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14-Hydroxylation of Opiates: Catalytic Direct Autoxidation of Codeinone to 14-Hydroxycodeinone

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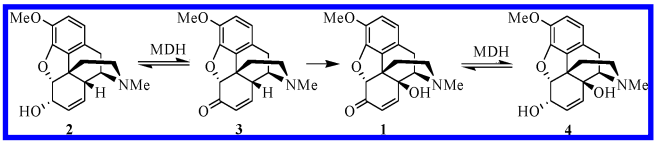
The introduction of a 14-hydroxyl group to the morphine alkaloid structure is known to improve its pharmacological properties.[1] This group of drugs includes oxycodone, naloxone, oxymorphone, and naltrexone with well-established clinical uses for analgesia, antitussive, and treatment of drug abuse and alcohol addiction.[2] The common starting material for the synthesis of many 14-hydroxylated opiates is thebaine, a minor component of natural opium extracts, which is converted by peracid oxidation to 14-hydroxycodeinone (**1**), the key 14-hydroxylated intermediate.[3] Because codeine (**2**) and morphine are much more available than thebaine, many approaches have been reported for their conversion to 14-hydroxylated opiates.[4] However, each of these methods is plagued by low yields, the formation of difficult-to-separate byproducts, or the use of undesirable heavy metals. For instance, significant effort has not resulted in practical success for the preparation of 14-hydroxycodeinone directly from codeinone (**3**), which can be easily prepared from codeine.[5] Indeed, it was previously concluded after a systematic study that "the direct conversion of [codeinone] to [14-hydroxycodeinone] cannot be performed with the vast majority of common oxidizing agents".[6]

The development of a bioprocess for the 14-hydroxylation of morphine alkaloids has also attracted great interest.[7] A number of microorganisms have been identified for the 14-hydroxylation of codeine and morphine.[7] The strain that has received the most attention in the past decade is *Pseudomonas putida* M10, which converts codeine and morphine to 14-hydroxycodeine (**4**) and 14-hydroxymorphine, respectively.[8] Interestingly, this microbial transformation has been demonstrated to be the result of a sequence of reactions (Scheme 1). Briefly, codeine is converted to **3** by morphine dehydrogenase (MDH), followed by hydroxylation of **3** to form **1**, which is then converted to **4** by the same MDH. While the MDH has been well characterized, cloned, and expressed, the key hydroxylation activity was barely detected.[7a]

During our study of opiates, we identified *Mycobacterium neoaurum* (MTP650) to be an efficient microorganism for the biotransformation of **2** to **4** and a few other derivatives (see Supporting Information), similar to those reported for *P. putida* M10. We report here our evidence that the 14-hydroxylation of **3** by *M. neoaurum* is a chemical hydroxylation, rather than an enzyme-catalyzed reaction. More importantly, we have discovered a simple chemical reaction for the direct conversion of **3** to **1** in aqueous solution, providing an attractive strategy for the synthesis of 14-hydroxylated opiates starting from codeine.

The presence of a dehydrogenase in *M. neoaurum* was implicated by the identification of **1** as one of the bioconversion products and confirmed by the conversion (7%) of **1** to **4** with a cell-free extract (CFE) in the presence of NADPH. To verify that the dehydrogenase

Scheme 1



and **3** are directly involved in the formation of **4**, a sample of 95% enriched [6-D]codeine was subjected to biotransformation by *M. neoaurum*. ^{1}H NMR analysis of the resulting isolated **4** indicated a complete loss of the deuterium label during the conversion. A sample of the remaining codeine was also recovered from the same experiment, and ^{1}H NMR analysis found the majority (83%) of the deuterium label retained, indicating the deuterium loss due to the equilibrium catalyzed by the dehydrogenase is slow. Thus, this deuterium labeling experiment strongly supports that **3** is an intermediate during the biotransformation of **2** to **4** by *M. neoaurum*. We also observed a significantly slow turnover rate for the deuterated substrate (4 days, 21%) compared to that for unlabeled codeine (3 days, ~35%). This kinetic isotope effect suggests that the dehydrogenase-catalyzed reaction is slow, and the 14-hydroxylation step must be fast. The absence of **3** from the fermentation medium during the reaction, along with the initial accumulation and resulting disappearance of **1**, also supports the hypothesis that the hydroxylation of **3** is much faster than the dehydrogenase-catalyzed oxidation of **2** to **3**.

A CFE and membrane fractions of *P. putida* M10 have been reported to catalyze the 14-hydroxylation of **3** without the requirement of any cofactors, and the activity was found to be extremely heat-stable.[7a] We found that a CFE of *M. neoaurum* was unable to catalyze the 14-hydroxylation of **3**. Conversely, excellent activity was detected in the spent medium, which was prepared from a 72 h fermentation broth after removal of all cells. When **3** (2 mM) and the spent medium (4 mL, adjusted to pH 8.0) were kept in a shaker at 29 °C and 300 rpm for 2 h, HPLC analysis indicated that nearly all **3** was converted to **1** (~60%) and other products. Similar activity was also observed after the spent medium was boiled for 30 min. Size-exclusion fractionation experiments determined that the activity was in a fraction with low molecular weight (<1 kD). These facts strongly support that the 14-hydroxylation of **3** is not catalyzed by an enzyme as previously believed. When the reaction was carried out using fresh medium (pH 8.0), a complex mixture was generated with **1** only as a minor component (<5%). Clearly, a component(s) of the spent medium facilitates the 14-hydroxylation reaction.

