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Transformations of Morphine Alkaloids by *Pseudomonas putida* M10

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The oxidation of morphine by washed-cell incubations of *Pseudomonas putida* M10 gave rise to a large number of transformation products including hydromorphone (dihydromorphinone), 14β-hydroxymorphine, 14β-hydroxymorphinone, and dihydromorphine. Similarly, in incubations with oxymorphone (14β-hydroxydihydromorphinone) as substrate, the major transformation product was identified as oxymorphol (14β-hydroxydihydromorphine). The identities of all these biological products were confirmed by mass spectrometry and ^{1}H nuclear magnetic resonance spectroscopy. This is the first report describing structural evidence for the biological synthesis of 14β-hydroxymorphine and 14β-hydroxymorphinone. These products have applications as intermediates in the synthesis of semisynthetic opiate drugs.

A prime motive for studying microbial transformations of morphine alkaloids is the prospect of specifically modifying opiate drugs to improve their analgesic qualities. Such biotransformations were studied in the 1960s, and this early work included transformations by the basidiomycete *Trametes sanguinea*, which converted thebaine into 14β-hydroxycodeinone by monooxygenation and *O* demethylation (9, 10, 20, 21). Several species of the fungus genus *Cunninghamella* were more recently shown to *N* demethylate codeine, and the enzyme catalyzing this reaction was identified as a cytochrome P-450 type monooxygenase (7, 17). A few bacterial transformations of morphine alkaloids have also been reported: for example, transformation of morphine to 14β-hydroxymorphine in low yields by an *Arthrobacter* sp. (13) and transformation of codeine to 14β-hydroxycodeine and *N*-norcodeine by *Streptomyces griseus* (12). Early attempts to prepare novel alkaloid analogs using microorganisms were limited to exploring the range of transformations available, relying on fortuitous side reactions due to enzymes with broad substrate specificities. More recently, work has concentrated on the degradation of morphine alkaloids by soil bacteria and the use of specific enzymes that initiate the metabolism of morphine for the synthesis of existing clinically useful opiate drugs and new analogs (1).

The initial steps in the metabolism of morphine and codeine by *Pseudomonas putida* M10 involve oxidation of the C-6 hydroxy group and subsequent reduction of the 7,8-olefinic bond, forming hydromorphone (dihydromorphinone) and hydrocodone (dihydrocodeinone), respectively (Fig. 1). These products have important industrial applications; hydromorphone is an analgesic some seven times more potent than morphine (14), while hydrocodone is widely used as an antitussive (15). These transformations by *P. putida* M10 are catalyzed by morphine dehydrogenase and morphinone reductase, respectively, which have been purified and characterized in some detail (2, 3, 5, 8). Existing methods for the synthesis of hydromorphone are unsatisfactory because of the difficulty in specifically oxidizing the C-6 hydroxy group. It seems worthwhile, therefore, to exploit these enzymes as biocatalysts. Accordingly, the structural genes for morphine dehydrogenase and morphinone reductase, designated *morA* and *morB*, have been cloned and overexpressed in *Escherichia coli* in order to provide recombinant strains that are capable of converting morphine and codeine into hydromorphone and hydrocodone, respectively, on an industrial scale (6, 19).

This study significantly extends our previous results by expanding the range of transformations available and identifying new routes for the biological synthesis of analgesics by *P. putida* M10.

MATERIALS AND METHODS

Chemicals. Morphine sulfate, hydromorphone hydrochloride, and dihydromorphine were kind gifts from Macfarlan Smith Ltd., Edinburgh, United Kingdom. All other reagents were the highest grade that could be obtained commercially. Oxymorphol was prepared by reduction of oxymorphone with $NaBH_4$ as described by Weiss and Daum (18).

Maintenance and growth of the organism. *P. putida* M10 was isolated as described by Bruce et al. (3). The organism was maintained and grown as described by Hailes and Bruce (8). Media generally contained 20 mM glucose or 50 mM sodium acetate, and growth was monitored by measuring optical density at 600 nm. Cells were harvested by centrifugation at 13,680 × *g* at 4°C. The pelleted cells were washed in 50 mM KH_2PO_4 buffer (pH 7.1), centrifuged, and resuspended in the same buffer at a concentration of 0.5 g (wet weight)/ml.

