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Naphthalene-dioxygenase-catalysed *cis*-dihydroxylation of azaarene derivatives Part 1: 2-Pyridones and 2-quinolones

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Abstract

The scope of the biotransformation of 2-pyridone- and 2-quinolone-derived compounds by recombinant whole-cells of *E. coli* JM109(DE3)(pDTG141) expressing the naphthalene-dioxygenase system from *Pseudomonas* sp. NCIB 9816-4 was explored, using a series of *N*- and *C*-substituted derivatives. Among them, only the *N*-methyl substituted compounds were good substrates for a regio- and stereoselective dihydroxylation reaction leading to *cis*-dihydroxydihydro pyridone derivatives, corresponding to the general pattern expected for this enzyme. In the absence of dihydroxylation reactions, *N*-dealkylations and monohydroxylations on external methyl groups were observed.

Keywords: *N*-Methyl-2-pyridone; *N*-Methyl-2-quinolone; *Pseudomonas* sp.; Dihydroxylation; Naphthalene-dioxygenase

1. Introduction

The bacterial metabolism of aromatic compounds often proceeds via an initial dioxygenase-catalysed dihydroxylation to yield *cis*-dihydrodiol derivatives. A large range of mono- and polycyclic substrates has been reported to form the corresponding enantiopure *cis*-dihydrodiol metabolites [1–3]. Multicomponent bacterial dioxygenases, mainly toluene dioxygenase (TDO) and naphthalene-dioxygenase (NDO), have been frequently utilized to produce hundreds of such *cis*-1,2-dihydrodiols, several of which have been used as chiral synthons for the synthesis of biologically active products and value-added chemicals [4].

However, to date, only a few data have been reported about the *cis*-dihydroxylation of compounds derived from heterocyclic arenes [1,5,6], including polycyclic azaarenes [5,7], quinoline [8,9], isoquinoline [9], quinoxaline [9], quinazoline [9], benzothiophene [6,10] and benzofuran [6,11]. Generally, dihydroxylation of such substrates occurs in the carbocyclic rings and only scarce examples of dihydroxylation in the heteroarene portion of the substrate or dihydrodiol metabolite formation from monocyclic heteroarenes had been described in Refs. [6,9]. However, in the recent years, direct evidence for the *cis*-dihydroxylation of a 2-pyridone ring by NDO has been reported in our laboratory and a major dihydroxylated metabolite, *N*-methyl-*cis*-5,6-dihydroxy-5,6-dihydro-2-pyridone was obtained from the corresponding *N*-methyl-2-pyridone substrate, together with small amounts of its 3,4-dihydroxy-dihydro isomer [12,13].

The present study was initiated to extend this observation by investigating the effect of new substituents on 2-pyridone and 2-quinolone rings (see Scheme 1) in the NDO-catalysed reaction and to elaborate new methods to directly and definitively determine the absolute configuration and enantiomeric

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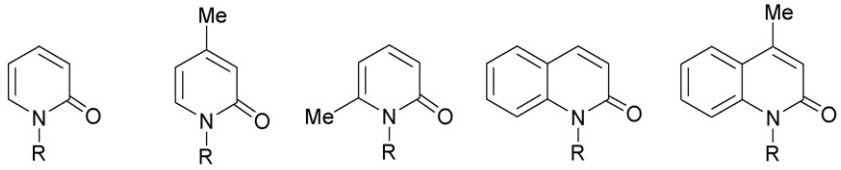


1a, R = Me
1b, R = CH_2-Phe
1c, R = CH_2-CH=CH_2

2a, R = Me
2b, R = CH_2-Phe

3a, R = Me
3b, R = CH_2-Phe

4a, R = Me
4b, R = CH_2-Phe

5a , R = Me

Scheme 1.

purity of the dihydrodiols, respectively, formed from *N*-methyl-2-pyridone and *N*-methyl-2-quinolone by this bacterial dioxygenase.

2. Experimental

2.1. Substrates and chemicals

Substrate **1a** was from commercial source. 4-Bromophenyl boronic acid was from Aldrich and 4-bromobenzoyl chloride from Fluka. (*R*)-Phenylglycine methyl ester hydrochloride was from Aldrich, (*S*)-phenylglycine methyl ester hydrochloride, and (*S*)-phenylglycine *t*-butyl ester hydrochloride were from Novabiochem. (*R*)-Phenylglycine *t*-butyl ester hydrochloride was from Bachem. Substrates **1b**, **1c**, **2a**, **2b**, **3a**, **3b**, **4a**, **4b** and **5a** were, respectively, prepared from 2-hydroxypyridine (**1b**, **1c**), 2-hydroxy-4-methyl pyridine (**2a**, **2b**), 2-hydroxy-6-methyl pyridine (**3a**, **3b**), 2-hydroxyquinoline (**4a**, **4b**) and 2-hydroxy-4-methylquinoline (**5a**), by literature procedures [14] and fully characterised by ESI-MS and NMR. Detailed complementary physical data of some compounds are given below.

N-Methyl-4-methyl-2-pyridone **2a**: $(M+H^+)$ 124; δ_H 250 MHz: 7.43 (1H, d, $J_{6,5}=6.9$, 6-H), 6.13 (1H, br.s, 3-H), 5.94 (1H, dd, $J_{5,6}=6.9$, $J_{5,3}=1.9$, 5-H), 3.39 (3H, s, N–CH_3), 2.07 (3H, s, 4-CH_3). δ_C 62.9 MHz: 162.5 (C2), 118.5 (C3), 151.1 (C4), 107.4 (C5), 138.7 (C6), 36.3 (CH_3–N), 20.6 (CH_3–C).

N-Benzyl-4-methyl-2-pyridone **2b**: $(M+H^+)$ 200; δ_H 250 MHz: 7.54 (1H, d, $J_{6,5}=7.0$, 6-H), 7.25–7.35 (5H, m, ArH), 6.26 (1H, br.s, 3-H), 6.05 (1H, dd, $J_{3,5}=1.9$, $J_{5,6}=7.0$, 5-H), 5.13(2H, s, N–CH_2), 2.15 (3H, s, CH_3). δ_C 62.9 MHz: 162.4 (C2), 119.3 (C3), 151.3 (C4), 108.1 (C5), 137.8 (C6), 51.3 (CH_2–N), 128–129 (Ar–C), 138.5 (Ar–C).

