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Transformations of morphine, codeine and their analogues by *Bacillus sp.*

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A bacterial strain belonging to the genus *Bacillus* islolated by enrichment culture technique using morphine as a sole source of carbon transforms morphine and codeine into 14-hydroxymorphinone and 14-hydroxycodeinone as major and 14-hydroxymorphine and 14-hydroxycodeine as minor metabolites, respectively. When the *N*-methyl group in morphine and codeine are replaced by higher alkyl groups, the organism still retains its ability to carry out 14-hydroxylation as well as oxidation of the C_6-hydroxyl group in these *N*-variants, although the level of metabolites formed are considerably low. The organism readily transforms dihydromorphine and dihydrocodeine into only dihydromorphinone and dihydrocodeinone, respectively, suggesting that the 7,8-double bond is a necessary structural feature to carry out 14-hydroxylation reaction. The cell free extract (20,000 × g supernatant), prepared from morphine grown cells, transforms morphine into 14-hydroxymorphinone in the presence of NAD^+, but fails to show activity against testosterone. However, the cell free extract prepared from testosterone grown cells contains significant levels of 17β- hydroxysteroid dehydrogenase but shows no activity against morphine.

n view of the difficulties associated with the synthesis of morphine alkaloids based pharmaceutically important compounds, several investigators explored the possibility of using microbes as an alternate method. Microbial transformations of morphine alka-loids have been studied using both bacterial and fungal strains. It was demonstrated earlier that *Trametes sanguinea* converts thebaine into 14-hydroxycodeinone and 14-hydroxycodeine[1]. The formation of these metabolites suggests the ability of fungi to carry out allylic oxygenation, and *O*-demethylation. Fungal strains were also shown to carry out *N*-demethylation of codeine[2], thebaine and its *N*-variants[3] and *N*-oxidation of codeinone[4]. Bacterial *N*-demethylation of codeine to norcodeine[5] has also been documented earlier. However, there are very few reports on the microbial transformation of morphine. Transformation of morphine into 14-hydroxymorphine by resting cells as well as cell-free system of *Arthrobacter sp.* has been reported[6]. Morphinone and dihydromorphinone have also been identified as intermediates in the metabolism of morphine by *Pseudomonas putida* M10[7]. The present paper describes the transformation of morphine, codeine, their *N*-variants, dihyromorphine and dihydro-codeine by a *Bacillus sp.* culture.

Materials and Methods

Microorganism. The organism used in this study was isolated from soil using morphine as the sole source of carbon. The organism was identified as a *Bacillus sp.*

Chemicals. Morphine **1**, codeine **2** and thebaine were procured from Government Opium and Alkaloid works, Ghazipur, India. *N*-Alkyl variants of morphine and codeine[8], dihydromorphine and dihydrocodeine[9], 14-hydroxycodeinone[10], 14-hydroxymorphine, 14-hydroxy-codeine and 14-hydroxymorphinone[11] were prepared as described earlier.

Analytical methods. Thin layer chromatographic (TLC) analyses were performed on silica gel-G plates (0.5mm) developed with either 15% methanol in chloroform (system I) or 8% methanol in chloroform (system II). HPLC analyses were carried out on a Water Associates ALC/GPC 244 series instrument using a μ-Bondapak C_{18} (ODS) analytical column (300 × 3.9 mm). The mobile phase used for the analysis was the same as reported earlier[12]. Steroids were analysed on normal phase silica gel column using 2% methanol in chloroform. The metabolites formed were quantified on the basis of area under each peak.

Protein was estimated by the method of Lowry *et al*[13].

Fermentation conditions. The organism was propagated on nutrient agar slants which contained 1% peptone, 0.5% beef extract and 2% agar. Although the organism used morphine as the sole source of carbon, the growth rate was slow. To enhance the growth rate, 0.1% glucose was added to the medium. Fermentations were carried out in a liquid mineral salts medium[14] containing 0.1% glucose and the substrate (0.03% of **1/2**; 0.02% of **3-8**; added in 0.2-0.3 mL methanol). Flasks were inoculated with 5% of 48hr grown culture (A_{660} = 1.3) and incubated on a rotary shaker (220 rpm) at 29-30 °C for 5 days.

Extraction of metabolites. At the end of the incubation period, the contents from all the flasks were pooled, basified to *p*H 9.0 using 2*N* NH_4OH and extracted with chloroform-methanol (2:1). The organic layer was dried over sodium suphate and concentrated *in vacuo*. The crude extract was then subjected to silica gel column chromatography.

Spectral studies. Nuclear magnetic resonance (NMR) and mass spectra (MS) were recorded as described earlier[15].

