alcohols to the conjugated 3-alkynylallyl compounds.[26] In this latter reaction, rearrangement of the initially formed 1-alkynylallyl carbonium, probably of stability comparable to that of II, is facilitated by formation of a resonance-stabilized secondary carbonium ion. Although there are no reports of allylic rearrangements of prostaglandin E_2 type compounds, Spraggins has observed that treatment of formate esters of 15-epiprostaglandin A_2 with a mixture of methanol and hydrochloric acid yielded 32% of the 13-hydroxy diastereoisomers of prostaglandin A_2.[27] Crystallographic data are not available on prostaglandin A_2. The crystal structure of prostaglandin A_1, however, has been determined.[28] These data indicate that the five-membered ring is nearly planar in prostaglandin A_1, whereas the ring in prostaglandin E_2 is well described as a C-9 half-chair.[29] Examination of molecular models reveals that the nonplanar ring in the E_2 prostaglandins leads to increased steric crowding at C-13 relative to that in prostaglandin A_2. The crowding is further increased by addition of a methyl group at the C-15 position. These steric considerations are probably the major factors in preventing allylic rearrangement during epimerization of R and S.

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Biosynthetic Conversion of Thebaine to Codeinone. Mechanism of Ketone Formation from Enol Ether in Vivo

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Abstract: Biosynthesis of the morphinan alkaloids proceeds by conversion of the enol ether of thebaine to the keto group of neopinone and thence to codeinone. To determine the mechanism of this transformation, [G-^{14}C,6-^{18}O]thebaine was fed to *Papaver somniferum* and the codeine and morphine were isolated. Comparison of the ^{18}O/^{14}C ratios in the codeine and morphine isolated with that of the thebaine fed showed that ~34% of the ^{18}O had been retained. Parallel feedings with [G-^{14}C,6-^{18}O]codeinone demonstrated that the loss was due to nonenzymic exchange. Thus, the mechanism of enol ether cleavage in thebaine is established as cleavage of the 6-*O*-methyl group with retention of the 6-oxygen in the codeinone.

The biosynthetic pathway for the opium alkaloids in *Papaver somniferum* has been shown[2-4] to proceed by the conversion of thebaine (**1**) to codeinone (**3**), probably via neopinone (**2**) and migration of the double bond into conjugation (see Scheme I). Codeinone (**3**) is then reduced to codeine (**4**) which subsequently is 3-O-demethylated to morphine (**5**). Morphine in turn is N-demethylated to normorphine (**6**), and the metabolic fate of the latter has yet to be determined.[5] Thus, in a formal sense, O-demethylations occur at two steps in the pathway: (a) the conversion of thebaine (**1**) to codeinone (**3**) and (b) the conversion of codeine to morphine.

It is reasonable to hypothesize that these two reactions may be catalyzed by different types of enzymes. Since reaction a involves an enol ether and reaction b an aromatic ether, different mechanisms may be involved in their cleavage, as is the case in vitro. Also, morphine and codeine are peculiar to *P.*

somniferum (except for very small amounts in *P. setigerum*) while thebaine (**1**) occurs in most other species as well, particularly *P. orientale* and *P. bracteatum*.[3] Oripavine (**7**) also occurs in these two species. These data strongly support the hypothesis that all three species contain an *O*-demethylase which is capable of cleaving the aromatic ether linkage, while *P. somniferum* is unique in that it contains an enzyme system which attacks the enolic ether as well. To establish the nature of the conversion of this enolic ether to ketone is the purpose of the present work.

O-Demethylation of aromatic methyl ethers is a frequent metabolic reaction in mammals, microorganisms, and plants, proceeding by a common oxidative mechanism in all biological systems.[6]

Definitive studies,[7] using $^{18}O_2$ and $H_2^{18}O$, showed that oxidative O-demethylation of *p*-methoxyacetanilide by liver

