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Biological Synthesis of the Analgesic Hydromorphone, an Intermediate in the Metabolism of Morphine, by *Pseudomonas putida* M10

ANNE M. HAILES AND NEIL C. BRUCE*

Institute of Biotechnology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QT, United Kingdom

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The morphine alkaloid hydromorphone (dihydromorphinone) was identified as an intermediary metabolite in the degradation of morphine by *Pseudomonas putida* M10. A constitutive NADH-dependent morphinone reductase capable of catalyzing the reduction of the 7,8-unsaturated bond of morphinone and codeinone, yielding hydromorphone and hydrocodone, respectively, was shown to be present in cell extracts. The structures have been identified by ^{1}H nuclear magnetic resonance and mass spectrometry. Morphinone reductase has been partially purified by anion-exchange and gel filtration chromatography. This enzyme has potential applications as a biocatalyst for the synthesis of the highly potent analgesic hydromorphone and the antitussive hydrocodone.

Microbial transformations of the morphine alkaloids were studied extensively in the 1960s, principally for the purpose of producing more effective analgesics from naturally available compounds. Iizuka et al. (6, 7) and Yamada et al. (19, 20) described transformations of the morphine alkaloids by fungi, especially by the basidiomycete *Trametes sanguinea*, which converts thebaine by allylic oxygenation and demethylation to 14-hydroxycodeinone, which is then reduced to 14-hydroxycodeine. Several species of the fungus genus *Cunninghamella* were found to demethylate codeine to norcodeine (17), and the *N*-demethylase responsible for this reaction was identified as a cytochrome P-450-type monooxygenase (5). Bacterial transformations of morphine have been described by Liras et al. (9), who used enzyme preparations from *Pseudomonas testosteroni* containing α- and β-hydroxysteroid dehydrogenases to produce 14-hydroxymorphinone via an intermediate that was tentatively identified as morphinone. The same authors in a separate communication showed that resting cells of an *Arthrobacter* species were capable of transforming morphine in low yields to 14-hydroxymorphine (10). N demethylation of codeine to norcodeine in *Streptomyces griseus* has also been described previously (8). There have been few reports on the microbial metabolism of the morphine alkaloids or the use of these catabolic pathways for the synthesis of new morphine alkaloids. Recently, a strain of *Pseudomonas putida* capable of utilizing morphine as a sole carbon and energy source was isolated from industrial waste liquors. This study resulted in the identification of morphinone as a metabolic intermediate in the dissimilation of morphine by *P. putida* M10 (4). Morphinone is difficult to synthesize chemically because of its sensitivity to strong acids and bases (1, 14) and is of interest as a possible parent compound for the synthesis of the high-value narcotic antagonists naloxone and naltrexone. The oxidation of morphine to morphinone in *P. putida* M10 was shown to be mediated by a highly specific $NADP^+$-dependent morphine dehydrogenase that catalyzes the oxidation of the C-6 hydroxyl of morphine, codeine, and ethylmorphine (3). Furthermore, the structural gene for morphine dehydrogenase, *morA*, has now been located on a large plasmid of 165 kb in *P. putida* M10, and curing experiments suggest that part, if not all, of the pathway for morphine dissimilation is encoded on this plasmid (18). To accomplish large-scale preparations of morphinone by biotransformations, a genetically engineered strain of *Escherichia coli* containing the cloned *morA* gene has been constructed (18).

This paper describes the identification of a second enzyme involved in the dissimilation of morphine, an NADH-dependent reductase that catalyzes the reduction of morphinone and codeinone to hydromorphone (dihydromorphinone) and hydrocodone (dihydrocodeinone), respectively. Hydromorphone is an important analgesic compound, some five to seven times more potent than morphine (11), while hydrocodone is used generally as an antitussive (12). Synthetic methods for these therapeutic morphine alkaloids are not satisfactory, as they suffer from low and variable yields and are generally hazardous. In view of the difficulties associated with the synthesis of hydromorphone and hydrocodone, it seems worthwhile to explore fully the use of this reductase as a biocatalyst.

MATERIALS AND METHODS

Chemicals. Morphine hydrochloride, hydromorphone hydrochloride, codeine hydrochloride, codeinone, and hydrocodone bitartrate were supplied by Macfarlan Smith Ltd. (Edinburgh, Scotland). Hydrocodone free base was obtained by dissolving the hydrocodone bitartrate in distilled water to give a final concentration of 2 mM and then adjusting the solution to pH 8.7 by the addition of 1 M NaOH. The resulting free base was extracted into an equal volume of chloroform and recovered by rotary evaporation. $NADP^+$ and NADH were obtained from Sigma (Poole, Dorset, United Kingdom). All reagents were of the highest grade that could be obtained commercially.