Preparation of cell-free extract. Erlenymeyer flasks containing mineral salts medium (100 mL), glucose (0.1%), morphine (0.015%) were inoculated from a 2-day grown inoculum (5%, A_{660}=1.3) and were incubated at 29-30 °C on a rotary shaker for 48hr. At the end of incubation period, the cells were harvested (3,000 × g, 20 min), washed with phosphate buffer (10 m*M*, *p*H 7.2) and suspended in phosphate buffer (50 m*M*, *p*H 8.0) containing 10% glycerol, 1m*M* DTT and 1 m*M* EDTA at a final concentration of 0.2 g/mL cell wet weight. The cell suspension was then sonicated using a Branson B-30 sonifier for 4 min. with intermittent cooling for every 30 sec. at maximum output. The sonicate was centrifuged at 3,000 × g for 30 min. The supernatant thus obtained was further centrifuged at 20, 000 ×g for 30 min. The 20,000 × g supernatant was dialyzed (18hr) against phosphate buffer (50 m*M*, *p*H 8.0) containing 10% glycerol, 1 m*M* DTT and 1 m*M* EDTA. The dialyzed 20,000 × g supernatant was referred as crude cell-free extract. In a similar way cell-free extracts were prepared from cells grown in the presence of testosterone (0.02%) and glucose (0.2%).

Incubation conditions. The incubation mixture contained 50 m*M* phosphate buffer (*p*H 8.0),10% glycerol, 1 m*M* DTT, 1 m*M* EDTA, morphine or testosterone (0,15 μmol in 50 μL of methanol), cell-free extract (4 mg) and NAD^+ (0.2 μmol) in a total volume of 2.0 mL. Reaction was started by the addition of NAD^+ and incubated for 1hr at 29-30 °C on a rotary shaker. At the end of the incubation period the reaction mixture was extracted with (2:1) chloroform-methanol (for alkaloids) or with methylene chloride (for steroids), dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue was subjected to TLC and HPLC analyses.

Results

Transformation of morphine 1. A batch of 30 flasks fermentation was carried out as descried in "Materials and Methods". At the end of incubation, the contents of the flasks were pooled and processed as described. The curde extract (860 mg) on examination by TLC (system I) revealed the presence of one major (R_f 0.72) and one minor (R_f 0.50) metabolites. The crude extract was subjected to column chromatography over silica gel (25 g) and the compounds with R_f 0.72 (**1a**) and 0.5 (**1b**) were eluted from the column using 2% and 5% methanol in chloroform, respectively. The compounds **Ia** and **1b** were further purified by preparative TLC (system I). Based on spectral characteristics (**Table 1**) and by comparing the spectral data with those of authentic samples, **1a** and **1b** were identified as 14-hydroxymorphinone and 14-hydroxymorphine, respectively (**Scheme I**).

Transformation of codeine 2. Transformation of **2** was carried out in 24 flasks as described in "Materials and Methods". The crude extract (804 mg) on TLC analysis (system II) revealed the presence of one major (R_f 0.78) and one minor (R_f 0.59) metabolites. The crude extract was subjected to column chromatography over silica gel (30 g) and the compounds with R_f 0.78 (**2a**, 200mg) and 0.59 (**2b**, 18 mg) were eluted from the column using 1% and 3% methanol in chloroform, respectively. These compounds (**2a** and **2b**) were further purified by preparative TLC (system II). The compounds **2a** and **2b** were identified as 14-hydroxycodeinone and 14-hydroxycodeine, respectively by comparing their spectral data (**Table I**) with those of authentic samples.

Transformation of morphine analogues 3-6. Transformations of *N*-ethylnormorphine **3**, *N*-proplynormorphine **4**, *N*-butylnormorphine **5** and *N*-ethylnorcodeine **6** were carried out as described in "Materials and Methods". The crude extracts (from a batch of 5 flasks) obtained from all these incubations (**3**:125mg; **4**:120mg; **5**:110mg; **6**:132 mg) upon TLC analyses (system I) revealed the presence of one metabolite (**3a, 4a, 5a**, and **6a**) in each case besides the unreacted substrate. Metabolites **3a-6a** were separated by silica gel column chromatography using 1-2% methanol in chloroform. On the basis of various spectral analyses (**Table I**), the metabolites were

identified as 14-hydroxy-*N*-enthylnormorphinone **3a**, 14-hydroxy-*N*-propylnormorphinone **4a**, 14-hydroxy-*N*-butylnormorphinone **5a** and 14-hydroxy-*N*-ethylnorcodeinone **6a (Scheme I)**.

