

## THE BIOSYNTHESIS OF THE BENZOYL MOIETY OF COCAINE\*†

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**Key Word Index**—*Erythroxylum coca*; Erythroxylaceae; cocaine; benzoic acid; hentriacontane; biosynthesis; Tween 80.

**Abstract**—[carboxyl- $^{13}\text{C}$ ,  $^{14}\text{C}$ ] Benzoic acid, [carbonyl- $^{13}\text{C}$ ,  $^{14}\text{C}$ ]- and [4- $^3\text{H}$ ]-benzoic acid, *S*-(2-acetamidoethyl)ester served as precursors of the benzoyl moiety of cocaine when fed, by leaf painting, to *Erythroxylum coca* plants. The high (10.5%) specific incorporation of the  $^{13}\text{C}$ -labelled thioester was established by  $^{13}\text{C}$ NMR. Aqueous Tween 80 was found to have a significant effect on the degree of incorporation of the benzoic acid, presumably facilitating the transport of the labelled compound through the leaf wax. A major component of the leaf wax is hentriacontane.

### INTRODUCTION

The benzoyl moiety of cocaine (3) is derived from phenylalanine [1, 2] in *Erythroxylum coca*. We have shown [3] that 3-benzoyloxy-2-carbomethoxy-2-tropene (6) (2,3-dehydrococaine) is not a direct precursor of cocaine. We presently consider that the final step in cocaine biosynthesis involves benzylation of methylecgonine (1), as illustrated in Fig. 1. Since the formation of oxyesters in nature usually involves the intermediate formation of thioesters with coenzyme A, we propose that the final enzyme controlled reaction for cocaine biosynthesis is between methylecgonine and benzoylcoenzyme A (2). We plan to examine this final step at the enzyme level, and as a preliminary to this work we now report feeding experiments with benzoic acid (4) and its *N*-acetylcysteamine thioester (5) with the intact *E. coca* plant. These compounds were labelled with  $^{13}\text{C}$  and  $^{14}\text{C}$  on the benzoyl carbonyl group, the former isotope being introduced so that potential incorporations could be monitored by  $^{13}\text{C}$ NMR spectroscopy. [carboxyl- $^{13}\text{C}$ ,  $^{14}\text{C}$ ] Benzoic acid [4] was converted to its thioester (5) by established methods [5]. [4- $^3\text{H}$ ] Benzoic acid was prepared by reduction of 4-iodobenzoic acid with Raney nickel alloy in tritrated water [6], and converted to the thioester in the same way.

### RESULTS AND DISCUSSION

The *N*-acetylcysteamine thioester of benzoic acid proved to be an excellent precursor of the benzoyl moiety of cocaine (Table 1), significantly better than benzoic acid which was fed as its ammonium salt in neutral water. The high (10.5%) specific incorporation of  $^{14}\text{C}$ , observed in experiment 3, was also apparent on observation of the  $^{13}\text{C}$ NMR spectrum of this enriched cocaine (Fig. 2). The assignment of signals in the  $^{13}\text{C}$ NMR of cocaine has been the subject of controversy. In one study [7] the C=O groups were misassigned. Our assignments of the  $^{13}\text{C}$ NMR spectrum of cocaine, based on 2D COSY and 2D HETCOR NMR, are in agreement with those of Carroll *et al.* [8]. The specific incorporation, calculated from the enhancement of the C-9 benzoyl signal was 8.9% in good agreement with the incorporation obtained by radioactive assay. It is very uncommon to obtain such high specific incorporations in biosynthetic studies involving intact higher plants. We were concerned that this result may have been an artifact, resulting from a non-enzymatic *in vitro* reaction of the *N*-acetylcysteamine thioester of benzoic acid with methylecgonine which is a fairly abundant alkaloid in fresh *coca* leaves. Accordingly the thioester of [4- $^3\text{H}$ ]benzoic acid, was mixed with methylecgonine and cocaine, and subjected to the same work-up procedure as that used to isolate cocaine from *coca* leaves. The re-isolated cocaine contained an insignificant amount of radioactivity compared with the conversions obtained in the feeding experiments in the intact plants. High specific incorporations were also observed in the feedings involving the [ $^3\text{H}$ ]-labelled thioester (Expts 4, 5). Others [9, 10] have also observed the efficiency of *N*-acetylcysteamine thioesters of carboxylic acids in studies on the biosynthesis of microbial polyketides.

