-(−) enantiomers from the morphine salts as previously described [8].

Feeding of precursors and isolation of cocaine. Feeding expts were carried out on intact *E. coca* Lamark plants growing in a greenhouse. Details of the amounts fed are recorded in Table 1. Cocaine was isolated and purified as previously described [12].

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REFERENCES

1. Gross, E. and Schütte, H. R. (1963) *Arch. Pharm. Weinheim Ger.* **296**, 1.
2. Leete, E. (1983) *Phytochemistry* **22**, 699.
3. Leete, E., Bjorklund, J. A. and Kim, S. H. (1988) *Phytochemistry* **27**, 2553.
4. Zenk, M. H. (1979) *Rec. Adv. Phytochem.* **12**, 139.
5. Yozaki, K., Heide, L. and Tabata, M. (1991) *Phytochemistry* **30**, 2233.
6. Kepler, J. A., Lytle, J. W. and Taylor, C. F. (1974) *J. Labelled Compounds* **10**, 683.
7. Schwab, J. M. and Klassen, J. B. (1984) *J. Am. Chem. Soc.* **106**, 7211.
8. Leete, E. (1983) *Phytochemistry* **22**, 933
9. Schöpf, C. and Wüst, W. (1959) *Justus Liebigs Ann. Chem.* **626**, 1950.
10. Kawaguchi, A. and Seyama, Y. (1987) in *Pyridine Nucleotide Coenzymes* (Dolphin, Polson, and Avramovic, eds), pp. 367–403. John Wiley.
11. Friedman, A. R. and Leete, E. (1963) *J. Am. Chem. Soc.* **85**, 2141.
12. Leete, E. (1982) *J. Am. Chem. Soc.* **104**, 1404.