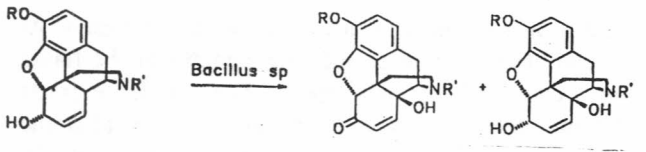
Transformation of dihydromorphine 7 and dihydrocodeine 8.

Incubations were carried out using 5 flasks for each substrate as described in "Materials and Methods". Substrate (0.02%) was added after 24hr of growth and incubation continued for a further period of 5 days. At the end of incubation period, the contents of the flasks were extracted as described. The crude extracts (**7**:96mg, **8**:85mg) on TLC examination revealed the presence of one metabolite (**7a**, R_f 0.31, system I; **8a**, R_f 0.39, system II) in each case besides the unreacted substrate. The metabolites were purified by silica gel column chromatography using 1-3% methanol in chloroform. Based on the spectral data (**Table I**) **7a** and

Table I.— Proton nuclear magnetic resonance (^{1}H NMR) and mass spectral data of the metabolites **1a-8a.**

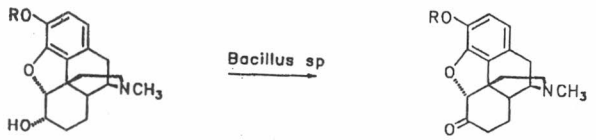
Compd	^{1}H NMR (δ) $CDCl_3$	MS (LR & HRMS)
1a	6.5-6.8(3H,m,Ar-H,H-8), 6.12(1H,d,10Hz,H-7), 4.68(1H,s,H-5) 3.2(2H,m, H-9, H_β-10), 2.6(1H,m, H_α-10), 2.5(3H,s,N-CH_3), 2.3(2H,t,H-16), 1.6(2H, m, H-15)	m/z 299 (M^+, 100%), 215 (M^+-$C_4H_4O_2$, 40%). HRMS: $C_{17}H_{17}NO_4$ requires 299.1158. Found 299.1161
1b	6.5(2H,*AB*$_q$, Ar-H), 5.8(1H,d,10.5Hz, H-7), 5.4(1H,dd,10.5Hz,H-8), 4.8 (1H ,d, 7.2Hz,H-5), 4.1(1H,m,H-6), 3.2(3H, m, H-9, H-10), 2.4(3H,s,*N*-CH_3), 2.3-2.6(2H,m,H-16), 1.7(2H,m, H-15)	m/z 301 (M^+, 100%), 215 (M^+-$C_4H_6O_2$, 30%). HRMS: $C_{17}H_{19}NO_4$ requires 301.1314. Found 301.1336
2a	6.5-6.7(3H,m,Ar-H,H-8), 6.15(1H,d,10.8Hz,H-7), 4.68(1H,s,H-5), 3.8(3H, s,-OCH_3), 3.32(1H,m, H-9), 3.04(2H,m,H-10), 2.44(3H,s,*N*-CH_3), 2.2-2.4 (2H,m, H-16)	m/z 313 (M^+, 100%), 299 (M^+-$C_4H_4O_2$, 40%)
2b	6.6(2H,*AB*$_q$, Ar-H), 5.9(1H,d,7.2Hz, H-7), 5.48(1H,dd, H-8), 4.88(1H,d, 7.2Hz,H-5), 4.64(1H,m,H-6), 3.84(3H,s,-OCH_3), 3-3.33(3H, m, H-9, H-10), 2.4(3H,s,*N*-CH_3), 2.2-2.3(2H,m,H-16), 1.6-1.8(2H,m, H-15)	m/z 315 (M^+, 100%), 299 (M^+-$C_4H_6O_2$, 38%)
3a	6.5-6.8 (3H, m, Ar-H, H-8), 6.2(1H, d, 9Hz, H-7), 4.7(1H, s, H-5) , 3.3(2H,m,H-9, H-10), 2.2-2.7(4H,m,H-16,*N*-CH_2), 1.7(2H,d,9Hz,H-15), 1.1(3H,t,CH_3 of *N*-Et)	m/z 313 (M^+, 100%), 299 (M^+-$C_4H_4O_2$, 50%)
4a	6.5-6.8(3H,m,Ar-H,H-8), 6.2(1H,d,9Hz,H-7), 4.7(1H,s,H-5), 3.3(3H, m,H-9, H-10), 2.2-2.7(4H,m,H-16,*N*-CH_2), 1.3-1.7(4H,m,H-15,-CH_2-of *N*-Pr), 0.95(3H,t,CH_3 of N-Pr)	m/z 327 (M^+, 100%), 243 (M^+-$C_4H_4O_2$, 30%)
5a	6.6-6.8(3H,m,Ar-H,H-8), 6.15(1H,d,9Hz,H-7), 4.7(1H,s,H-5) 3-3.3(3H, m, H-9, H-10), 2.2-2.7(4H,m,H-16,*N*-CH_2-), 1.2-1.7(6H,m,H-15,-CH_2-CH_2- of *N*-Bu), 0.9(3H,t,CH_3 of *N*-Bu)	m/z 341 (M^+, 100%), 257 (M^+-$C_4H_4O_2$, 33%)
6a	6.5-6.8(3H,m,Ar-H,H-8), 6.2(1H,d,9Hz,H-7), 4.7(1H,s,H-5), .85 (3H,s, OCH_3), 3-3.3(3H,m,H-9, H-10), 2.3-2.7(4H,m,H-16,*N*-CH_2-), 1.6-1.8(2H, m, H-15),1.8(3H,t,CH_3 of *N*-Et)	m/z 327 (M^+, 100%), 243 (M^+-$C_4H_4O_2$, 50%)
7a	6.64(2H,*AB*$_q$,Ar-H), 4.64(1H,s,H-5), 2.9-3.3(3H,m,H-9,H-10), 2.48 (3H,s,*N*-CH_3), 2.2-2.8(5H,m,H-7,H-14,H-16), 1.6-2(4H,m,H-8,H-15)	m/z 285 (M^+, 100%), 270 (M^+-CH_3 , 10%). HRMS: $C_{17}H_{19}NO_3$ requires 285.1365. Found 285.1359
8a	6.68(2H,*AB*$_q$, Ar-H), 4.66(1H,s,H-5), 3.92(3H,s,OCH_3), 2.6-3.3(4H,m,H-9,H-14,H-10), 2.28(3H,s,*N*-CH_3), 1.2-2.3(8H,m,H-7,H-8,H-15,H-16)	m/z 299 (M^+, 100%), 284 (M^+-CH_3 , 14%). HRMS: $C_{18}H_{21}NO_3$ requires 299.1522. Found 299.1524