N-Methyl-6-methyl-2-pyridone **3a**: $(M+H^+)$ 124; δ_H 250 MHz: 7.25 (1H, dd, $J_{4,3}=9.1$, $J_{4,5}=6.8$, 4-H), 6.27 (1H, d, $J_{3,4}=9.1$, 3-H), 6.10 (1H, d, $J_{5,4}=6.8$, 5-H), 3.48 (3H, s, N–CH_3), 2.38 (3H, s, 6-CH_3). δ_C 62.9 MHz: 163.9 (C2), 116.8 (C3), 139.4 (C4), 106.4 (C5), 148.2 (C6), 30.9 (CH_3–N), 20.6 (CH_3–C).

N-Benzyl-6-methyl-2-pyridone **3b**: $(M+H^+)$ 200; δ_H 250 MHz: 7.33 (1H, m, 4-H), 7.22–7.36 (5H, m, ArH), 6.36 (1H, d, $J_{3,4}=9.1$, 3-H), 6.06 (1H, d, $J_{5,4}=6.7$, 5-H), 5.36 (2H, s, N–CH_2), 2.27 (3H, s, C–CH_3). δ_C 62.9 MHz: 163.8 (C2), 118.1 (C3), 140.3 (C4), 106.7 (C5), 148.2 (C6), 47.4 (–CH_2–N), 127–129 (Ar–C), 138.4 (Ar–C).

2.2. Analytical procedures

^{1}H NMR and ^{13}C NMR spectra were recorded in d_6-acetone (unless stated otherwise) on Bruker ARX250 (250.13 MHz Larmor frequency for ^{1}H) or Bruker Avance500 (500.13 MHz Larmor frequency for ^{1}H) spectrometers. Chemical shifts (δ) are reported in ppm and coupling constants are given in Hz. ^{1}H signals were assigned using 2D homonuclear ^{1}H–^{1}H COSY and TOCSY experiments. The TOCSY and the COSY spectra were used to distinguish the spin groups and to confirm the assignments of the hydrogen resonances within each spin group. The $^3J_{1H,1H}$ coupling constants were determinated by homodecoupling experiments of 1D ^{1}H spectra. ^{13}C signals were assigned using 2D heteronuclear ^{1}H–^{13}C HSQC and HMBC experiments. Optical rotation measurements ($[\alpha_D]$) were carried out with a Perkin-Elmer 241 polarimeter at ambient temperature (ca 20 °C).

1,2-Dihydroxy-1,2-dihydronaphthalene determinations were performed at 40 °C with UV detection at 254 nm on a Lichrospher 100 RP18e column (Hewlett Packard, 4.6 mm × 250 mm, 5 μm), equilibrated with a water–acetonitrile mobile phase (60:40) and eluted with an acetonitrile gradient containing formic acid (999:1) at a 0.5 mL/min flow rate.

HPLC-MS analyses were performed on a Thermo Finnigan LCQ Advantage Instrument (electrospray positive mode) equipped for HPLC with a Polarity dC18 column (Waters, 4.6 mm × 250 mm, 5 μm) equilibrated with the appropriate water–acetonitrile mobile phase. Products were eluted with an acetonitrile gradient containing formic acid (999:1) at 40 °C and 0.4 mL/min flow rate.

Flash chromatography was performed on Kieselgel Si60 (Merck, 40–63 μm) columns equilibrated with CH_2Cl_2 and eluted with CH_2Cl_2–acetone–methanol in the appropriate ratios.

2.3. Microorganism and culture media

E. coli JM109(DE3)(pDTG141) was kindly provided by D.T. Gibson (Iowa University). This strain which contains the cloned nahAaAbAcAd genes encoding for the NDO components from *Pseudomonas* sp. strain NCIB 9816-4 [15] was used for all biotransformations. Cells were grown at 37 °C and 350 rpm stirring in a 6 L fermentor containing mineral salt broth [16] supplemented with yeast extract (Difco, 4 g L^{-1}), thiamine (1 mM), ampicillin (sodium salt, 100 mg L^{-1}) and glucose (0.36%). During the growth, pH was maintained at 7.2 by addition of ammonia

solution (6%), and glucose (30%, 50 mL) was added every hour. When OD_{600} reached 0.7, temperature was lowered to 27 °C and isopropyl-β-D-galactopyranoside (IPTG) (final concentration 1 mM) was added. Incubation was continued at 27 °C for 3 h. Microbial biomass was harvested by centrifugation (8000 rpm, 15 min), washed with BTDG (50 mM Bistris–HCl pH 7.2, 5% glycerol, 1 mM dithiothreitol) and stored at −80 °C.

The dihydroxylating activity of the cells toward naphthalene was routinely examined in initial rate conditions as described for analytical biotransformations (see below). Four values of cell concentrations (final OD_{600} 0.04–0.2) were used and aliquots withdrawn after 0.5, 1, 2, 3 and 6 h were analysed by HPLC. Activity was found to be about 0.8(±0.1) mM 1,2-dihydroxydihydronaphthalene formed/hour/OD_{600} unit.

2.4. General procedure for analytical biotransformations

In optimised conditions, frozen cells were suspended in 20 mL of 0.1 M phosphate buffer pH 7.2 (about 0.5 g of cells, OD_{600} about 7), containing sodium ampicillin (0.27 mM, 0.1 g L^{-1}) and glucose (0.2%) in 125 mL conical flasks. After addition of 5 mg of substrate, the biotransformation was conducted at 27 °C with orbital shaking at 250 rpm for 30 h. Aliquots (0.7 mL) were withdrawn at intervals, centrifuged and microfiltered for direct HPLC-MS analysis.