Analytical techniques. High-performance liquid chromatography (HPLC) separations were performed on a Waters component system (Millipore Waters U.K. Ltd., Watford,

* Corresponding author.

United Kingdom) consisting of a model 510 pump, a model 712 WISP autoinjector, and a Waters 994 programmable photodiode array detector set to 230 nm and 285 nm, 0- to 10-V fsd. Separation of samples (50 μl) was achieved on a 5-μm C18 Spherisorb octyldecyl silane column (4.6 by 250 mm) (Anachem Ltd., Luton, United Kingdom) with an isocratic solvent system, delivered at a flow rate of 1 ml/min. The mobile phase consisted of 15 mM KH_2PO_4 (adjusted to pH 3.5 with 1 M H_3PO_4) in 30% (vol/vol) HPLC-grade acetonitrile (13). Integrations were performed by using Maxima 820 software, and UV absorbance scans of resultant peaks were measured between 190 and 350 nm with the Waters 994 programmable photodiode array detector. Thin-layer chromatography (TLC) was performed with 200-μm-thick plastic plates precoated with silica gel (Kiesegel 60 F_{254}; Merck) developed with solvent I (methanol-NH_4OH, 100:1.5 [vol/vol]) or solvent II (chloroform-methanol, 80:20 [vol/vol]). Plates were dipped in 0.1 M KOH in methanol prior to use if developed in solvent I. Metabolites were purified by preparative TLC on glass plates coated with silica gel (250-μm thickness; Whatman 60 A F_{254}). Compounds were detected by monitoring the UV absorbance and color formation by using Ludy Tenger reagent (12).

^{1}H nuclear magnetic resonance (^{1}H-NMR) was performed at 250 MHz on a Bruker spectrometer with trimethylsilane as an internal standard and deuterated chloroform as the solvent. Positive-ion fast atom bombardment mass spectrometry was performed on a Kratos model MF890 mass spectrometer with methanol-dichloromethane as the solvent. ^{1}H-NMR and mass spectrometry were performed by the Department of Chemistry, University of Cambridge.

Organism and growth of cells. The organism used in this investigation was *P. putida* M10, which was originally isolated from industrial waste liquors by enrichments with morphine as the sole source of carbon and energy (4). Cultures of *P. putida* M10 were grown in 2-liter Erlenmeyer flasks containing 750 ml of sterile defined mineral medium, consisting of Na_2HPO_4 (4.33 g), KH_2PO_4 (2.65 g), NH_4Cl (2.0 g), and nitrilotriacetic acid (0.1 g), and 4 ml of mineral salts, all per liter (16). The defined minimal medium was supplemented with either 50 mM acetate and 5 mM morphine, 50 mM acetate, or 10 mM glucose. Cells were harvested by centrifuging at 12,000 × *g*, washed by resuspension in 50 mM potassium phosphate buffer (pH 7.0), again centrifuged, and resuspended in buffer at 0.5 g (wet weight) per ml of buffer. Bulk cultures of *P. putida* M10 grown on 10 mM glucose were as described by Bruce et al. (4). The additional strains of *P. putida* ATCC 17464, *P. testosteroni* ATCC 17454, and *Pseudomonas fluorescens* NCIB 9815 were supplied by the National Collections of Industrial and Marine Bacteria Limited Aberdeen, Scotland.

Whole-cell studies. The degradation of hydromorphone by washed cells of *P. putida* M10 grown at the expense of 50 mM acetate and 5 mM morphine was assayed by monitoring the decrease in concentration by HPLC. The reaction mixture contained, in a total volume of 3 ml, 50 mM potassium phosphate buffer (pH 7.0), washed cells of *P. putida* M10 (500 μl), and 5 mM substrate. The mixture was incubated at 30°C on an orbital shaker, samples (300 μl) were removed at intervals, the cells were removed by centrifugation in a microcentrifuge, and the supernatant was analyzed by HPLC. All incubations were performed in duplicate.