Compounds **1a-6a** showed IR (nujol)ν_{max} at 3300-3600 for hydroxyl groups and at 1677-1686 cm^{-1} for conjugated carbonyl groups. **7a** and **8a** showed IR (nujol)ν_{max} at 1720-1730 cm^{-1} for carbonyl groups.



	R	R'	Yield (%)	
1	H	CH_3	94 (**1a**)	2 (**1b**)
2	CH_3	CH_3	53 (**2a**)	9 (**2b**)
3	H	$CH_2\,CH_3$	43 (**3a**)	–
4	H	$CH_2.\,CH_2\,CH_3$	20 (**4a**)	–
5	H	$CH_2(CH_2)_2\,CH_3$	20 (**5a**)	–
6	CH_3	$CH_2\,CH_3$	10 (**6a**)	–

Scheme I — Transformation of morphine **1a** codeine **2** and their analogues **3-6** by *Bacillus sp.*



	R	Yield (%)
7	H	95 (**7a**)
8	CH_3	82 (**8a**)

Scheme II— Transformation of dihydromorphine **7** and dihydrocodeine **8** by *Bacillus sp.*

8a were identified as dihydromorphinone and dihyrocodeinone, respectively (**Scheme II**).

Metabolism of intermediates. Flasks containing 100 mL of mineral salts medium and 0.1% glucose were inoculated and incubated as described in "Materials and Methods". After 24hr, to one flask 14-hydroxycodeine (**2b**, 10 mg in 0.1mL methanol) and to another flask codeinone (10 mg in 0.1mL methanol) were added and incubation continued for a further period of 3 days. At the end of the incubation period, the contents of the flasks were processed as described in "extraction of metabolites". The crude extracts on HPLC analyses indicated that 75% of the added codeinone was transformed into 14-hydroxycodeinone **2a**, whereas only 10% of the added 14-hydroxycodeine **2b** was transformed into 14-hydroxycodeinone **2a**.