2.5. General procedure for preparative biotransformations

Frozen cells (about 12 g) were suspended in 500 mL of 0.1 M phosphate buffer (pH 7.2) containing sodium ampicillin (0.27 mM) and glucose (0.2%) in 2 L conical flasks. After addition of 150 mg of substrate, biotransformations were conducted at 27 °C with orbital shaking at 250 rpm for 30 h. Aliquots (0.7 mL) were withdrawn at intervals, centrifuged and microfiltered for direct HPLC-MS analysis. The final yields of the biotransformations were calculated by comparing the integrated peaks areas of products to that of the remaining substrate in the HPLC-MS analysis, at the end of the incubation.

After centrifugation of the microbial biomass, the bioproducts were isolated by freeze-drying of the supernatant followed by extraction of residual material by acetone at room temperature. Purification of the metabolites was achieved by column chromatography and their structure established by ^{1}H and ^{13}C NMR spectroscopy.

2.6. Characterisation and identification of biotransformation metabolites

2.6.1. Biotransformation of N-methyl-2-pyridone

In the optimised conditions only a single product was isolated by flash chromatography as colourless crystals (35–40% yield) and identified as the *cis*-(5*S*,6*S*)-dihydroxy-5,6-dihydro-1-methyl-2-pyridone (**6a**). $[\alpha]_D$ +41 (c 0.017, MeOH) (lit [13] +41, MeOH). $M+H^+$ 144. δ_H 500 MHz: 6.37 (1H, ddd, $J_{4\text{-}3}=10$, $J_{4\text{-}5}=2$, $J_{4\text{-}6}=1.7$, 4-H), 5.71 (1H, dd, $J_{3\text{-}4}=10$, $J_{3\text{-}5}=2.5$, 3-H), 4.93 (1H, dt, $J_{6\text{-}5}=4.4$, $J_{6\text{-}4}=1.7$, $J_{6\text{-}OH}=5.6$, 6-H), 4.57 (1H, br.m, $J_{6\text{-}5}=4.4$, $J_{5\text{-}4}=J_{5\text{-}3}=2.2$, 5-H), 4.23 and 4.99 (2H, 5-OH and 6-OH), 2.94 (3H, s, CH_3). δ_C 125 MHz: 164.3 (C2), 143.1 (C4), 123.6 (C3), 84.3 (C6), 67.3 (C5), 32.8 (N–CH_3).

(4-Bromo)phenylboronate derivative (**D6a**): *cis*-(5*S*,6*S*)-dihydroxy-5,6-dihydro-1-methyl-2-pyridone **6a** (20 mg, 0.139 mmole) and 4-bromophenyl boronic acid (30 mg, 1.1 equiv.) were reacted in CH_2Cl_2 (5 mL) at room temperature during 1 h. The product formed was purified by flash chromatography with CH_2Cl_2 as eluent. Crystals suitable for X-ray analysis were grown by slow evaporation from the CH_2Cl_2 solution. δ_H 250 MHz: 7.51–7.62 (4H, m, Ar–H), 6.58 (1H, dd, $J_{4\text{-}3}=10$, 4-H), 6.04 (1H, d, $J_{6\text{-}5}=7.4$, 6-H), 5.94 (1H, d, $J_{4\text{-}3}=10$, 3-H), 5.39 (1H, d, $J_{5\text{-}6}=7.7$, 5-H), 3.09 (3H, s, CH_3).

For the determination of its enantiomeric excess, **6a** (3.2 mg, 0.022 mmole) in d_6-acetone (0.5 mL) and 2-formyl phenylboronic acid (3.4 mg, 1 equiv.) in $CDCl_3$ (0.5 mL) were mixed, then the solvent was evaporated. The boronic ester was solubilised in $CDCl_3$ in the presence of 4 Å molecular sieve and the completion of the reaction was checked by NMR. (*S*)-Phenylglycine *t*-butylester hydrochloride (5.5 mg, 1 equiv.) and $CsCO_3$ (8 mg, 1.1 equiv.) were suspended in $CDCl_3$ (0.5 mL) and stirred at room temperature until complete transformation to free aminoester, then the suspension was filtered on a celite pad. The resulting solution was added to the boronic ester to give the ternary derivative. The same protocol was used with all aminoesters and *rac*-1,2-dihydroxy-1,2-dihydronaphthalene.

2.6.2. Biotransformation of N-allyl-2-pyridone

Flash chromatography afforded two products (5–6% yield) which were only partially separated and respectively identified as the *cis*- and the *trans*-5,6-dihydroxy-5,6-dihydroderivatives.

Cis-(5*S*,6*S*)-dihydroxy-5,6-dihydro-1-allyl-2-pyridone (**6c**). $M+H^+$ 170. δ_H 500 MHz: 6.37 (1H, dt, $J_{4\text{–}3}=10$, $J_{4\text{–}5}=J_{4\text{–}6}=2$, 4-H), 5.77 (1H, dd, $J_{3\text{–}4}=10$, $J_{3\text{–}5}=2.5$, 3-H), 4.89 (1H, dt, $J_{6\text{–}5}=4.7$, $J_{6\text{–}4}=2$, 6-H), 4.58 (1H, br.m, $J_{5\text{-}6}=4.7$, $J_{5\text{-}3}=2.5$, $J_{5\text{–}4}=2.1$, 5-H), 4.30 (1H, d, $J_{5\text{-}5OH}=8.6$, 5-OH), 5.01 (1H, d, $J_{6\text{-}6OH}=6$, 6-OH), 5.92 and 5.87 (2H, 2m, –C_b=C_cH_2), 5.85 (1H, m, –C_bH=), 4.53 and 3.65 (2H, 2 m, N–C_aH_2–). δ_C 125 MHz: 162.5 (C2), 143.5 (C4), 135 (C_b),125 (C_a), 122 (C3), 80 (C6), 66 (C5), 46 (C_c).

Trans-5,6-dihydroxy-5,6-dihydro-1-allyl-2-pyridone.
$M+H^+$ 170. δ_H 500 MHz: 6.62 (1H, ddd, $J_{4\text{-}3}=9.8$, $J_{4\text{-}5}=5.2$, $J_{4\text{-}6}=1.5$, 4-H), 5.92 (1H, d, $J_{3\text{-}4}=9.8$, 3-H), 4.96 (1H, dt, $J_{6\text{–}5}=2.1$, $J_{6\text{-}4}=1.7$, 6-H), 4.16 (1H, dd, $J_{5\text{-}4}=5.2$, $J_{5\text{–}6}=2.1$, 5-H), 5.14 (b.m, 5-OH), 4.55 (b.m, 6-OH), 5.31 and 5.11 (2H, 2m, –C_b=C_cH_2), 5.82 (1H, m, –C_bH=), 4.54 and 3.70 (2H, 2 m, N–C_aH_2–). δ_C 125 MHz: 162 (C2), 138 (C4), 134 (C_b), 124 (C3), 116 (C_a), 83 (C6), 66 (C5), 45 (C_c).

