Microbiological Systems in Organic Synthesis: Preparation of Racemic Prenalterol Utilizing *Cunninghamella echinulata*

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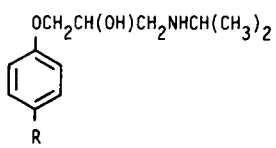
Abstract □ The fungal microorganism *Cunninghamella echinulata* was utilized to *para*-hydroxylate the synthetic substrate (±)-1-isopropylamino-3-phenoxy-propan-2-ol (**1**). The resulting product, (±)-1-(4-hydroxyphenoxy)-3-isopropylamino-propan-2-ol, or (±)-prenalterol, (**2**) was formed in >85% yield. Extracts from incubates were derivatized with trifluoroacetic anhydride and the concentrations of **1** and **2** were determined by GC on a fused silica methyl silicone capillary column with nitrogen–phosphorus detection. At substrate concentrations of 100 and 200 mg/L, the biotransformation proceeds with apparent first-order kinetics. With higher concentrations, the kinetics of prenalterol formation and substrate uptake appear to be nonlinear with a K_m value of 427.8 μg/mL and a V_{max} value of 232.3 μg/mL/d. This suggests that the biotransformation may proceed through a single-capacity limited pathway. The microbial product was isolated and identified as (±)-prenalterol by comparison (mp, IR, MS, ^{1}H NMR) with an authentic specimen.

Microorganisms have been utilized extensively for the biotransformation of drugs or their precursors.[1-4] Of current interest is their use as alternatives to animal (mammalian) models, particularly for initial drug metabolism studies.[5] A significant advantage in this respect is that preparative quantities of otherwise difficult to synthesize metabolites may be obtained by scale-up of the fermentation process.

Microorganisms (especially fungi) serve as effective models for mammalian metabolism since the cytochrome P-450-linked mono-oxygenase enzymes are often similar to those found in mammalian liver.[6-9] Cytochrome P-450-mediated aromatic hydroxylation[7,10] is frequently an important pathway for the mammalian and microbial metabolism of drugs containing an activated aryl ring; the metabolism of β-adrenoceptor antagonists, such as propranolol, is illustrative.[11] The 4-hydroxy metabolite of propranolol appears to contribute significantly to the β-blocking effects of the latter drug in both humans and animals.[12] The aryloxypropanolamine structural feature found in propranolol and its 4-hydroxy metabolite is also present in Hassle compound H80/62 [(±)-1-(4-hydroxyphenoxy)-3-isopropylamino-propan-2-ol] which is known as (±)-prenalterol (**2**). Interestingly, **2** is not a β-antagonist, but rather shows cardioselective β_1-adrenoceptor agonist properties and is potentially useful in the treatment of cardiac failure and β-blocker overdosage.[13] It has been shown that the pharmacological activity of **2** is due to the (−)-isomer, also known as prenalterol.[14-16]



1 R=H

2 R=OH

Ongoing studies in our laboratories suggested that the fungal organism *Cunninghamella echinulata* was an effective *para*-hydroxylator of β-blocking drugs; propranolol and toliprolol were transformed completely and exclusively to the respective 4-hydroxy metabolites.[17] The purpose of the present study was to utilize this micro-organism for the synthesis of (±)-prenalterol (**2**) from (±)-1-isopropylamino-3-phenoxy-propan-2-ol (**1**).

Experimental Section

Chemicals and Reagents—Dehydrated Czapek Dox broth was obtained from Difco Labs (Detroit, MI) and was reconstituted in distilled water (35 g/L). *Cunninghamella echinulata* UAMH 4145 was provided by the University of Alberta Mold Herbarium (Edmonton, Canada). Solvents were reagent grade and glass distilled prior to use. Phenol, epichlorhydrin, and isopropylamine were purchased from Aldrich Chemical Co. (Milwaukee, WI), while monobenzone was purchased from Lancaster Synthesis (Windham, NH). Trifluoroacetic anhydride was obtained from Pierce Chemical Co. (Rockford, IL). Standard stock solutions of the hydrochlorides of **1** and **2** (equivalent to 1 mg of free base/mL) were prepared in distilled water and stored at 4 °C. Test solutions (100 μg/mL in Czapek Dox broth) were prepared just prior to derivatization. The internal standard (propranolol) solution (10 μg/mL) was also prepared in broth. The buffer was prepared from 2 M sodium carbonate which was adjusted to pH 9.9 with sodium bicarbonate.