Preparation of crude extract. Cells were disrupted by sonication in an MSE Soniprep (Fisons Instruments, FSA Ltd.) with 15-s bursts alternated with 30 s of cooling in melting ice, for a total sonication period of 3 min. Cell debris and unbroken cells were removed by centrifugation at 48,000 × *g* for 20 min at 4°C in a Sorvall RC-5C centrifuge, to give a clarified cell extract.

Enzyme assays. Morphinone reductase activity was determined by monitoring the oxidation of NADH at 340 nm in 50 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 7.0, containing 3 mM codeinone, 0.25 mM NADH, and enzyme in a final volume of 1 ml. One unit of enzyme activity is defined as the amount of enzyme necessary to oxidize 1 μmol of NADH per min at 30°C. Morphine dehydrogenase activity was determined by the method of Bruce et al. (3). Protein was measured by the method of Bradford (2).

Purification of morphinone reductase. All steps were performed at 4°C. Cell extract (47 ml), obtained from 20 g (wet weight) of glucose-grown cells of *P. putida* M10, was applied to a DEAE-Sephacel (Pharmacia/LKB Biotechnology) column (1.3 by 13 cm), preequilibrated with buffer A (50 mM MOPS, pH 7.0). The column was washed with buffer A at a flow rate of 15 ml/cm^2/h until no further elution of protein could be detected at 280 nm. After adsorption, morphinone reductase was eluted with a linear gradient of NaCl (0 to 0.5 M) in 200 ml of buffer A and then washed with 100 ml of buffer A containing 0.5 M NaCl. Fractions (6 ml) were collected and assayed for morphinone reductase activity and protein. Enzyme activity eluted at approximately 0.5 M NaCl. The fractions (65 to 70) containing the highest reductase activity were pooled and concentrated to a final volume of 2.2 ml in an Amicon ultrafiltration cell fitted with a YM10 membrane. The concentrate was applied to a Sephacryl S-300 (Pharmacia/LKB Biotechnology) column (0.8 by 55 cm) preequilibrated with buffer A. Elution of the enzyme was performed at 4 ml/cm^2/h. Fractions (1.4 ml) were collected and assayed for morphinone reductase activity and protein.

Purification of morphine dehydrogenase. Morphine dehydrogenase was purified from glucose-grown cells of *P. putida* M10 as described by Bruce et al. (3). The enzyme was judged homogeneous by both denaturing and nondenaturing polyacrylamide gel electrophoresis (3).

RESULTS AND DISCUSSION

Metabolism of morphine and codeine by *P. putida* M10. *P. putida* M10 was capable of using morphine or codeine to support growth; although both compounds could be utilized as sole carbon and energy sources, the growth rates were exceptionally low, with a lag phase of approximately 200 h for morphine, which eventually supported a doubling time of approximately 180 h. Figure 1 shows that *P. putida* M10 was capable of cometabolizing morphine when cultures were supplemented with an additional carbon source, such as acetate. HPLC analysis of the morphine-metabolizing culture revealed the appearance of an accumulating metabolite with a retention time of 3.94 min, identical to that of morphinone and hydromorphone, that correlated with the disappearance of morphine (retention time = 3.48 min). Further support for the identity of this compound was obtained from the UV spectrum of this metabolite, which matched closely the spectrum of authentic hydromorphone and was clearly distinguishable from that of morphinone. The compounds in the culture supernatant were isolated by adjusting the pH to 8.7 by the addition of $NaHCO_3$ and 1 M NaOH and extracting with chloroform. The extracted compounds were resolved by TLC on silica plates in solvent I and separated into three components, with R_f values of 0.66, 0.44, and 0.31, when viewed by UV absorbance or sprayed