Scheme I
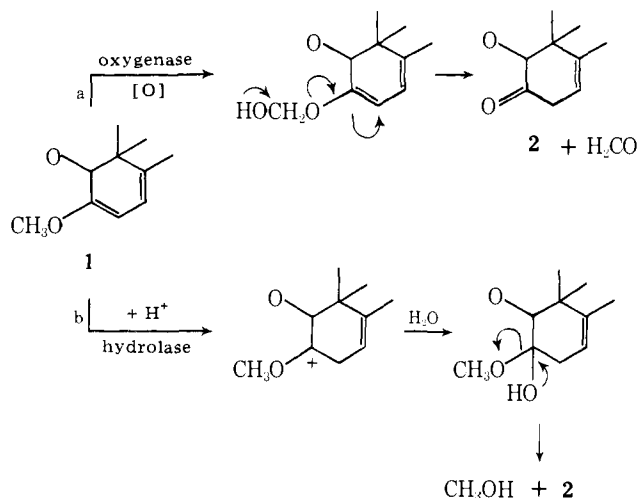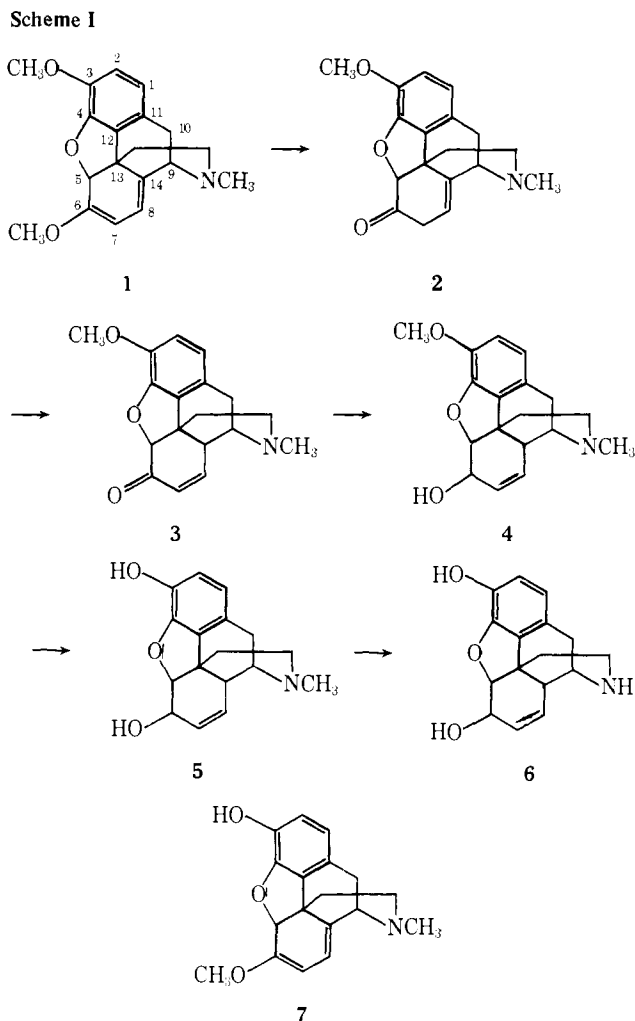
ploratory experiments showed that [6-^{18}O]codeinone in 6 h at physiological pH (7.2) lost about one-third of its isotopic enrichment. This rather facile exchange coupled with the dilution which would result from alkaloids already present in the plant dictated that a double-label procedure was needed. ^{14}C was chosen as the other label since ^{14}C-labeled opium alkaloids are readily obtained by biosynthesis with ^{14}CO$_2$.[4]

The experimental design was to feed [G-^{14}C,6-^{18}O]thebaine to *P. somniferum* and, after a 24-h growth period, to isolate the codeine. From the ^{18}O/^{14}C ratios of precursor and product, loss or retention of ^{18}O would be obvious since the ^{14}C content would remain constant, there being no way for ^{14}C to leave the molecules except through the pendant methyl groups. Provision for these methyl groups is considered below. As a control, parallel feedings would proceed with [G-^{14}C,6-^{18}O]codeinone; this would also establish the extent of ^{18}O loss by chemical exchange. Morphine also would be isolated and its isotopic ratio determined as a check on the codeine.

Preparation of Labeled Substrates

All the labeled alkaloids used in the present work were isolated from *P. somniferum* grown in an atmosphere of ^{14}CO$_2$.[4] The thebaine thus produced by a general labeling biosynthesis contained ^{14}C at all its carbon atoms. Since the comparison products, codeine and morphine, do not contain the 6-*O*-methyl and the 3- and 6-*O*-methyls, respectively, of thebaine it was necessary to determine the amount of ^{14}C activity in these two methyl groups. This was done by stepwise demethylation following the reported[2] procedure via 14-bromocodeinone (8), neopine (9), dihydrocodeine (10),[11] and dihydromorphine (11) (Scheme II). Significant improvements were made, the purity of each intermediate was rigorously established, and radioactivity determinations were made throughout on pure, weighed samples. In separate experiments feeding [G-^{14}C]-thebaine, we found a constant ratio of NCH$_3$ to nuclear radioactivity[2] in thebaine, codeine, and morphine, establishing that the NCH$_3$ group remained intact.