Upon consideration of the possible reaction mechanisms and the requirement of molecular oxygen for the 14-hydroxylation of **3**, we envisioned that a peroxide intermediate might be involved. The spent medium may contain a reducing equivalent that can reduce the peroxide intermediate to form the product. Because the fresh medium contains no reductant, a peroxide intermediate may undergo

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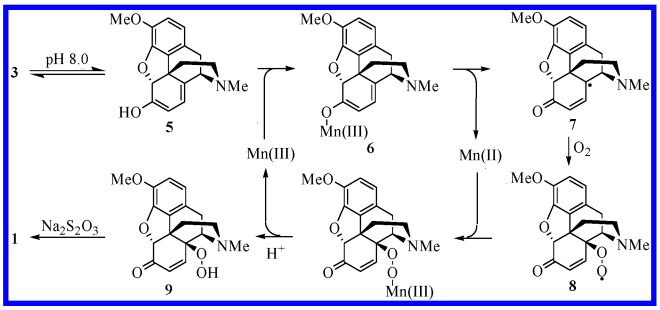
Scheme 2



further complex chemistry. To test this hypothesis, a number of simple reductants were individually included in the reaction mixture. When sodium thiosulfate (5 mM) was included in a reaction mixture of **3** (2 mM) in fresh medium (4 mL, pH 8.0), **1** was found to be the main product (~85%) after the mixture was kept at 29 °C and 300 rpm for 2 h. A control experiment with sodium thiosulfate in phosphate buffer (100 mM, pH 8.0) resulted in unchanged **3**. Obviously, the reaction is catalyzed by a component(s) of the fresh medium. Because the fresh medium (Mineral Salts Broth) is chemically defined, deconvolution of medium components identified $MnSO_4$ and $CuSO_4$ as the most active catalysts for the hydroxylation of **3** in the presence of molecular oxygen and thiosulfate. The concentrations of $MnSO_4$ and $CuSO_4$ in the medium are only 7.4 and 1.3 μM, respectively, indicating they are very effective catalysts.

A typical reaction mixture (5 mL) consisted of 2 mM codeinone, 2.5 mM thiosulfate, and 7 μM $MnSO_4$ in phosphate buffer (75 mM, pH 8.0). The reaction was completed in about 3 h at 29 °C and 300 rpm in a rotary shaker, provided that sufficient O_2 was included in the reaction vessel. Manganese reagents with other valencies were also found to be effective catalysts for the reaction, including $Mn(OAc)_3$, MnO_2, $KMnO_4$, and even $Mn_2(CO)_{10}$. In the presence of O_2 and thiosulfate, all these manganese reagents may be converted to the same catalytic species, such as Mn(III). The reaction could be explained by the initial coordination of Mn(III) with codeinone dienol (**5**) at slightly basic conditions (Scheme 2). The dienolate (**6**) transfers one electron to manganese to generate a stabilized tertiary radical species (**7**), followed by the incorporation of O_2 to form a peroxy radical species (**8**), which may obtain one electron back from the manganese to yield a peroxy anion and to regenerate the catalytic species. The peroxy anion then picks up a proton to form 14-hydroperoxycodeinone (**9**), which is reduced to **1** by thiosulfate. This mechanism is supported by the positive detection of peroxide species in the reaction mixture using a peroxide paper when the reaction was carried out in the absence of a reductant. Such a radical mechanism is also consistent with those of other autoxidation reactions,[9] including the Mn(III)-catalyzed α-hydroperoxidation of 1,3-dicarbonyl compounds[10] and the γ-hydroxylation of α,β-unsaturated ketones.[11] It was intriguing to find that there is a lag phase of about 1 h for the reaction with $MnSO_4$ (see Supporting Information), whereas no obvious lag phase was observed for the copper-catalyzed reaction. One possible explanation is that the Mn(II) species needs to be oxidized to the Mn(III) species by **8** initially generated from spontaneous autoxidation of **3**, and this is known to be very slow.[7a] The reaction with $KMnO_4$ completes in about 1 h and does not have a lag phase, perhaps because the Mn(III) species is formed directly by the thiosulfate reduction of permanganate.

The addition of an appropriate reductant is critical for the hydroxylation reaction because it may facilitate the reduction of the peroxy intermediate generated during the reaction and affect the oxidation state of the catalytic metal species. Thiourea was found to be another efficient reductant capable of replacing thiosulfate, whereas a variety of other reagents could also be used for the reaction with somewhat decreased yield, including sodium iodide, sodium thiocyanate, 2-ketoglutarate, methionine, *N*-acetylmethionine, and alanine. The identification of these reductants suggests that the reaction using spent medium is likely accomplished with the combined reduction by a number of reducing agents, such as amino- and sulfur-containing compounds generated during the fermentation.

In summary, we elucidated the key 14-hydroxylation step during the microbial transformation of **2** to **4**. Our results clearly suggest that the 14-hydroxylation of **3** is a chemical reaction, rather than an enzymatic reaction, as previously believed. Most importantly, we have developed an efficient method for the direct 14-hydroxylation of **3**, thereby allowing the more abundant codeine to be employed as the starting material for the synthesis of 14-hydroxylated opiate drugs. Because of the use of cheap inorganic reductant, free oxidant, and very low amounts of more environmentally benign metal catalysts in an aqueous solution, the catalytic hydroxylation presents a great opportunity to significantly improve process economics, especially through decreased waste treatment/disposal costs.

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Supporting Information Available: Experimental procedures and profiles of biotransformation and lag phase of $MnSO_4$. This material is available free of charge via the Internet at http://pubs.acs.org.

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