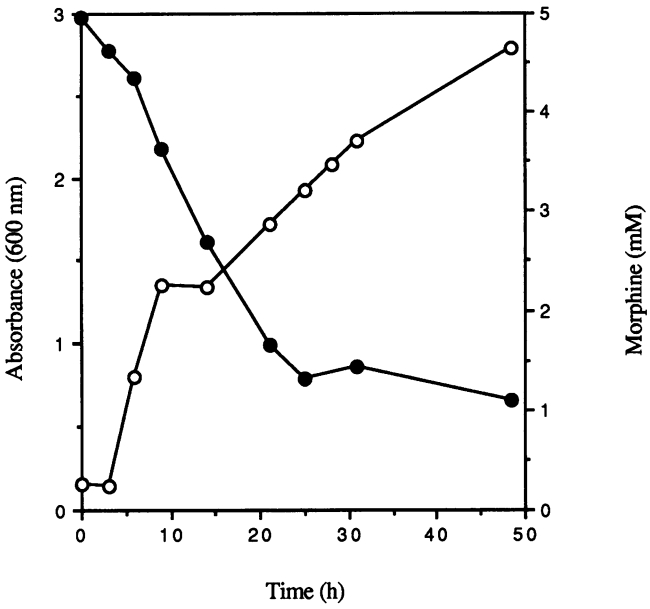

FIG. 1. Degradation of morphine by *P. putida* M10. Erlenmeyer flasks containing minimal media supplemented with 50 mM acetate and 5 mM morphine were inoculated with late-exponential-phase cultures pregrown with 50 mM acetate as the sole carbon source. Morphine (●) utilization was quantified by HPLC analysis, and growth was monitored by measuring the A_{600} (○).

with Ludy Tenger reagent. The R_f value of 0.31 corresponded with that of authentic hydromorphone, while the component(s) with an R_f of 0.44 coincided with authentic morphine and morphinone, which comigrate in solvent I. The compound with an R_f value of 0.66 is currently unknown. Washed cells of *P. putida* M10 grown with 50 mM acetate supplemented with 5 mM morphine were able to degrade authentic hydromorphone. Furthermore, *P. putida* M10 was capable of degrading 5 mM hydromorphone when cultures were supplemented with 50 mM acetate.

Enzyme activities. The results of the growth studies prompted us to evaluate the further metabolism of codeinone in cell extracts (morphinone is not readily available). Growth of *P. putida* M10 at the expense of a variety of substrates, such as glucose, acetate, and acetate supplemented with morphine, elicited in extracts high levels (0.25 U · mg of protein^{-1}) of a constitutive NADH-dependent activity that was seen by HPLC analysis to degrade codeinone rapidly with the accumulation of a compound (retention time = 6.7 min) corresponding to authentic hydrocodone. Further degradation was not observed in vitro, and no degradation of codeinone was observed when boiled extract was used in the reaction mixture or when NADH was omitted. No activity was displayed when NADH was replaced by NADPH in the reaction mixture. Chloroform extracts of the basified reaction mixture resolved two compounds, with R_f values of 0.41 and 0.33, when examined by TLC in solvent II. These compounds corresponded to authentic codeinone and hydrocodone, respectively.

Purification of morphinone reductase. To verify the identity of the reaction products of codeinone and morphinone degradation, the morphinone reductase preparation was purified by anion-exchange and gel filtration chromatography. Morphinone reductase was partially purified 17-fold from 20 g (wet weight) of glucose-grown cells of *P. putida* M10, with a 23% overall recovery. Partially purified morphinone reductase had a specific activity of 4.23 U · mg of protein^{-1}.