Washed-cell incubations. Transformations of the morphine alkaloids were performed by washed-cell suspensions of *P. putida* M10 grown on either 20 mM glucose or 50 mM acetate as a sole source of carbon. Reaction mixtures (50 to 200 ml) typically contained 50 mM KH_2PO_4 buffer (pH 7), cell suspension (5 to 10 mg [dry weight]/ml), and 10 mM substrate. Reaction mixtures were incubated at 30°C, and samples (300 μl) were removed at timed intervals. Cells were removed by centrifugation using an MSE Microcentaur microcentrifuge, and the supernatants were analyzed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC).

Extraction of transformation products. Alkaloid compounds were isolated from culture broth and washed-cell incubations by adjusting the pH to 8.7 with addition of $NaHCO_3$ followed by 1 M NaOH and extracting with 3 equal volumes of ethyl acetate or chloroform. The organic phase was then dried with anhydrous Na_2SO_4, and the solvent was removed in vacuo. The extracted material was generally redissolved in a small volume of methanol.

Chromatography. Reversed-phase HPLC was performed on a Waters component system (Millipore Waters U.K. Limited, Watford, United Kingdom) consisting of a model 712 WISP autoinjector, 510 pump, and a Waters 994 programmable photodiode array detector set to 235 nm, 0 to 1 V full-scale deflection. Separation was achieved on a 5-μm C_{18} Spherisorb ODS column (4.6 by 250 mm) (Anachem, Luton, United Kingdom) with a mobile phase consisting of 10 mM KH_2PO_4 (adjusted to pH 3.5 with 1 M H_3PO_4) in either 20% HPLC grade acetonitrile (mobile phase I) or 30% acetonitrile (mobile phase II), both of which were delivered at a flow rate of 1 ml/min (modified from the method of Poochikian and Craddock [16]). Integrations were performed with Maxima 820 software, and UV absorbance scans of the resultant peaks were measured between 190 and 350 nm with a 994 programmable photodiode array detector. Preparative-scale HPLC for the purification of 1 to 3 mg of material was performed on the same analytical column. Those fractions containing the compound

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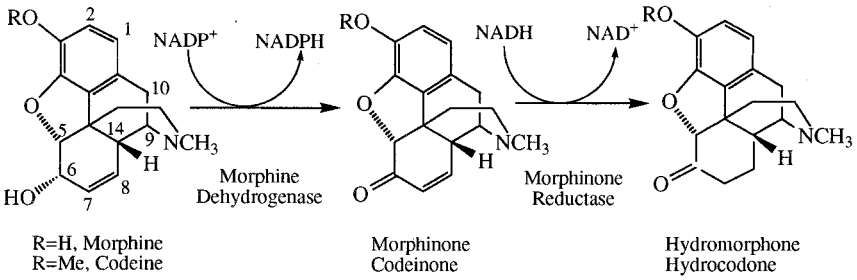

FIG. 1. Initial steps in the metabolism of morphine and codeine by *P. putida* M10.

of interest were collected, and the solvent was removed in vacuo. The compounds were extracted from the phosphate buffer by increasing the pH to 8.7 and extracting with 3 equal volumes of chloroform as described above.

TLC was performed with plastic-back plates (thickness, 200 μm) precoated with Kieselgel 60 F_{254} (Merck) developed with solvent I (methanol-NH_4OH [100:1.5, vol/vol]), solvent II (methanol-glacial acetic acid [100:1, vol/vol]), solvent III (chloroform-methanol-NH_4OH [90:9:1, vol/vol]), or solvent IV (chloroform-methanol [80:20, vol/vol]).

Transformation products were purified either by preparative TLC or by silica gel column chromatography. Preparative TLC plates were coated with Kieselgel 60 A F_{254} (thickness, 250 μm). Compounds were detected by their UV absorbance and color formation using the Ludy Tenger reagent (15). Columns (2 by 10 cm) were packed with Kieselgel 60, washed with 2 volumes of the initial eluting solvent, and loaded with up to 50 mg of extract from washed-cell incubations. Compounds were then eluted with solvent system II or III and detected by TLC in the systems described above.

Spectral analysis. ^1H nuclear magnetic resonance (^1H NMR) spectroscopy was performed at 200, 250, or 400 MHz on Bruker spectrometers with tetramethylsilane as an internal standard and deuterated chloroform as the solvent unless otherwise stated. Further structural evidence was provided by ^1H spectra with D_2O exchange of hydroxy groups. Chemical shifts (δ) are in parts per million, and coupling constants (*J*) are in hertz. Electron impact (EI) and laser desorption time of flight mass spectrometry were performed on Kratos model MF890 and Kompact Maldi III mass spectrometers, respectively.