Generally labeled [^{14}C]codeinone (3) was prepared from the biosynthetic [G-^{14}C]codeine by oxidation with silver carbonate or by the Oppenauer procedure.[12] The activity in the 3-*O*-methyl was determined by reduction to dihydrocodeine (10) and demethylation to dihydromorphine (11), as in the case of thebaine.

Highly ^{18}O-enriched codeinone was prepared by merely dissolving codeinone in an H$_2$^{18}O solution of oxalic acid. After 2 days at room temperature, [6-^{18}O]codeinone was isolated by extraction and sublimation.

To introduce ^{18}O specifically at C-6 of thebaine was more complex, but here we took advantage of the two distinctly different methoxyl groups at C-6 in codeinone dimethyl ketal

microsomes involved the cleavage of the oxygen–methyl bond with neither incorporation of oxygen into the phenolic product nor displacement of the intact methoxy group by a hydroxyl group. The reports[8] that a partially purified *O*-demethylase from a *Pseudomonas* which demethylated *p*-anisate could hydroxylate *p*-toluic acid to *p*-carboxybenzyl alcohol indicated that hydroxylation of the methyl group was the primary event preceding demethylation. Similar O-demethylation results were obtained with plant cell cultures.[9] These experiments establish the commonality of O-demethylation of aromatic methyl ethers by oxidation to the hydroxymethyl ether (hemiacetal) which then reverts to formaldehyde and the phenol.

Enol ethers are rarely found in nature, the more common being enolpyruvates, intermediates in aromatic ring and muramic acid syntheses, and plasmalogens. Neither is particularly pertinent to the question at hand.

Experimental Protocol

In considering the conversion of thebaine (1) to neopinone (2), two paths are conceivable, an oxidative path a and a hydrolytic path b. Path a would mimic that already established for biosynthetic cleavage of aromatic ethers, while path b proceeds by the mechanism established for in vitro hydrolysis of enol ethers.[10] The two paths are clearly distinguishable by the fact that a leads to retention of the 6-oxygen of thebaine in the subsequent alkaloids while b leads to its loss.

Thus a delineation between these two mechanisms might be realized by simply feeding [6-^{18}O]thebaine to *P. somniferum* and determining the ^{18}O content of the isolated codeine. However, these initial hopes were quickly dispelled when ex-

Table I. Feeding Experiments[a] with ^{18}O,^{14}C-Labeled[b] Thebaine and Codeinone

Compd fed	Amount fed			% retention of ^{18}O in compd isolated	
	% ^{18}O	dpm/mmol × 10^{-6}	% ^{18}O to ^{14}C dpm/mmol × 10^{-6}	Codeine	Morphine
Thebaine	14.4	12.6	1.14	37	32
Codeinone	16.7	2.92	5.7	33	32

[a] The *Papaver somniferum* plants used were prebudding and preelongation and of the same morphological age; chronological age, 60–100 days. [b] Percent ^{18}O is the amount of ^{18}O present above natural abundance; ^{14}C radioactivity is corrected to the morphine carbon skeleton.