A result of considerable significance was the effect of Tween 80 on the incorporation of ammonium benzoate into cocaine. In experiment 1 the *coca* leaves were painted with the aqueous solution of ammonium benzoate (pH 7) containing 5% Tween 80 for one day only. The resultant incorporation of activity into cocaine was very

\* Part 40 in the series 'Chemistry of the Tropane Alkaloids and Related Compounds'. This paper is dedicated to the memory of Tony Swain who I first met in 1954 when I was a young instructor in the Department of Chemistry at the University of California, Los Angeles, and he was a postdoctoral fellow in Ted Geissman's research group. We accompanied Ted on several field trips in the Southern California desert, collecting Compositae. Tony also introduced me to the rotary evaporator. He had a home-made model, which had been invented in the Low Temperature Research Laboratory, Cambridge, U.K. We were amazed that it worked. Tony had an infectious love of secondary natural products and, like many of us, was eager to find out why nature produces such a diverse collection of organic structures.

† Contribution No. 207 from this laboratory.

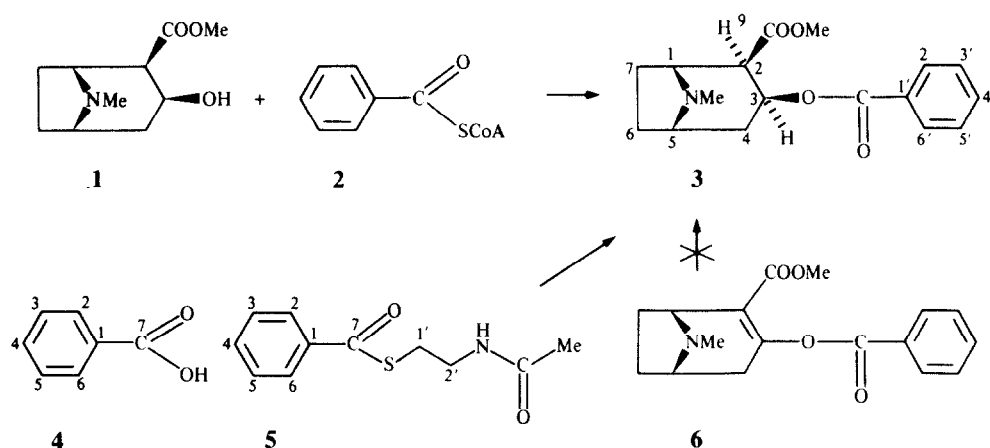


Fig. 1.

Table 1. Precursors fed to *Erythroxylum coca* and activity of cocaine

Exp. no.	Precursor (mmol, total activity, dpm)	Duration of feeding (days)	Specific incorporation* (%) of $^{14}\text{C}$ or $^3\text{H}$ into cocaine
1	[carboxyl- $^{13}\text{C}$ , $^{14}\text{C}$ ] Benzoic acid, $\text{NH}_4^+$ salt (0.34, $3.31 \times 10^8$ )	24	0.07
2	[carboxyl- $^{13}\text{C}$ , $^{14}\text{C}$ ] Benzoic acid, $\text{NH}_4^+$ salt (0.45, $1.61 \times 10^8$ )	14	2.1
3	[carbonyl- $^{13}\text{C}$ , $^{14}\text{C}$ ] Benzoic acid, S-(2-acetamidoethyl)ester (0.23, $2.13 \times 10^8$ )	14	10.6
4	[4- $^3\text{H}$ ] Benzoic acid, S-(2-acetamidoethyl)ester† (0.27, $2.81 \times 10^6$ )	10	4.1
5	[4- $^3\text{H}$ ] Benzoic acid, S-(2-acetamidoethyl)ester‡ (0.38, $3.96 \times 10^6$ )	14	8.5

\* Specific inc. = dpm/mmol in cocaine/dpm/mmol in precursor.

† Administered along with DL-[5- $^{14}\text{C}$ ]ornithine (specific inc. of  $^{14}\text{C}$  into methyl egonine moiety: 0.035%).