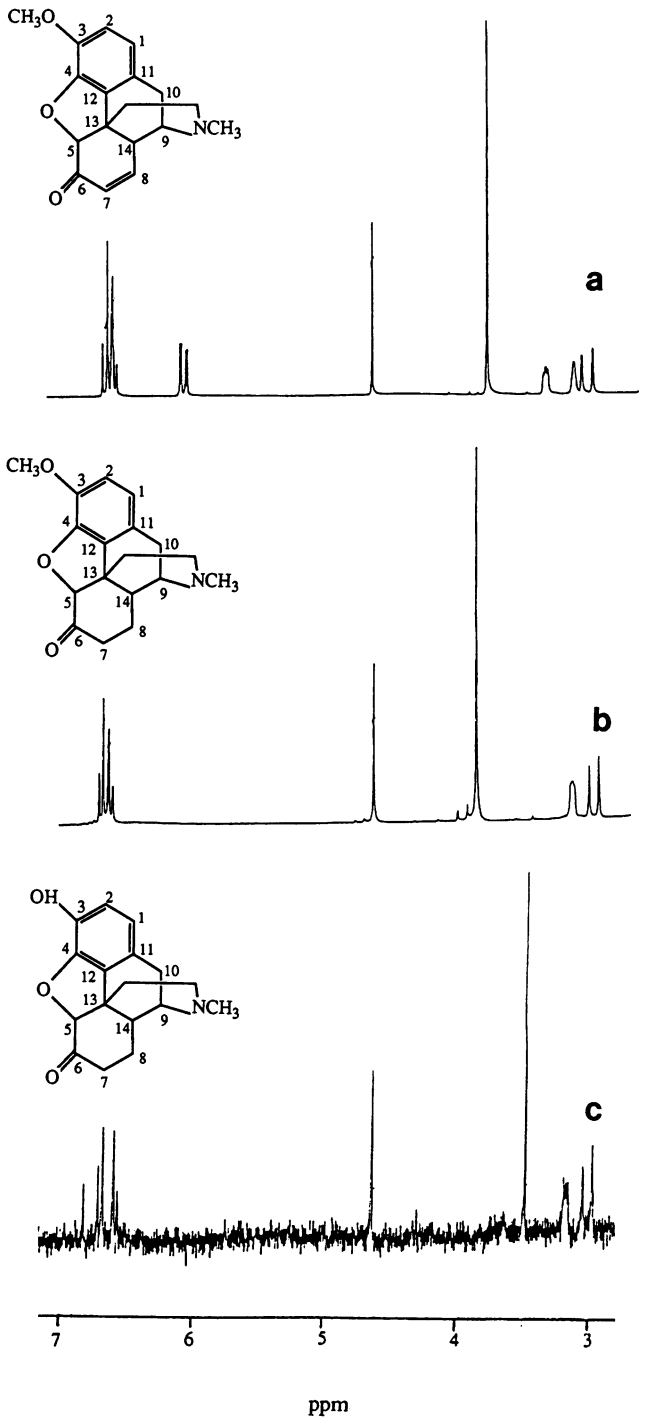

FIG. 2. ^{1}H-NMR spectra of authentic codeinone (a), biological hydrocodone (b), and biological hydromorphone (c) recovered from reaction mixtures containing partially purified morphinone reductase.

Metabolite isolation and identification. The reaction product of codeinone degradation was generated in mixtures containing 3 U of partially purified morphinone reductase, 2 mM codeinone, 2 mM NADH, and 50 mM Tris-HCl buffer,

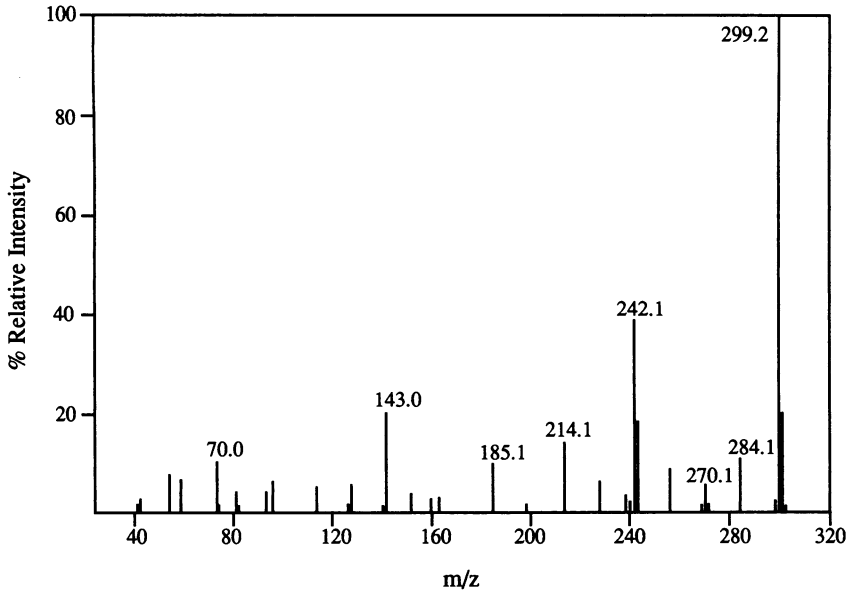

FIG. 3. Mass spectrum of biological hydrocodone.