Scheme II
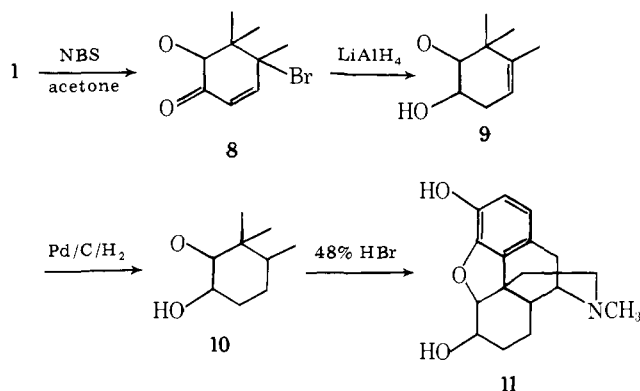
Scheme III
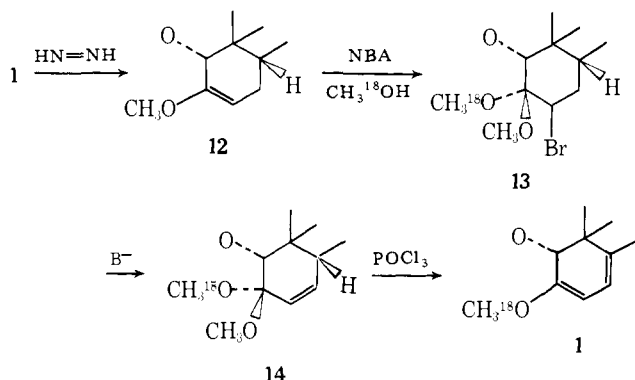
(**14**). Using ^2H labels, it has previously been established that addition to Δ^6-dihydrothebaine (**12**) and elimination from codeinone dimethyl ketal (**14**) are stereospecific.[13] This observation was conveniently applied to the synthesis of [6-^{18}O]thebaine by effecting the addition of methyl hypobromite to **12** in the minimum amount of [^{18}O]methanol.[14] Elimination of hydrogen bromide then gave **14** and treatment with phosphorus oxychloride resulted in stereospecific cis elimination of methanol to give [6-^{18}O]thebaine (see Scheme III).

Feeding Procedures

The *P. somniferum* plants used in the feeding experiments were approximately the same morphological age, but their chronological ages varied from 60 to 100 days. Administration of the precursors was examined by three routes: root feeding through hydroponic growth, wick feeding using an unwaxed dental floss wick, and direct injection with a motor-driven syringe. The latter method proved the most effective for rapid uptake of precursor and was used throughout. Injections were made both into the leaf midvein and into the hypocotyl. While leaf vein injections were effective, they were difficult to perform and reproduce. Therefore, injection into the hypocotyl became our method of choice in all feeding experiments.

The optimum amount of material to feed was examined using thebaine as precursor and determining its incorporation

into codeine and morphine. Increased incorporation would make subsequent analyses easier, but there appeared to be a control mechanism operating which limited the amount of thebaine that could be incorporated. For plants of the age we used, 6 mg of thebaine per plant was about optimum; larger amounts gave no greater incorporation.

The feeding solution for both thebaine and codeinone was buffered at pH 5.5. This minimum acidity was necessary to keep the alkaloids in solution. The total injection time was 1.5 h; under these conditions thebaine is completely stable and the 6-oxygen of codeinone exchanges very little. Biosynthesis was allowed to continue for 24 h; longer times led to less isotope in both the codeine and morphine.

Results and Conclusions

The results are summarized in Table I. By comparing the $^{18}O/^{14}C$ ratio of the thebaine fed with that of the codeine isolated, it was determined that 37% of the 6-^{18}O of thebaine had been retained in the 6-OH of codeine. This value was confirmed by a similar determination for the isolated morphine which showed 32% retention.

These data by themselves could be interpreted as supporting dual mechanisms for thebaine (**1**) to codeinone (**3**) conversion: path a in which the 6-oxygen of thebaine is retained and path b in which it is lost. However, the control experiment with [6-^{18}O]codeinone showed 33% of the ^{18}O was retained in the isolated codeine and 32% in the morphine, in striking agreement with the values of 37 and 32%, respectively, found for thebaine. These results, coupled with our previous observation of exchange of the carbonyl oxygen of codeinone at pH 7.2, clearly establish that loss of ^{18}O was due to nonenzymatic exchange.

Thus, we conclude that biosynthetic enol ether cleavage of thebaine proceeds by methyl cleavage with retention of the 6-oxygen. Although we have no independent supporting data, we suggest that this conversion probably involves an oxygenase, path a, as has been established for aromatic methyl ether cleavage. However, another path consistent with the data could involve direct methyl transfer by attack of a nucleophile on **1** or a protonated intermediate. Direct nucleophilic O-demethylation has precedent in vitro but has not been observed in vivo.