‡ Administered along with [1- $^{13}\text{C}$ ,  $^{14}\text{C}$ ,  $^{15}\text{N}$ ]-4-methylaminobutanal diethyl acetal (specific inc. of  $^{14}\text{C}$  into methylegonine moiety: 0.32%).

low (0.07%). In experiment 2, the precursor was fed in the same way, but on the subsequent 13 days of the experiment, the leaves were painted with a 5% solution of Tween 80 in water, resulting in a much higher (2.1%) incorporation of  $^{14}\text{C}$  into cocaine. The Tween 80 is apparently facilitating the transfer of organic compounds from the surface of the leaf into the internal cells of the *coca* leaf where the biosynthesis of cocaine takes place. The surface of leaves contain plant wax and we have found that one of the major components of the lipid fraction isolated from *coca* leaves is the normal alkane, hentriacontane ( $\text{C}_{31}\text{H}_{64}$ ). Its structure was established by mass spectrometry and comparison with an authentic specimen. This hydrocarbon is a common component of plant wax [11]. It is presumably formed by decarboxylation of the corresponding  $\text{C}_{32}$  fatty acid. In our case the hentriacontane was isolated from *coca* leaves which had

been fed sodium [1- $^{14}\text{C}$ ] acetate, and the activity (0.15% absolute incorporation) found in the alkane is consistent with this biosynthetic origin. The same feeding technique was followed in Expts 3–5. Since the thioester is only sparingly soluble in water, it was dissolved in ethanol, and then diluted with water containing 5% Tween 80 to afford a solution containing 10% ethanol. On subsequent days after the initial feeding the leaves were painted with the same aqueous solution containing 3–5% Tween 80 and 10% ethanol.

#### EXPERIMENTAL

*General methods.* Radioactive materials were assayed by liquid scintillation counting using dioxane-EtOH as the solvent with the usual scintillators [12]. NMR spectra were determined at 300 and 75.5 MHz for  $^1\text{H}$  and  $^{13}\text{C}$  respectively, with the