2.6.3. Biotransformation of N-methyl-4-methyl-2-pyridone

HPLC-MS analysis of the final incubation product revealed two major metabolites with *m/z* 158 and *m/z* 110 which were, respectively, identified as a dihydroxydihydroderivative and a *N*-desmethylated compound (11% yield) identical to 4-methyl-2-hydroxy-pyridine. After chromatographic purification, the dihydroxy dihydroproduct (4–5% yield) was fully characterized by ^{1}H and ^{13}C NMR as *cis*-5,6-dihydroxy-5,6-

dihydro-1,4-dimethyl-2-pyridone (**8a**). $[\alpha]_D + 17.5$ (c 0.016, MeOH). $M+H^+$ 158. δ_H 500 MHz: 5.55 (1H, quint., $^4J_{3\text{-}4Me} = {}^4J_{3\text{-}5} = 1.5$, 3-H), 4.98 (1H, d, $^3J_{6\text{-}6OH} = 4.3$, 6-OH), 4.92 (1H, dd, $^3J_{5\text{–}6} = 4.2$, $^3J_{6\text{-}6OH} = 4.3$, 6-H), 4.42 (1H, bm, $^4J_{3\text{-}5} = {}^4J_{4Me\text{-}5} = 1.5$, $^3J_{5\text{-}5OH} = 8.9$, $^3J_{5\text{-}6} = 4.2$, 5-H), 4.11 (1H, d, $^3J_{5\text{-}5OH} = 8.9$, 5-OH), 2.92 (3H, s, N–CH_3), 1.90 (3H, t, $^4J_{3\text{-}4Me} = {}^4J_{4Me\text{-}5} = 1.5$, 4-$CH_3$). δ_C 125 MHz: 163.9 (C2), 151.7 (C4), 118.8 (C3), 83.3 (C6), 68.6 (C5), 31.3 (N–CH_3), 17.4 (4-CH_3).

2.6.4. Biotransformation of N-methyl-6-methyl-2-pyridone

HPLC-MS analysis of incubation medium revealed two metabolites. One of them (*m/z* 110) was identified by comparison with an authentic sample as 6-methyl-2-hydroxy-pyridine. The major one (*m/z* 140) was purified by silicagel chromatography (16% yield) and identified by ^1H and ^{13}C NMR analysis as 6-hydroxymethyl-1-methyl-2-pyridone. $M+H^+$ 140. δ_H 250 MHz: 6.29–6.32 (2H, m, 3-H, 5-H), 7.31 (1H, dd, 4-H), 4.58 (2H, s, –CH_2–O–), 3.50 (3H, s, N–CH_3). δ_C 62.9 MHz: 164.0 (C2), 152.0 (C6), 139.6 (C4), 118.9 (C3), 104.8 (C5), 61.6 (–CH_2–OH), 30.0 (N–CH_3).

2.6.5. Biotransformation of N-methyl-2-quinolone

HPLC-MS analysis of incubation medium revealed two metabolites; one of them (*m/z* 146) was identified by comparison with an authentic sample as 2-hydroxy-quinoline. The other one (*m/z* 194) was purified by silicagel chromatography (28% yield) and identified by ^1H and ^{13}C NMR analysis as *cis*-3,4-dihydroxy-3,4-dihydro-1-methyl-2-quinolone **7a**. Colourless crystals. $M+H^+$ 194; $[\alpha]_D + 19.4$ (c 0.31, MeOH); δ_H 7.42 (1H, m, $^3J_{7\text{-}8} = {}^3J_{6\text{–}7} = 7.5$, $^4J_{5\text{–}7} = 1.6$, 7-H), 7.40 (1H, m, $^3J_{5\text{-}6} = 7.5$, $^4J_{5\text{–}7} = 1.6$, 5-H), 7.19 (1H, dd, $^3J_{7\text{-}8} = 7.5$, $^4J_{6\text{-}8} = 1.0$, 8-H), 7.12 (1H, td, $^3J_{5\text{-}6} = {}^3J_{6\text{-}7} = 7.5$, $^4J_{6\text{-}8} = 1.0$, 6-H), 4.74 (1H, dd, $^3J_{3\text{-}4} = 3.2$, $^3J_{4\text{-}4OH} = 2.6$, 4-H), 4.40 (1H, br,s, $^3J_{4\text{-}4OH} = 2.6$, 4-OH), 4.28 (1H, t, $^3J_{3\text{-}4} = {}^3J_{3\text{-}3OH} = 3.2$, 3-H), 4.23 (1H, br d, $^3J_{3\text{-}3OH} = 3.2$, 3-OH), 3.39 (3H, s, N–CH_3). δ_C: 161.8 (C2), 139.6 (C8a), 129.7 (C5), 129.5 (C7), 126.0 (C4a), 123.1 (C6), 115.4 (C8), 71.2 (C3), 69.2 (C4), 29.1 (N–CH3).

Bis(*p*-bromo)benzoyl derivative (**D7a**): 0.336 mmole of **7a**, TEA (2.2 equiv.) and DMAP (0.2 equiv.) were solubilised in 10 mL CH_2Cl_2, then 4-bromobenzoyl chloride (2.2 equiv.) dissolved in 5 mL CH_2Cl_2 was added dropwise at 0 °C. After 12 h, the product was isolated and purified by flash chromatography with CH_2Cl_2-cyclohexane (1:1) as eluent. Crystals suitable for X-ray analysis were obtained by slow evaporation at room temperature from a methanol–anisole solution. δ_H 250 MHz: 7.59–7.72 (4H, m, Ar–H), 6.58 (1H, m, 4-H), 6.04 (1H, d, 6-H), 5.94 (1H, d, 3-H), 5.40 (1H, d, 5-H), 3.09 (3H, s, N–CH_3).