RESULTS AND DISCUSSION

Accumulation of transformation products. Transformations of morphine alkaloids by washed-cell incubations were analyzed directly by HPLC and TLC. Incubations were performed with glucose-grown cells of *P. putida* M10 and 10 mM morphine as substrate. HPLC analysis with mobile phase I revealed degradation of morphine (R_t = 3.9 min) after 19 h coincident with the accumulation of a metabolite which matched closely authentic hydromorphone (R_t = 4.6 min) and a number of unknown compounds. The three major peaks had retention times of 3.5, 3.8, and 4.6 min (Fig. 2). The UV

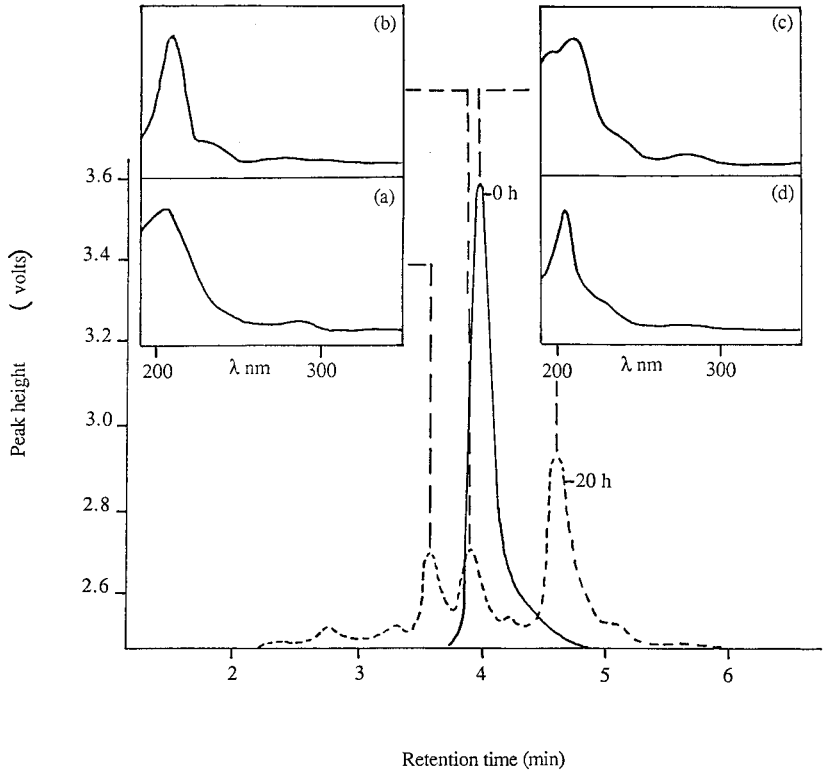

FIG. 2. HPLC analysis of the metabolites (20-h incubation) of morphine with the associated UV spectra. The incubation mixture (10 ml) contained glucose-grown *P. putida* M10 with 10 mM morphine. Samples (300 μl) were removed after 0 and 19 h of incubation, and the supernatants were diluted (20-fold) in mobile phase and subjected to HPLC analysis. Specific conditions are described in Materials and Methods. The spectra correspond to an unknown compound (compound 1) (a), dihydromorphine (compound 2) (b), morphine (c), and hydromorphone (d).

spectrum of the unknown compound (R_t = 3.5 min) designated compound 1 did not match those of any of the authentic morphine alkaloids available, while the UV spectrum of the unknown compound (R_t = 3.8 min) designated compound 2 matched closely that of authentic dihydromorphine. Analysis by TLC (solvent III) revealed the appearance of at least three major compounds with R_f values of 0.54, 0.36, and 0.28. The compound with an R_f value of 0.36 corresponded to authentic hydromorphone, while the compound with an R_f value of 0.28 coincided with authentic dihydromorphine. The unknown compound (R_f = 0.54), corresponding to compound 1, was detectable under UV light (254 nm) and by its reaction with the Ludy Tenger reagent (see Figure 3 for the structures of the transformation products, identified by compound number).