Experimental Section

Demethylation Procedures. Thebaine (**1**) was 6-O-demethylated by conversion to 14-bromocodeinone (**8**) with *N*-bromosuccinimide in acetone.[15] Lithium aluminum hydride reduction to neopine (**9**) was followed by hydrogenation to dihydrocodeine[2] (**10**), the first degradation compound to be counted. This gave the activity in the 6-O-methyl. The dihydrocodeine (**10**) was 3-O-demethylated with 48% hydrobromic acid to give dihydromorphine (**11**).[16]

14-Bromocodeinone (7). Thebaine (200 mg, 0.644 mmol) was placed in a 25-mL round-bottomed three-necked flask fitted with an N_2 bubbler and stirring bar. After addition of 4 mL of 19/1 (v/v) acetone/water, purified[17] *N*-bromosuccinimide (120 mg, 0.67 mmol) dissolved in 8 mL of the acetone/water solvent was added to the stirred and cooled (15–18 °C) thebaine suspension. The addition was made by syringe during 14 min, transferring the residual NBS in the syringe to the reaction flask with 2 mL of the acetone/water solvent. After

the reaction mixture was stirred for 40 min, the pale yellow mixture was poured into 10 mL of $CHCl_3$ and shaken with 20 mL of 50% saturated aqueous $NaHCO_3$. The aqueous phase was extracted 3 times with 10-mL portions of $CHCl_3$, the combined organic extract was dried in the dark and evaporated, and the resulting pale yellow foam, dissolved in 1 mL of $CHCl_3$, was column chromatographed in system A. After evaporation of the benzene, a glassy yellow solid (159 mg, 62%) remained and was used immediately in the next reaction.

Neopine (9). The 14-bromocodeinone (8, 149 mg, 0.397 mmol) was dissolved in 125 mL of ether in an N_2 atmosphere and refluxed for 2 h with excess $LiAlH_4$. After cooling, the excess $LiAlH_4$ was destroyed by careful addition of ethyl acetate and the solution was acidified to pH 1 with 1 N HCl. The ether phase was separated, and the aqueous solution was adjusted to pH 14 with a 1/1 (v/v) mixture of 10% KOH and 20% Rochelle salt (potassium sodium tartrate·$4H_2O$). Extraction of the solution with eight 25-mL portions of $CHCl_3$ afforded, after drying and evaporating, a pale yellow oil (118.5 mg, ca.100%) which was used immediately for reduction to dihydrocodeine.

Dihydrocodeine (10). To neopine (9, 118.5 mg, 0.396 mmol), dissolved in 100 mL of 5% acetic acid, was added 300 mg of 10% Pd/C and the mixture was hydrogenated in a Parr apparatus for 12 h at 50 psi. The catalyst was filtered off and was washed with three 10-mL portions of pyridine which were added to the filtrate. Evaporation of the solution left a residue which was dissolved in warm benzene and column chromatographed in system B. The resulting product was further purified by preparative TLC in system C and sublimation at 120 °C (100 μm). It was washed off the cold finger with anhydrous methanol and dried to constant specific activity at 60 °C 100 μm) for 12 h. The resulting dihydrocodeine (102.5 mg, 85%) showed one spot on TLC in system D with R_f 0.15, and one sharp peak at 6.6 min on GC.

Dihydromorphine (11). Dihydrocodeine (10, 46 mg, 0.153 mmol), dissolved in 1 mL of twice-distilled 47% HBr, was heated in an oil bath from 24 to 117 °C (1 h) and then at 117–125 °C (1 h) with light excluded and in an N_2 atmosphere. After cooling, the solution was diluted (20/1) with water, the pH was adjusted with 10% KOH to 12.3, and the solution was extracted three times with CH_2Cl_2. It was then adjusted to pH 9.3 with 1 N HCl and extracted three times with $CHCl_3/i$-PrOH (3/1). The solution was readjusted to pH 9.3 and extracted three times with $CHCl_3/i$-PrOH (3/1) and the process repeated once again. The $CHCl_3/i$-PrOH extract was dried and evaporated, leaving 37.4 mg, 87% yield, of dihydromorphine which was purified by preparative TLC in system C. The isolated material (R_f 0.29–0.41) was sublimed at 140 °C (100 μm), washed off the cold finger with anhydrous methanol, and dried to constant specific activity at 60 °C (100 μm) for 12 h. The resulting dihydromorphine showed one spot in TLC system C at R_f 0.31 and one peak at 7.9 min on GC.

[6-^{18}O]Thebaine was synthesized following the reported[13] procedure except that the preparation of 7-bromodihydrocodeinone dimethyl ketal (13) required modification in using a limiting amount of $CH_3^{18}OH$.