2.7. X-ray crystal data

Crystal structure diffraction data of the 4-bromophenylboronate (**D6a**) and di-4-bromobenzoate (**D7a**) derivatives were recorded on a CCD Nonius diffractometer operating the Mo Kα wavelength (λ = 0.7107 Å). In each case, a full sphere of diffraction was recorded. Data reductions were performed as usual but not merged. The two structures were solved by direct methods and their two absolute configurations were refined independently. The X-ray statistics and results are reported in Table 1. The two structure files were deposited with the Cambridge Data Centre as ref. Number CCDC639623 for the (4-bromo)phenylboronate derivative **D6a** and CCDC 639624 for the bis(*p*-bromo)benzoyl derivative **D7a** [17].

Table 1
X-ray diffraction results

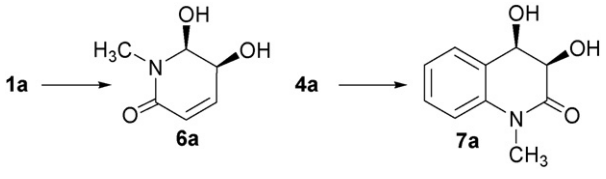
	D6a	D7a
Formula	$C_{12}BBrH_{11}NO_3$	$C_{24}Br_2H_{17}NO_5$
Molecular weight	307.94	559.21
Space group	$P2_12_12_1$	$P2_1$
Parameters (Å, °)		
a	6.882(1)	10.231(1)
b	15.743(1)	17.125(1)
c	22.867(1)	12.734(1)
β	–	93.10(3)
Volume (Å^3)	2477.5(4)	2227.9(2)
Z	8	4
Nb. of reflection measured	5631	9921
Completeness (%)	99.5	99.8
R sym (%) overall	4.1	3.9
Nb. of reflections in refinements	2551	6338
Nb. of observed reflections (*)	2343	5403
R factor (observed *F* data)	0.031	0.051
R factor (all *F* data)	0.037	0.066
R factor (observed F^2 data)	0.062	0.122
Flack index (direct/inverted)	0.02(1)/0.98(2)	0.01(1)/0.98(2)
Nb. of parameters	325	577
Min/max (e^-) in last electron density	−0.241/+0.184	−0.370/+0.307

*) Criteria for observation: $F \geq 2s(F)$.

3. Results

3.1. Biotransformation of N-methyl-2-pyridone

The previously used *E. coli* JM109(DE3)(pDTG141), a strain that overexpresses the NDO system from *Pseudomonas* sp. NCIB 9816-4, was used, as a whole-cell biocatalyst, in order to establish the scope of the biotransformation of various 2-pyridone and 2-quinolone derivatives differently substituted on the nitrogen or on the carbon atoms of the heterocyclic ring (Scheme 2). First of all, an optimisation of the production of *N*-methyl-*cis*-5,6-dihydroxy-5,6-dihydropyridone (**6a**) from *N*-methyl-2-pyridone (**1a**) was undertaken in order to obtain good and reproducible conversion yields with a minimum of contaminants arising from the self-metabolism of the cells, that impaired the purification of the desired metabolite. Several



Scheme 2.

buffers were tested (MES, phosphate, Tris, Bistris) at various pHs. The best results were obtained using 0.1 M phosphate buffer, 7.2 < pH < 7.5. A significant effect of decreasing the incubation temperature (up to 25 °C) was observed, allowing to obtain in 30 h-incubations the *cis*-5,6-dihydrodiol **6a**, identical to that previously described [13], in 35–40% isolated yield, without any noticeable presence of the 3,4-dihydrodiol, any *trans*-isomer or any dehydration product. However, compared to naphthalene hydroxylation in the same conditions, the best dihydroxylation rate of *N*-methyl-2-pyridone (μmoles/mg fresh biomass) remained in a low range, approximating 200:1. Non-*N*-substituted 2-pyridone (2-hydroxy-pyridine) is not transformed by NDO in any conditions [13].

3.2. Biotransformation of N-methyl-2-quinolone

When *N*-methyl-2-quinolone (**4a**) was incubated in the same conditions, the final transformation yield was higher (see Table 2): fair yield (28%) of a single *cis*-diol product was obtained but a large amount of *N*-dealkylation product (34%), characterised by comparison with authentic 2-hydroxy-quinoline, was observed. The diol was unambiguously identified by mass spectrometry and ^{1}H and ^{13}C NMR as the *N*-methyl-*cis*-3,4-dihydroxy-3,4-dihydro-2-quinolone **7a**. No evidence for any mono- or dihydroxylated product in the carbocyclic ring could be obtained, contrarily to the reported results of the TDO-catalysed transformation of quinoline [8]. Non-*N*-substituted 2-quinolone (2-hydroxy quinoline) was not a substrate for any transformation by NDO.

3.3. Absolute configuration and enantiomeric purity of N-methyl-5,6-dihydroxy-cis-5,6-dihydro-2-pyridone (6a)

In the previously cited work [12], a (5*S*,6*S*) absolute configuration was tentatively assigned to this *cis*-diol, on the basis of relative chemical shifts in NMR determinations of a cyclic chiral boronate derivative, from a delicate analysis of direct X-ray crystal structure of the underivatized diol, and from a comparison of circular dichroism spectra with the corresponding 2-quinolinone derivative.

In order to confirm and ascertain this stereochemical assignment, the 4-bromophenylboronate cyclic derivative of the 5,6-*cis*-dihydrodiol **6a** was easily prepared in a stoichiometric reaction with 4-bromophenylboronic acid and suitable crystals for X-ray diffraction were subsequently obtained by slow evaporation of a chloroform solution. Analysis of the structure, facilitated by the heavy atom substituent and based on a clear indication of the Flack index [18] during the refinements [19], unequivocally revealed a *cis*-diaxial (5*S*,6*S*)-configuration for this derivative as shown in Fig. 1. The crystal structure analysis also indicated that the sample was enantiopure.