HPLC analysis in mobile phase II of whole-cell incubations and *P. putida* M10 with 10 mM hydromorphone as substrate revealed the complete disappearance of hydromorphone (R_t = 4.1 min) after 21 h, coincident with the accumulation of an unknown compound (R_t = 3.3 min). The UV spectrum and chromatographic behavior of this compound were identical to those of compound 2 described above and, therefore, it also corresponded closely to dihydromorphine.

Similarly, HPLC analysis in mobile phase II of whole-cell incubations with 10 mM oxymorphone as substrate revealed the disappearance of oxymorphone (R_t = 3.8 min) with the concurrent accumulation of an unknown compound (R_t = 3.2 min), designated compound 3. Two compounds were resolved by TLC in solvent IV, corresponding to authentic oxymorphone (R_f = 0.44) and compound 3 (R_f = 0.30); both were detected under UV light (254 nm) and by their reaction with the Ludy Tenger reagent.

Purification of transformation products. The alkaloid components from washed-cell incubations, with morphine as substrate, were extracted into chloroform. Purification of compound 1 was achieved by silica gel column chromatography in solvent III. Fractions (1 ml) were collected and analyzed by TLC in solvent I. Those containing compound 1 (fractions 40 through 52) were pooled, and the solvent was removed in vacuo. HPLC analysis of the resultant residue in methanol revealed the presence of a minor impurity (R_t = 7.5 min); this was effectively removed by preparative HPLC. Similarly, compound 2 was extracted from washed-cell incubation mixtures with morphine as substrate. Purification of this compound was achieved by passage through two Kieselgel 60 columns with solvents III and II, respectively. Fractions (7 through 9) from the second column, containing compound 2, were evaporated to dryness, and the residue was redissolved in a small volume of methanol.

Analysis of the concentrated chloroform extract by TLC revealed the presence of a number of unknown compounds, previously unseen because of their low concentrations in incubation mixtures. An unknown compound (R_f = 0.59 in solvent III), designated compound 4, was detected under UV light (254 nm) and by its reaction with the 2,4-dinitrophenylhydrazine reagent and Ludy Tenger reagent. Purification of compound 4 was achieved by silica gel column chromatography in solvent III as described above. Fractions (1 ml) containing compound 4 (fractions 22 through 32) were pooled, and the solvent was removed in vacuo. Preparative HPLC of this mixture in the system described previously effectively purified compound 4. On one occasion, compound 4 was converted fortuitously during purification largely into a methanol adduct.

The alkaloids from washed-cell incubations with oxymorphone as substrate were extracted into chloroform after 20 h and purified by preparative TLC in solvent I. The band corresponding to compound 3 was eluted in a small volume of methanol, the silica was removed by centrifugation, and the solvent was removed in vacuo.

Identification of transformation products (Fig. 3). The morphine metabolites were identified by ^{1}H NMR spectroscopy and mass spectrometry. When possible, spectra were compared with those of reference samples (see the article by Weiss and Daum [18]).

(i) Compound 1, 14β-hydroxymorphine. The ^{1}H spectrum (400 mHz) gave, inter alia, the following signals, which unambiguously identified the structure (for the proton assignments see Fig. 1, which gives the morphine numbering system): δ_H 6.63 (1H, d, *J* 8.1, 2-H), 6.51 (1H, d, *J* 8.1, 1-H), 5.89 (1H, dt, *J* 9.9 and 1.4, 7-H), 5.49 (1H, dd, *J* 9.9 and 3.1, 8-H), 4.89 (1H, dd, *J* 6.5 and 1.1, 5-H), 4.67 (1H, ddd, *J* 6.6, 3.0, and 1.9, 6β-H), 3.15 (1H, d, *J* 18.7, 10β-H), 3.00 (1H, d, *J* 6.5, 9-H), and 2.40 (3H, s, NMe). The high-resolution mass spectrum (EI) gave a molecular ion, *m/z* 301.1292, for $C_{17}H_{19}NO_4$ (calculated, 301.1314).

(ii) Compound 2, dihydromorphine. This product was identified by comparison of its ^{1}H NMR spectrum with that of a reference sample. Structurally diagnostic signals were observed at δ_H (200 MHz) 6.59 (1H, d, *J* 8.1, 2-H), 6.49 (1H, d, *J* 8.1, 1-H) 4.53 (1H, d, *J* 5.2, 5-H), 3.94 (1H, q, *J* 5.3, 6-H), 3.22 (2H, br s, OH, exchangeable with D_2O), 3.05 (1H, dd, *J* 5.6 and 2.6, 9-H), 2.90 (1H, d, *J* 18.5, 10β-H), and 2.33 (3H, s, NMe). The mass spectrum (EI) gave a molecular ion, *m/z* 287.1522, for $C_{17}H_{21}NO_3$ (calculated, 287.1521).