[6-α-^{18}O]-7-Bromodihydrocodeinone Dimethyl Ketal (14). Δ^6-Dihydrothebaine (400 mg, 1.28 mmol) was suspended in 12 mL of $CH_3^{18}OH$ and the mixture was heated to reflux and then sonicated for 15 min while cooling to give finely divided crystals. After addition of N-bromoacetamide (210 mg, 1.52 mmol) in 1 mL of $CH_3^{18}OH$, the resulting mixture was sonicated for 1 h at room temperature in the dark. Water (3 mL) was added, the mixture was placed at 0 °C for 20 h, the supernatant was decanted, and the solid residue was dissolved in $CHCl_3$. The $CHCl_3$ solution was dried and evaporated to give 433.6 mg, 80.5% of the ketal which is somewhat unstable to light and heat and was immediately converted to [6-α-^{18}O]codeinone dimethyl ketal (14).[13]

[^{18}O]Codeinone. Oxalic acid·$2H_2O$ (126 mg, 1.0 mmol) and codeinone (300 mg, 1.0 mmol) were added to 4 mL of 50% $H_2^{18}O$. The mixture (pH 2.7) was warmed on a steam bath to effect solution and placed in the dark under argon for 50 h, after which it was brought to pH 9.2 with 8 M K_2CO_3 and extracted with $CHCl_3$. The extract was dried and evaporated and the residue was sublimed to give 209 mg, 70% of [6-^{18}O]codeinone showing an ^{18}O atom percent excess of 24%.

Procedure for Feeding Precursors. The plants to be fed were removed from the hydroponic tank and their roots were placed in a

beaker of water. [G-^{14}C,6-^{18}O]Thebaine (71.5 mg) was dissolved in 1.7 ml of 1 M H_3PO_4 and the solution adjusted to pH 5.5 with 0.255 mL of 8 M KOH. Aliquots (109 μ, 4 mg) were injected into the hypocotyl at the stem–root junction of 12 plants with a no. 341 Sage syringe pump at the rate of 1.3 μL/min. After completion of the injection (1.5 h) the plants were returned to the hydroponic tank and allowed to grow for a 24-h period, starting from the initial injection. In a similar manner, 90 mg of [G-^{14}C,6-^{18}O]codeinone was fed.

Isolation Procedure. The alkaloids were isolated by the usual procedures,[4] resulting in two fractions: the phenolic and nonphenolic alkaloids. Morphine was separated from the phenolic fraction by TLC (system C) in the thebaine experiment and by recrystallization from $CHCl_3/i$-PrOH (3/1) in the codeinone experiment. Codeine was separated and purified from the nonphenolic fraction by column chromatography in system B followed by preparative TLC in systems D and C. Both compounds showed one spot on TLC and one peak on GC.

Chromatography Systems. Column Chromatography. Woelm alumina in benzene was used in 25-mm o.d. glass columns as follows: (A) 11 g of neutral alumina, activity 2.5; eluent 300 mL of benzene; (B) 115 g of basic alumina, activity 3; eluent successively, 400 mL of benzene/$CHCl_3/i$-PrOH (88.5/10/1.5), 600 mL of benzene/$CHCl_3/i$-PrOH/MeOH (87.5/10/1.5/1).

Thin-layer chromatography was on Eastman precoated plastic sheets for analysis and on Camag silica gel preparative plates for isolation. Solvent systems used were (C) $CHCl_3/CH_3OH/NH_4OH$ (75/25/1) and (D) benzene/CH_3OH (4/1).

Gas chromatography was performed on 6 ft × 6 mm glass columns, packed with 3% OV-17 on Varaport 30 (100–120 mesh), at 230 °C using helium at 40 mL/min as the carrier gas and a hydrogen flame detector. The retention times with a typical column were: thebaine, 14 min; codeine, 8 min; morphine, 12 min; codeinone, 9.5 min; dihydrocodeine, 7 min; dihydromorphine, 10 min.

Isotope Analyses. Radioactivity was determined by liquid scintillation counting of a weighed sample. **Atom percent excess of ^{18}O** was determined by nonoxidative pyrolysis of a sample to CO and analysis of the CO by mass spectrometry.[18] Allowance was made for natural abundance (0.23%) and a multiplication factor of three was used since the metabolites contain three oxygen atoms per mol; the data are given in Table I.

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