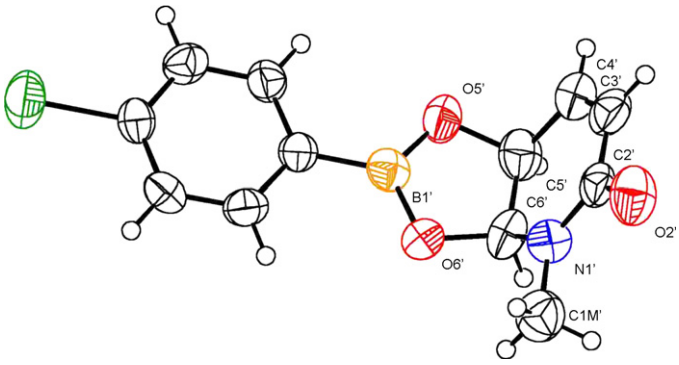


Fig. 1. X-ray structure of the 4-bromophenylboronate derivative of *cis*-5,6-dihydroxy-5,6-dihydro-1-methyl-2-pyridone.

The enantiomeric purity of the *cis*-dihydrodiol was additionally confirmed by using an original method deriving from a ^{1}H NMR three-component derivatization protocol recently described for the analysis of the enantiomeric purity of primary amines [20]: a cycloadduct of 2-formylphenylboronic acid with a chiral *cis*-diol (*S*-BINOL) is able to react with an amine to give quantitatively the corresponding iminoboronate ester. In particular this has been tested with *rac*-2-phenylglycine methyl ester and *rac*-2-phenylglycine *t*-butyl ester, resulting in a large splitting of several characteristic protons of the diastereoisomeric complex. This induced us to use in turn this "three-component" protocol to determine the enantiomeric purity of our *cis*-dihydrodiols [21] taking advantage of the commercial availability of (*R*)- and (*S*)-2-phenylglycine methyl and *t*-butyl esters. We first assayed the method using *rac*-1,2-dihydroxy-1,2-dihydronaphthalene with (*S*)-2-phenylglycine methyl ester and (*S*)-2-phenylglycine *t*-butyl ester and obtained for each of the two aminoesters a rapid and quan-

Table 2
Biotransformations of 2-pyridone and 2-quinolone derivatives by the NDO system from *Pseudomonas* sp. NCIB 9816-4 (27 °C, 30 h)

	Transformation (%)[a]	Dihydroxylation (%)	*N*-dealkylation (%)	Monohydroxylation (%)
N-Methyl-2-pyridone (**1a**)	35–40	35–40 (**6a**)	–	–
N-Benzyl-2-pyridone (**1b**)	n.d.[b]	–	–	–
N-Allyl-2-pyridone (**1c**)	5–6	5–6 (**6c**)	–	–
N-Methyl-4-methyl-2-pyridone (**2a**)	18	4–5 (**8a**)	11	3
N-Benzyl-4-methyl-2-pyridone (**2b**)	n.d.[b]	–	–	–
N-Methyl-6-methyl-2-pyridone (**3a**)	22	<1	6	16
N-Benzyl-6-methyl-2-pyridone (**3b**)	n.d.[b]	–	–	–
N-Methyl-2-quinolone (**4a**)	62	28 (**7a**)	34	–
N-Benzyl-2-quinolone (**4b**)	80	<1	80	<1
N-Methyl-4-methyl-2-quinolone (**5a**)	11	–	9	2

[a] Estimated from HPLC/MS measurement of residual substrate.
[b] n.d.: No detectable products.

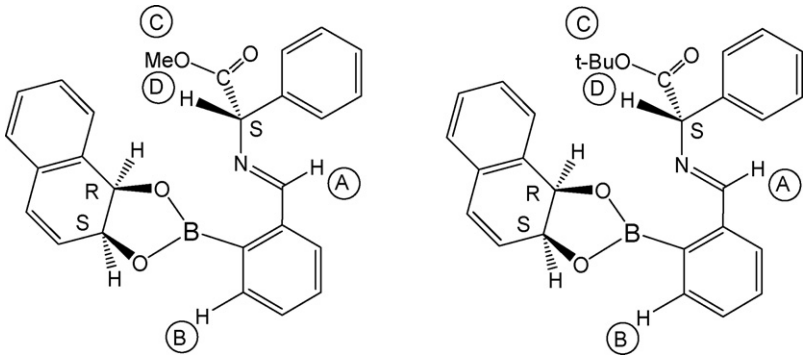


Fig. 2. Ternary compounds formed with (1*R*,2*S*)-naphthalenedihydrodiol and (*S*)-phenylglycine methyl ester or (*S*)-phenylglycine *t*-butyl ester, respectively.

titative formation of the ternary compound (Fig. 2). With the methyl ester, at 250 MHz in $CDCl_3$, H(A) protons are not resolved, H(B) and H(D) partially overlap with the resonance of the naphthalenedihydrodiol protons, but H(C) are clearly separated with $\Delta(\delta) = 0.259$ ppm. With the *t*-butyl ester, at 250 MHz in $CDCl_3$, H(A) protons are separated with $\Delta(\delta) = 0.048$ ppm, H(B) and H(D) partially overlap with the resonance of the naphthalenedihydrodiol protons, but again H(C) are clearly separated with $\Delta(\delta) = 0.204$ ppm (see Fig. 2 for the numbering of the characteristic protons).

The same method was applied to the *cis*-dihydrodiol **6a**, using separately (*R*)- and (*S*)-phenylglycine esters (as no racemic diol was available) for the preparation of the ternary diastereoisomeric complex. However, with (*R*)- and (*S*)-phenylglycine methyl esters derivatives, no sufficiently significant shift of any proton could be observed. On the contrary, with (*R*)- and (*S*)-phenylglycine *t*-butylester (Fig. 3), $\Delta(\delta)[(R)/(S)]$ as large as −0.30 ppm for H(A) and + 0.21 ppm for H(C) were measured. In both spectra, none of the corresponding signals for the opposite diastereoisomer was detected, indicating an enantiomeric purity higher than 98% for **6a**.