Experiments with cell-free extracts. Assays carried out using cell-free extracts prepared from morphine grown cells clearly showed the formation of 14-hydroxymorphinone from morphine in the presence of NAD^+. TLC analysis showed the formation of one metabolite with R_f 0.72(system I) which is same as that of **1a**. It was further confirmed by HPLC analysis where the enzymatically formed compound had the same retention time as that of authentic 14-hydroxymorphinone (Rt 3.5 min). HPLC analysis also indicated that about 25% morphine was transformed into 14-hydroxymorphinone. However, the cell free extract did not contain any 17β-hydroxysteroid dehydrogenase activity as evidenced by its inability to transform testosterone into androstenedione in the presence of either NAD^+ or $NADP^+$. On the contrary, cell-free extracts prepared from cells grown in the presence of testosterone readily converted testosterone to androstenedione in the presence of NAD^+, whereas it showed no activity against morphine. The enzymatically formed compound with R_f 0.5 (system I) was identified as androstenedione by comparing with an authentic sample. It was further confirmed by HPLC analysis where the enzymatically formed compound had the same retention time (Rt 7.0 min) as that of authentic androstenedione. HPLC analysis indicated that over 90% of testosterone was transformed into androstenedione. The cell-free extracts prepared from cells grown only in the presence of glucose did not show any activity against morphine as well as testosterone suggesting that morphine dehydrogenase as well as 17β-hydroxysteroid dehydrogenase are induciable in nature. Both morphine dehydrogenase and 17β-hydroxysteroid dehydrogenase of *Bacillus sp.* showed absolute requirement for NAD^+ for oxidation and NAD^+ could not be replaced by $NADP^+$.

Discussion

The present studies have clearly demonstrated that the *Bacillus sp.* effectively carries out the oxidation of the secondary alcoholic group at C-6 and 14-hydroxylation in both morphine **1** and codeine **2** yielding 14-hydroxymorphinone **1a** and 14-hydroxycodeinone **2a**, respectively as major transformation products (**Scheme I**). The minor transformation products are 14-hydroxymorphine **1b** and 14-hydroxycodeine **2b.** Since codeinone is transformed at significantly faster rate than 14-hydroxycodeine **2b** into 14-hydroxycodeinone **2a**, it is reasonable to assume that the oxidation of the secondary alcoholic group at C-6 takes place prior to allylic hydroxylation. Similar sequence of reactions has been noticed earlier in the conversion of morphine and codeine into 14-hydroxymorphinone and 14-hydroxycodeinone, respectively by *Pseudomonas testosteroni*[16]. In contrast, the ability of *Pseudomonas putida* M10 to oxidise morphine and codeine into morphinone and codeinone, respectively has been reported[6]. It was further demonstrated that a constitutive NADH-dependent morphinone reductase catalyses the reduction of the 7,8-double bond of morphinone and codeinone[17]. However, the organism failed to carry out 14-hydroxylation reaction. The *Bacillus sp.* used in the present studies is devoid of morphinone reductase activity. When dihydromorphine **7** and dihydrocodeine **8** were used as substrates, the

organism readily transformed them only into dihydromorphinone **7a** and dihydrocodeinone **8a** respectively (**Scheme II**). This suggests that the 7,8-double bond is a necessary structural feature to carry out 14-hydroxylation reaction.

We were interested to find out whether *Bacillus sp.* still retains its ability to carry out oxidation of the secondary alcoholic group at C-6 and hydroxylation at C-14 if *N*-methyl group in morphine and codeine is replaced by higher alkyl groups. It is known that the physiological properties of morphine are altered greatly with substitution on the N-atom and the length of the *N*-alkyl chain is critical for exhibiting analgesic or antimorphine properties[18]. The present studies clearly indicate that the organism retains its ability to carry out oxidation of secondary hydroxyl group at C-6 position and hydroxylation at C-14 position in all the *N*-variants of morphine and codeine tested (**Scheme I**) although these compounds are not good substrates for *Bacillus sp.*

The hydroxysteroid dehydrogenase of *Pseudomonas testosteroni*[16] has been shown to transform morphine into morphinone in the presence of NAD^+. This is different to what has been observed in the present studies. The cell-free extract prepared from morphine grown cells of *Bacillus sp.* contained an NAD^+ - dependent dehydrogenase active against morphine and codeine, but failed to act on testosterone. However, cells grown in the presence of testosterone contained high levels of NAD^+- dependent 17β-hydroxysteroid dehydrogenase but devoid of morphine dehydrogenase activity. These observations suggest that the NAD^+-dependent morphine dehydrogenase and hydroxysteroid dehydrogenase in *Bacillus sp.* are different from each other. Similar observations have been made earlier with cell-free extracts prepared from morphine grown cells of *Pseudomonas putida* M10[19]. Interestingly, morphine dehydrogenase from *Bacillus sp.* and from *Pseudomonas putida* M10 are having different pyridine nucleotide specificity.

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