(iii) Compound 3, oxymorphol (14β-hydroxydihydromorphine). The ^{1}H NMR spectrum was indistinguishable from that of a sample prepared from oxymorphone by reduction with $NaBH_4$. Owing to the low solubility of this metabolite in $CDCl_3$, the spectrum was obtained for a solution in CD_3OD: δ_H (250 MHz) 6.61 (1H, d, *J* 8.1, 2-H), 6.50 (1H, d, *J* 8.1, 1-H), 4.50 (1H, d, *J* 4.7, 5-H), 4.12 (1H, dt, *J* 12.1 and 4.2, 6β-H), 3.15 (1H, d, *J* 18.6, 10β-H), 2.78 (1H, d, *J* 6.5, 9-H), 2.56 (1H, dd, *J* 18.4 and 6.6, 10α-H), and 2.36 (3H, s, NMe). For a discussion of the stereochemistry of ring C see the article by Crouch et al. (4). The mass spectrum (laser description time of flight) showed a molecular ion, *m/z* 303, for $C_{17}H_{21}NO_4$. Previous studies indicated that oxymorphone was a substrate for morphine dehydrogenase (1), which could account for the formation of oxymorphol in washed-cell incubations.

(iv) Compound 4, 14β-hydroxymorphinone. The ^{1}H NMR spectrum was clearly that of a 14-substituted, ring C enone: δ_H (200 MHz) 6.70 (1H, d, *J* 8.2, 2-H), 6.65 (1H, d, *J* 10.3, 8-H), 6.59 (1H, d, *J* 8.2, 1-H), 6.18 (1H, d, *J* 10.1, 7-H), 4.73 (1H, s, 5-H), 3.22 (1H, d, *J* 18.6, 10β-H), 3.04 (1H, d, *J* 5.9, 9-H), and 2.45 (3H, s, NMe). The mass spectrum (EI) gave a molecular ion, *m/z* 299.1143, for $C_{17}H_{17}NO_4$ (calculated, 299.1158). Moreover, the presence of an αβ-unsaturated, ring C ketone and of hydroxy groups was confirmed by the infrared spectrum: ν_{max} ($CHCl_3$)/cm^{-1}, 3568, 3334, and 1683.

On one occasion the same chromatographic fraction yielded a small quantity of 14β-hydroxymorphinone mixed with a much larger amount of an artifact arising apparently by addition of methanol to the enone double bond: δ_H (400 MHz) 6.72 (1H, d, *J* 8.2, 2-H), 6.62 (1H, d, *J* 8.2, 1-H), 4.63 (1H, s, 5-H), 3.33 (1H, d, *J* 5.9, 9-H), 3.31 (3H, s, MeO), 3.19 (1H, d, *J* 18.6, 10β-H), and 2.42 (3H, s, NMe); *m/z* (EI) 331.1423 (calculated for $C_{18}H_{21}NO_5$, 331.419).

The addition of a 14-hydroxyl group to the morphine alkaloid structure has been found to dramatically increase potency (11). However, the synthesis of 14-hydroxymorphine from morphine has not been accomplished chemically, and present syntheses of derivatives hydroxylated at C-14 rely on thebaine, which is a minor component of opium poppy alkaloids, as a