3.4. Absolute configuration and enantiomeric purity of *N*-methyl-*cis*-5,6-dihydroxy-5,6-dihydro-2-quinolone (**7a**)

Any attempt to prepare a crystalline derivative by condensation of **7a** with 4-bromophenylboronic acid was unsuccessful, even by heating. A similar behaviour was previously observed in the attempted esterification of the *cis*-3,4-dihydrodiol resulting from a TDO-catalysed 2-quinolone oxidation [9], confirming the assumption that the 3-hydroxy group was in a strong (hydrogen bond) interaction with the carbonyl group. This also precluded any further attempt to react this dihydrodiol with an asymmetric boronate or the ternary complex formation method in order to measure its enantiomeric purity. However, as a consequence and as deduced from the recovery of intact **7a**, it appeared that the dihydroxyderivative was fairly stable to dehydration, so it was possible to use a simple derivatization reaction with excess 4-bromobenzoylchloride, following conventional conditions, to obtain quantitatively the dihydrodiol diester which was purified by silicagel chromatography. Crystals suitable for X-ray diffraction were obtained by slow evaporation at room temperature from a methanol–anisole solution. Resolution of the structural data obtained clearly indicated a (3*R*,4*R*) configuration of the diester as shown in Fig. 4. Analysis of the crystal structure also suggested the presence of an enantiopure compound.

3.5. Biotransformation of differently N-substituted substrates

In the perspective of using the *cis*-diol derived from 2-pyridone and 2-quinolone as potential chiral precursors for hydroxylated piperidine compounds, some of which have been demonstrated as inhibitors of glycosidases, or as chiral building blocks for the synthesis of alkaloids or azasugars, it was important to evaluate the possibility to change the *N*-methyl group to a more easily deprotected substituent. To this end, the *N*-benzyl (**1b**, **2b**, **3b**, and **4b**) and the *N*-allyl (**1c**) derivatives

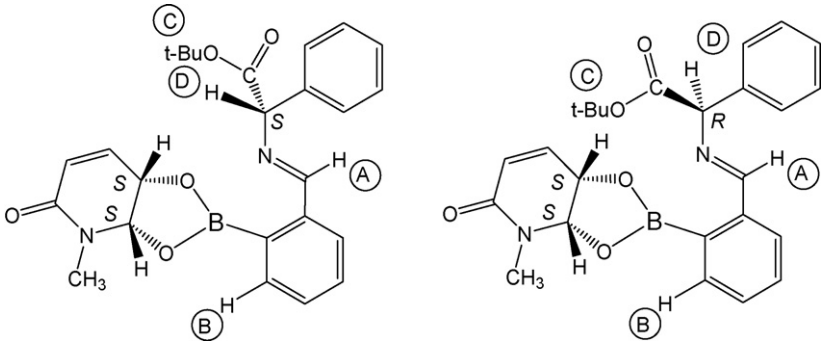


Fig. 3. Ternary compounds formed with the *cis*-(5*S*,6*S*)-dihydroxy-5,6-dihydro-1-methyl-2-pyridone **6a** and (*R*)- or (*S*)-phenylglycine *t*-butyl ester.

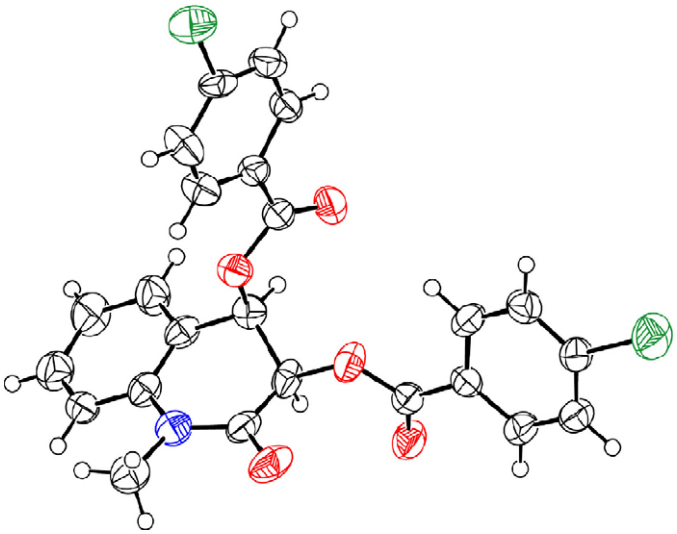


Fig. 4. X-ray structure of the bis(4-bromophenylbenzoate) derivative of *cis*-3,4-dihydroxy-3,4-dihydro-1-methyl-2-quinolone.

were prepared and submitted to the biotransformation in the previously optimised conditions. As shown in Table 2 no transformation (<1%) could be detected with the *N*-benzyl derivatives **1b**, **2b**, **3b**, and **4b**. Concerning the *N*-allyl substituted derivative **1c**, only a weak transformation (about 5%) into two dihydrodiol compounds was observed. After chromatographic separation and ^{1}H and ^{13}C NMR analysis, they were, respectively, identified as the corresponding 5,6-*cis*- and 5,6-*trans* dihydrodiols, in a 6:1 approximate ratio. Due to the very low yield of the metabolites, it was not possible to carry out further characterisation. However, by analogy with the corresponding *N*-methyl derivative **6a**, it was assumed that the major *N*-allyl-*cis*-dihydrodiol **6c** was a single enantiomer with a (5*S*,6*S*) configuration.

3.6. Biotransformation of ring-substituted substrates

In order to extend the scope of this reaction, it was advisable to assay other ring-substituted 2-pyridone and 2-quinolone compounds as potential substrates of NDO. The *N*-methyl-derivatives of 4-methyl- (**2a**) and 6-methyl-2-pyridone (**3a**) were prepared and submitted to the biotransformation in the previously optimised conditions. Both compounds were metabolised (about 20%) but only a dihydrodiol of the 4-methyl compound **2a** could be detected and isolated (about 5%). After chromatographic separation, it was identified by ^{1}H and ^{13}C NMR as the *N*-methyl-4-methyl-*cis*-5,6-dihydroxy-5,6-dihydro-2-pyridone **8a**. No dihydrodiol was detected in the biotransformation of **3a**. In both cases (see Table 1), a significant amount of *N*-dealkylation and *C*-methyl hydroxylation products were observed and characterised by comparison with authentic compounds or MS and NMR data.