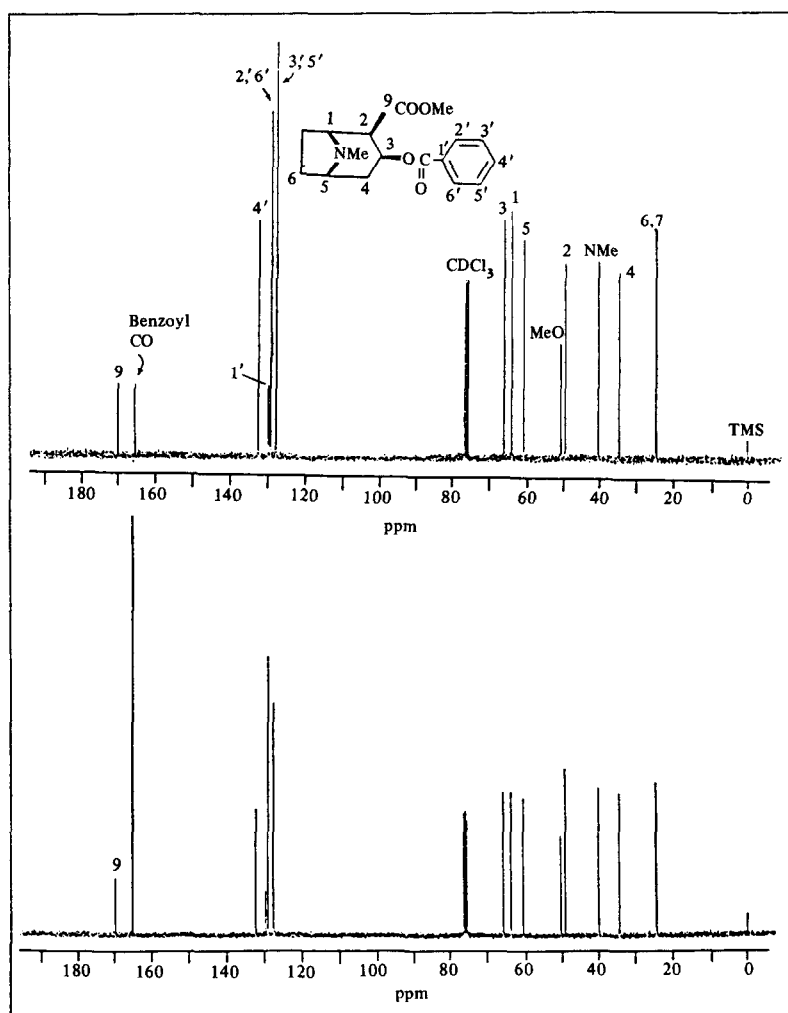


Fig. 2.  $^{13}\text{C}$  NMR spectrum of cocaine in  $\text{CDCl}_3$ . Upper spectrum is natural cocaine. Lower spectrum is cocaine derived from  $[\text{carbonyl-}^{13}\text{C}, ^{14}\text{C}]$ benzoic acid, *S*-(2-acetamidoethyl)ester.

assistance of Dr S.B. Philson. All recorded spectra are ppm from TMS. Mass spectra were determined by Dr E. Larka and his assistants at the University of Minnesota. Gas chromatography was carried out in a Hewlett-Packard model 5890A gas chromatogram on a 25 m glass capillary column coated with cross-linked methyl silicone (0.52  $\mu\text{m}$  thick) internal diameter 0.31 mm, using the following instrument parameters: He flow rate 1 ml/min, injection temp. 250°, initial oven temp. 50°, equilibration time 4 min, rate of temp. increase 30°/min, final temp. 250°. Retention times (min) are reported: GC  $R_t$ . Elemental analyses were carried out by M.H.W. Laboratories, Phoenix, Arizona.

$[\text{carbonyl-}^{13}\text{C}, ^{14}\text{C}]$ Benzoic acid, *S*-(2-acetamidoethyl)ester (5).  $[\text{carboxyl-}^{13}\text{C}]$ Benzoic acid (>99%  $^{13}\text{C}$ ) was prepared by reaction of phenyl magnesium bromide with  $^{13}\text{CO}_2$  generated from  $\text{Ba}[^{13}\text{C}]\text{CO}_3$  with conc.  $\text{H}_2\text{SO}_4$  [4]. This  $[\text{carbonyl-}^{13}\text{C}]$ benzoic acid had the following  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 172.70 (C-7, enhanced signal with small satellites symmetrically arranged,  $^1J_{1-7}$  72.5 Hz), 133.70 (C-4), 130.27 (d, C-2, 6,  $^2J_{2,6-7}$  2.3 Hz), 129.37 (d, C-1,  $^1J_{1-7}$  72.2 Hz), 128.53 (d, C-3, 5,  $^3J_{3,5-7}$  4.7 Hz). This  $[\text{carboxyl-}^{13}\text{C}, ^{14}\text{C}]$ benzoic acid (72.5 mg) was mixed with

$[\text{carboxyl-}^{14}\text{C}]$ benzoic acid (0.5 mg) affording material with a specific activity of  $9.86 \times 10^8$  dpm/mmol. A solution of this  $[\text{carboxyl-}^{13}\text{C}, ^{14}\text{C}]$ benzoic acid (73 mg, 0.593 mmol) in  $\text{CH}_2\text{Cl}_2$  (10 ml) was stirred at 25° with diphenylphosphoryl azide (0.2 ml, 1.2 mmol) and trimethylamine (0.33 ml) for 0.5 h. *N*-Acetylcysteine [5] (4.39 g, 3.69 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 ml) was then added and the mixture stirred at 25° for 4 hr.  $\text{Et}_2\text{O}$  (60 ml) was then added and the solution washed with 5% citric acid and satd aq.  $\text{Na}_2\text{CO}_3$ . Evapn of the dried ( $\text{Na}_2\text{SO}_4$ )  $\text{Et}_2\text{O}$  afforded an oil which was dissolved in EtOAc and subjected to chromatography on a column of silica gel (2  $\times$  26 cm). Elution with toluene,  $\text{CHCl}_3$  and finally 5%  $\text{Me}_2\text{CO}$  in  $\text{CHCl}_3$  afforded  $[\text{carbonyl-}^{13}\text{C}, ^{14}\text{C}]$ benzoic acid, *S*-(2-acetamidoethyl)ester which crystallized from EtOAc in colourless needles (109 mg, 83%) mp 84.5–85°. IR(KBr): thioester  $^{13}\text{C}=\text{O}$  1625  $\text{cm}^{-1}$  (1624  $\text{cm}^{-1}$  was expected due to the  $^{13}\text{C}$  isotope effect on the  $^{12}\text{C}=\text{O}$  absorption at 1661  $\text{cm}^{-1}$ ) amide  $\text{C}=\text{O}$  1649  $\text{cm}^{-1}$ ;  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ):  $\delta$ 192.19 (C-7, enhanced signal with satellites,  $^1J_{1-7}$  61.5 Hz), 170.4 (amide  $\text{C}=\text{O}$ ), 136.68 (d, C-1,  $^1J_{1-7}$  61.6 Hz), 133.71 (C-4), 128.72 (d, C-3, 5,  $^3J_{3,5-7}$  4.2 Hz), 127.28 (d, C-2, 6,  $^2J_{2,6-7}$  3.0), 39.65 (C-2'), 28.57 (C-1'), 25.23 (Me). Assignments of C-1' and C-