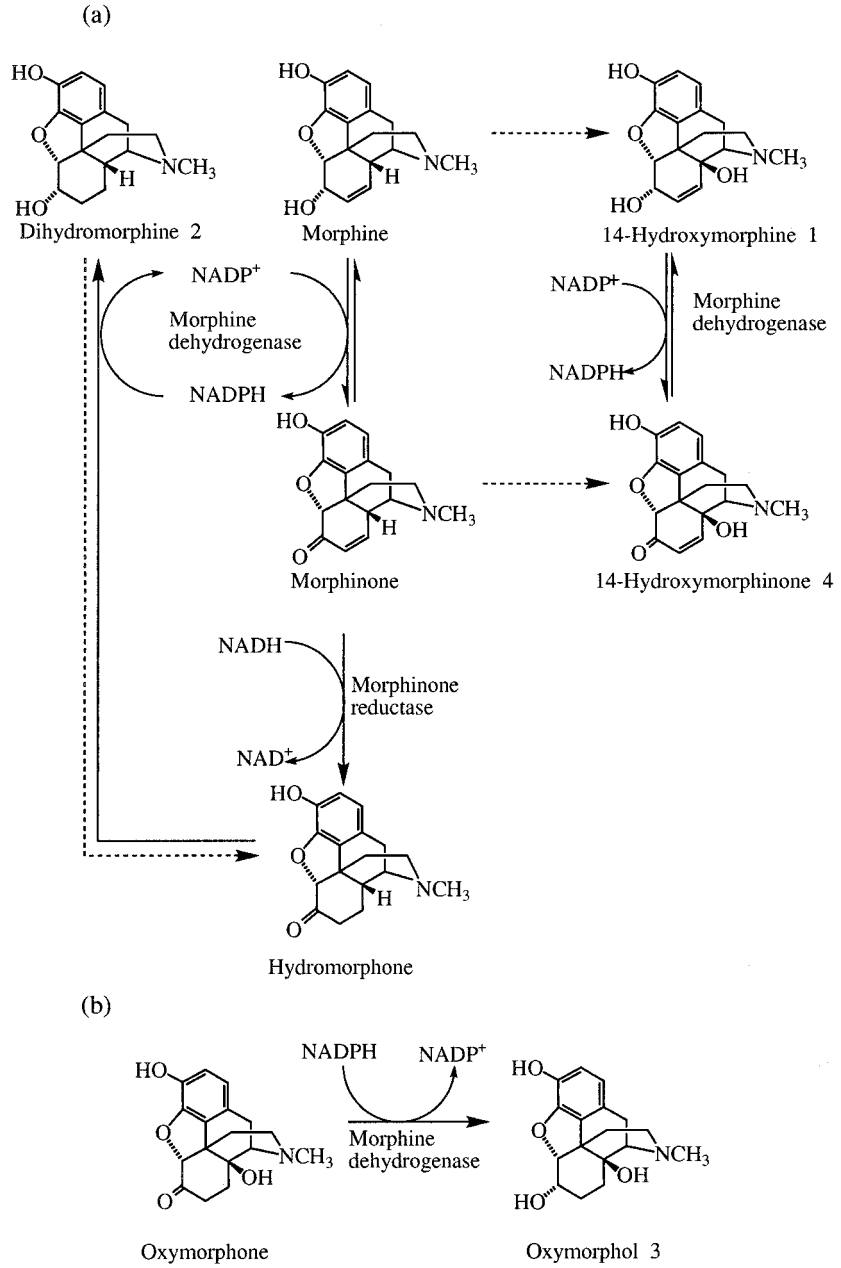

FIG. 3. Products of transformation of morphine (a) and oxymorphone (b) by *P. putida* M10.

starting material. This has been attributed to the saturated and therefore unreactive nature of the carbon at C-14 in morphine and codeine, whereas in thebaine this carbon is olefinic and readily undergoes hydroxylation (12). Biologically catalyzed reactions would, therefore, greatly facilitate these syntheses since morphine and codeine could be utilized as starting materials. The oxidation of codeine to 14β-hydroxycodeine by *S. griseus* (12) is the only reported transformation of this type by a bacterium in which the product has been fully identified by ^{1}H NMR and mass spectral analyses. Such a biotransformation with morphine as substrate has never been fully established. 14-Hydroxymorphine and 14-hydroxymorphinone are likely to be substrates for morphine dehydrogenase in *P. putida* M10, and this would account for the appearance of both of these compounds in incubations. Figure 3 summarizes the transformations identified so far.

In summary, a number of potentially useful transformations of morphine alkaloids by *P. putida* M10 have been identified. Morphine was transformed into hydromorphone, dihydromorphine, 14β-hydroxymorphine, and 14β-hydroxymorphinone by uninduced washed-cell incubations along with a number of other, as yet unidentified, metabolites. Dihydromorphine is a narcotic analgesic, while 14β-hydroxymorphine and 14β-hydroxymorphinone could be used as pivotal intermediates in the synthesis of other analgesic agents. In view of the difficulties associated with chemical syntheses of morphine alkaloid derivatives, it seems logical to explore fully the use of bacteria such as *P. putida* M10 for biotransformations.

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