The *N*-methyl derivative of 4-methyl-2-quinolone (**5a**) was similarly submitted to the biotransformation with NDO (about 10% transformation). No dihydroxylation could be detected but again some *N*-dealkylation and *C*-methyl hydroxylation were observed (see Table 2) and similarly characterised.

4. Discussion

Despite the relatively low level of biotransformation observed with our substrates, it is surprising that such a simple monocyclic pyridone derivative such as **1a** can be recognised as a dihydroxylation substrate by NDO, which is known to act more generally on bicyclic or polycyclic arenes. The activity of NDO in the dihydroxylation of the 2-quinolone derivative **4a** is more understandable. Surprising too is the reported absence of any significant activity of TDO on the monocyclic 2-pyridone derivative [12], while this dioxygenase is fully active in the transformation of 2-quinolone and quinoline derived compounds [9,12]. As a matter of fact, the occurrence of a resonance pattern and thus the aromaticity of such *N*-substituted-2-oxo substrates can be questioned. Moreover, NDO is completely inactive in the biotransformation of non-*N*-substituted 2-pyridone and 2-quinolone, which are mostly present as truly aromatic 2-hydroxy-pyridine or 2-hydroxy-quinoline forms.

The absolute configuration of the *cis*-dihydrodiol major metabolite of *N*-methyl-2-pyridone fits correctly with the general stereochemical model earlier described for the preferred *cis*-diol enantiomers obtained using TDO or NDO [12,22], where steric factors are considered as preeminent over electronic factors for directing the regio- and stereoselectivity of the dihydroxylation reaction (see Fig. 5).

The same template can be applied to the observed *cis*-dihydroxylation of the corresponding 2-quinolone substrate (**4a**), as shown in Fig. 5. This substrate is more similar to a benzocycloalkene substrate such as 1,2-dihydronapthalene, which affords the same absolute configuration (1*R*,2*S*) in the NDO-catalysed dihydroxylation [23].

Interestingly, dihydroxylated TDO-reaction products formed from 1,2-dihydronapthalene [23,24], benzochromene [10,25] or benzothiochromene [10] exhibit opposite configurations compared to that found in the *cis*-dihydroxylation of bicyclic arenes in general [1]. On the contrary, all the data available suggest that a general structure of (benzo)cycloalkene can be recognized by NDO and dihydroxylated in a uniform stereochemical pattern responding to the model previously described for arene compounds (Fig. 5).

It is remarkable that the substitution of the nitrogen atom of the pyridone ring with a methyl group is the only one which allows a significant dihydroxylation activity of NDO, preventing the use of classical deprotection groups (*N*-benzyl, *N*-allyl) for preparative purposes. Preparation of other type of *N*-derivatives such as *N*-carboxyesters from 2-hydroxy pyridine or 2-hydroxy quinoline compounds, is hampered by competition with a preeminent hydroxyl reactivity. As expected, *C*-substitution by a simple methyl group in the pyridine ring abolishes all dihydroxylation activity when the substitution occurs in one of the usual dihydroxylation positions. When the methyl group is located in a different position, some activity can still be observed, as exemplified with the preparation in low yield of the dihydrodiol **8a**.

However, all derivatives suffer some biotransformation by NDO in our conditions (see Table 1). When the dihydroxylation reaction does not constitute the most active transformation,

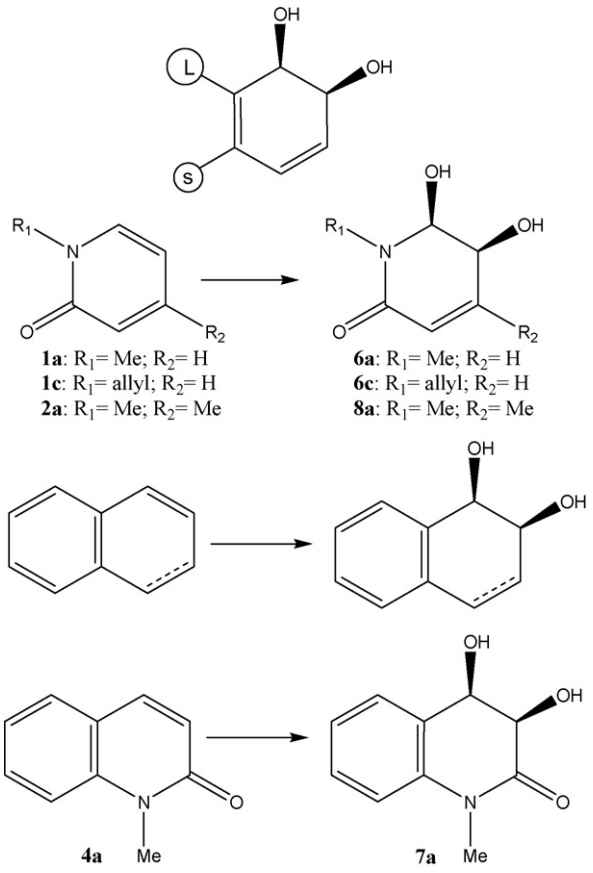


Fig. 5. Preferred regio- and stereoisomers of *N*-substituted mono- and bicyclic 2-pyridone derived *cis*-dihydrodiols obtained with NDO. For comparison, the general template [1,12] observed with mono- and polyclic arenes is also illustrated.

N-dealkylation and methyl (benzylic) monohydroxylation can be observed, corresponding to previously reported uncommon monooxygenase activities catalysed by NDO and Rieske-type non-heme ion oxygenases in general [22,26–28].

5. Conclusion

The biotransformation of non-aromatic 2-pyridone and 2-quinolone derivatives by the NDO system from *Pseudomonas* sp. 9816-4 appears to be considerably limited by the presence and nature of the substituents on the pyridinic ring. Except for *N*-methyl derivatives unsubstituted on the pyridinic ring carbons, which can be slowly but regio- and stereoselectively dihydroxylated by NDO, no (or unsignificant amounts) of dihydroxylated products could be obtained, precluding any preparative utilisation. More work is necessary to understand such a limitation in a system which apparently is able to accommodate a number of polycyclic azaarene compounds.

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