2' were made on the basis of 2D-HETCOR NMR.  $^1\text{H}$ NMR ( $\text{CDCl}_3$ ) on unlabelled thioester:  $\delta$  1.94 (3H, s, Me), 3.19 (2H, t, C-1'), 3.39 (2H, q, C-2'), 6.27 (1H, br s, NH), 7.41 (2H, t, meta, H), 7.56 (1H, t, para H), 7.91 (2H, dt, ortho-H). In the  $^{13}\text{C}$ thioester the triplet signal at 3.19 changed to a doublet of triplets, and the ortho H's at 7.91 became a complex multiplet. MS  $m/z$  (rel. int.) 224 ( $\text{M}^+$ ) (4.1), 106 ( $\text{C}_6\text{H}_5$   $^{13}\text{C}\equiv\text{O}^+$ ) (100), 77 ( $\text{C}_6\text{H}_5^+$ ) (100), 43 ( $\text{MeC}\equiv\text{O}^+$ ) (68). Analysis of the various peak intensities indicated that this material contained  $\approx 99\%$   $^{13}\text{C}$  at C-7. (Elemental analysis (on unlabelled sample) Found: C: 58.94, H: 6.05, N: 6.46, S: 14.14.  $\text{C}_{11}\text{H}_{13}\text{O}_2\text{NS}$  requires C: 59.17, H: 5.87, N: 6.27, S: 14.36%).

**Feeding of precursors to *Erythroxylum coca* and isolation of cocaine.** The amounts of the benzoic acid and its thioester which were fed are recorded in Table 1. The thioester was dissolved in 1 ml of EtOH, prior to diln with  $\text{H}_2\text{O}$  (9 ml) containing 5% Tween 80. As previously described [13] these solutions were painted upper sides of the *coca* leaves with an artist's paint brush. At the end of the feeding period the leaves were removed and extracted to yield crude cocaine hydrochloride [13]. Capillary GC indicated that this material contained *cis*- and *trans*-cinnamoylcocaines. (GC  $R_f$  for cocaine, *cis*-, and *trans*-cinnamoylcocaines being 18.51, 22.47, and 26.91 min. respectively). Purification to afford cocaine, 99% pure by GC, was accomplished by crystallization of the free base from heptane. In all these feeding experiments the plants were 6–8 years old. The yield of purified cocaine was 30–60 mg from 20–100 g of the fresh *coca* leaves. The GC  $R_f$  times of other alkaloids found in fresh *coca* leaves were: hygrine (9.06), methylecgonine (11.69), cuscohygrine (12.92), and dihydrocuscohygrine (13.12).