pH 8.0, in a final volume of 50 ml at 30°C. The reaction was monitored spectrophotometrically at 340 nm, and after 100 min the enzymatic conversion reached completion. The reaction mixture was adjusted to pH 8.7 and extracted with three equal volumes of cooled chloroform. The chloroform extract was dried over molecular sieves (4 Å [0.4 nm]), and then the solvent was removed by rotary evaporation, yielding an oily residue. This residue was redissolved in 1 ml of chloroform and resolved by preparative TLC developed in solvent II. The band corresponding to hydrocodone was eluted from the silica with methanol, and the solvent was removed by rotary evaporation. Figure 2 shows the ^{1}H-NMR signals for the compound's aromatic AB system due to the protons positioned at C-1 and C-2 centered at δ 6.64 ppm and δ 6.57 ppm. A singlet at δ 4.7 ppm occurred because of the proton at C-5. The spectrum differs from that of codeinone in the loss of the multiplet assigned to the hydrogen atom bonded to C-8 at δ 6.6 ppm which overlapped the aromatic AB system and the loss of the multiplet assigned to the hydrogen atom bonded to C-7 at δ 6.12 ppm. This provides strong evidence that the enzyme has reduced the 7,8-unsaturated bond of codeinone. In all respects the ^{1}H-NMR spectrum was identical to that of authentic hydrocodone. Mass spectrometric analysis indicated that the molecular ion, m/z = 299, and the fragmentation pattern were indistinguishable from those of authentic hydrocodone (Fig. 3).

To identify the immediate product of morphinone degradation, morphinone was synthesized from morphine by using purified morphine dehydrogenase from *P. putida* M10. The reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 2 mM morphine, 2 mM $NADP^+$, and 8 U of morphine dehydrogenase in a total volume of 50 ml. The reaction was monitored by HPLC and after 70 min approached completion, at which time 2 mM NADH and 3 U of partially purified morphinone reductase were added. The spectrum of the newly generated reaction product (retention time = 3.94 min) matched exactly that of hydromorphone. TLC analysis of the reaction mixture resolved two compounds in solvent I with R_f values of 0.44 and 0.31, corresponding to morphine and hydromorphone, respectively. The compound with an R_f value of 0.31 was isolated and confirmed as hydromorphone by ^{1}H-NMR analysis (Fig. 2).

Several other species of pseudomonads (*P. putida*, *P. fluorescens*, and *P. testosteroni*) were screened for morphinone reductase activity. None of the organisms were capable of growth on morphine as the sole carbon source (4), and in each case, when the cells were grown on acetate supplemented with 5 mM morphine, no traces of morphinone reductase activity could be detected. Thus, the ability of *P.*

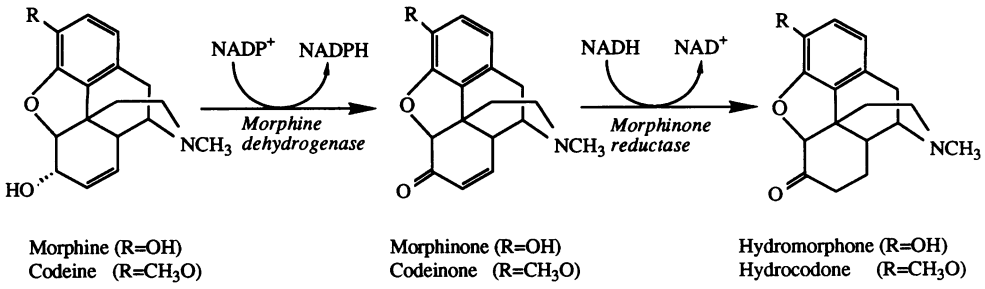

FIG. 4. Initial products of morphine and codeine metabolism in *P. putida* M10.

putida M10 to degrade morphinone further appears to be a property peculiar to this strain of *Pseudomonas* sp.

In summary, the isolation of metabolites from growth media and fractionation of cell extracts demonstrate that morphinone is transformed further to hydromorphone in the degradation of morphine by *P. putida* M10 (Fig. 4). Cell extracts contained high levels of a constitutive NADH-dependent reductase that was capable of saturating the olefin bond of morphinone and codeinone. The present study suggests that morphinone reductase is the second enzyme mediating the catabolism of morphine and codeine. However, since both morphine dehydrogenase and morphinone reductase are expressed constitutively, only an appropriately blocked mutant in *P. putida* M10 can demonstrate unequivocally their involvement in morphine catabolism.

Hydromorphone is a highly potent analgesic that can be prepared by catalytic hydrogenation of dihydromorphine followed by an Oppenauer oxidation using metal-*t*-butoxide and a suitable ketone (15). Chemical synthesis of hydromorphone directly from morphine via morphinone has been precluded because of the extreme sensitivity of morphinone to acids and bases (14). In view of the difficulties associated with the synthetic routes for hydromorphone, experiments are planned to examine further the biological transformation of morphine to hydromorphone with appropriate blocked mutants of *P. putida* M10 or genetically engineered strains of *E. coli*.

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