**Isolation of hentriacontane from *coca* leaves.** Fresh *coca* leaves (230 g) from plants which had been fed Na [ $1\text{-}^{14}\text{C}$ ]acetate ( $2.0 \times 10^9$  dpm) were chopped up in a Waring blender with  $\text{CHCl}_3$  (1 l) and 10%  $\text{NaCO}_3$  (200 ml). The mixture was filtered and the  $\text{CHCl}_3$  layer separated and evapd to small bulk, diluted with  $\text{Et}_2\text{O}$  (200 ml), which was then extracted with 0.5 N HCl (4  $\times$  100 ml) to remove alkaloids. The dark green  $\text{Et}_2\text{O}$  solution was evapd to dryness and the residue sublimed ( $140^\circ$ ,  $10^{-4}$  mmHg) to afford a white sublimate (840 mg). Crystallization from EtOH afforded colourless plates of hentriacontane (520 mg,  $2.59 \times 10^6$  dpm/mmol  $\pm 0.15\%$  absolute inc.), mp  $67\text{--}68^\circ$ , mass spectrum was typical of a normal alkane with clusters of peaks separated by 14 mass units. High resolution MS: 436.5040,  $\text{C}_{31}\text{H}_{64}$  requires: 436.5017. TLC on silica gel, developing with  $\text{CHCl}_3\text{--C}_6\text{H}_6\text{--HOAc}$  (16:4:1) indicated that  $>99\%$  of its  $^{14}\text{C}$  activity was coincident with a spot ( $R_f$  0.95) where authentic hentriacontane appears.

[4- $^3\text{H}$ ]Benzoic acid. 4-Iodobenzoic acid (2.48 g, 10 mmol) was dissolved in triturated  $\text{H}_2\text{O}$  (40 ml, having an activity of  $7.5 \times 10^8$  dpm/ml  $\equiv 1.35 \times 10^7$  dpm/mmol) containing NaOH (2.0 g). Raney Ni alloy (2.0 g) was added to the solution which was stirred at  $25^\circ$  for 24 hr. Additional Ni/Al alloy (2.0 g) was added and the stirring continued for another 24 hr. The mixture was filtered and the residual Ni washed with hot  $\text{H}_2\text{O}$ . The filtrate was made acid with HCl and extracted with  $\text{Et}_2\text{O}$ . The residue obtained on evapn of the dried ( $\text{Na}_2\text{SO}_4$ ) extract was crystallized from hexane and then from hot  $\text{H}_2\text{O}$  to afford [4- $^3\text{H}$ ]benzoic acid (720 mg, 59%)  $>99\%$  pure by GC ( $R_f$  9.66) with an activity of  $1.04 \times 10^7$  dpm/mmol. This was converted to

its *N*-acetylcysteamine thioester as described for the [ $^{13}\text{C}$ ]benzoyl derivative.

**Investigation of the possible formation of cocaine by reaction of the thioester (5) with methylecgonine in vitro.** Cocaine HCl (0.5 g, 1.65 mmol) was refluxed in MeOH containing HCl for 24 hr. The solution was evapd, the residue made basic with  $\text{Na}_2\text{CO}_3$  and extracted with  $\text{CHCl}_3$  to yield a mixture of cocaine and methylecgonine (9:11). This mixture, of cocaine and methylecgonine, [4- $^3\text{H}$ ]benzoic acid *S*-(2-acetaminoethyl-ester) (53 mg, 0.237 mmol, total act.  $2.46 \times 10^6$  dpm) and Tween 80 (0.5 g) were added to  $\text{CHCl}_3$  (500 ml) and 10% aqueous  $\text{Na}_2\text{CO}_3$  (250 ml) and the mixture shaken. After 23 hr the  $\text{CHCl}_3$  layer was separated, dried ( $\text{Na}_2\text{SO}_4$ ) and evapd to 10 ml. After 6 hr this  $\text{CHCl}_3$  solution was washed with 0.5 N HCl (40 ml). The aqueous solution was washed with  $\text{Et}_2\text{O}$  and then made basic with  $\text{Na}_2\text{CO}_3$  and the alkaloids extracted with  $\text{CHCl}_3$ . Cocaine was isolated by prep TLC on  $\text{SiO}_2$  and crystallized from heptane. It had an activity of 20 dpm/mg, representing a 0.06% conversion of the labelled thioester into cocaine.

**Determination of the  $^{13}\text{C}$  NMR spectrum of cocaine from feeding expt 3.** The  $^{13}\text{C}$  NMR spectra illustrated in Fig. 2 were obtained on 69 mg of cocaine dissolved in 0.5 ml of  $\text{CDCl}_3$  in a 5 mm tube. Parameters for both the enriched and natural abundance spectra were: scans 273, acquisition time 0.8 sec.

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