Instrumentation—A 5703A gas chromatograph equipped with a nitrogen–phosphorus detector, a 18740B capillary column controller, and a 3390A integrator–recorder (Hewlett-Packard, Palo Alto, CA) was used in the analysis of samples obtained from the uptake studies. The IR spectra (KBr pellet) were determined with a Nicolet 5DX spectrometer (Nicolet Instrument Canada, Mississauga, Canada). Chemical ionization and high-resolution electron-impact mass spectra were recorded with AEI MS 12 and MS 50 spectrometers (Associated Electrical Industries, Manchester, England), respectively. The GC–mass spectra were recorded with a VG 7070E system (Analytech Instrumentation and Service, St. Laurent, Canada). The ^{1}H NMR spectra were obtained with a Bruker AM 300 (Bruker Spectrospin Canada, Milton, Canada) using acetone-d_6 as the solvent and Me_4Si as the internal standard. A model G-25 gyratory incubator shaker (New Brunswick Scientific, Edison, NJ) was used for incubations. Solvents were evaporated under reduced pressure using a 100H Savant Speed Vac Concentrator/Evaporator (Emerston Instruments, Scarborough, Canada).

Chromatographic Conditions—The fused silica capillary column (12 m × 0.2 mm ID) was coated (film thickness 0.33 μm) with a high-performance cross-linked methyl silicone film (Hewlett-Packard). The operating conditions were as follows: the column head pressure was maintained at 0.85 bar; the injector was maintained at 250 °C; the column was maintained at 100–230 °C (32 °C/min); the detector was maintained at 300 °C; and the gas flow rates were maintained at 2 mL/min helium (carrier gas), 3 mL/min H_2, and 50 mL/min air (auxiliary gas).

Sample Analysis and Determination of Calibration Curves—A 25-μL aliquot of cell-free broth was diluted with 200 μL of buffer, and 20 μL of internal standard solution was added. The sample was vortexed and extracted with 3 mL of toluene. To ensure complete

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extraction of 1, the aqueous phase was mixed with 50 μL of 1 M NaOH and extracted with 3 mL of toluene. The organic phases were combined and the solvent was evaporated to dryness under reduced pressure. The resulting residue was reconstituted with 50 μL of ethyl acetate to which was added 200 μL of trifluoroacetic anhydride. The mixture was vortexed for 30 s and immediately evaporated to dryness. The residual material was reconstituted in 200 μL of toluene and a 1-μL portion was analyzed by GC.

To determine the quantities of 1 and/or 2 in the microbial cells, an accurately weighed portion of the mycelium, dried to constant weight (~50 mg), was homogenized in 2 mL of 0.4 M $HClO_4$, and an aliquot of the supernatant phase was analyzed as described above.

For the purposes of calibration, 100-μL portions of cell-free broth (diluted with 250 μL of buffer) were spiked with various aliquots of standard solutions of 1 and 2 to give final concentrations of 0.125, 0.25, 0.5, 1, 2, 5, 10, and 20 μg/mL. Following the addition of 20 μL of the internal standard solution, the derivatization procedure was carried out as described above.

Treatment of Data—The mean (n = 5) concentrations of 1 and 2 were plotted versus time. For the uptake of 1, the rate-disappearance constant, k_1, of the log–linear phase was calculated using regression analysis. The amount of prenalterol remaining to be formed (ARF) was calculated by subtracting the observed prenalterol concentrations at time t from the maximum observed concentrations. From the plot of ARF versus time, the formation rate constant, k_2, was calculated. The Michaelis–Menten constants K_m and V_{max} were estimated from the 400-mg/L concentration–time plot using the equations: $K_m = C_o/2.303 \log (C_o^*/C_o)$ and $V_{max} = \text{slope} \cdot 2.303 \cdot K_m$; where C_o is the observed concentration at time zero, and C_o^* is the concentration obtained by extrapolating the log–linear phase to time zero.[18]

Chemical Syntheses—The microbial substrate 1 was synthesized from phenol according to a procedure reported for metoprolol.[19] The HCl salt was recrystallized from acetonitrile to give a white solid, mp 107 °C (reported, 110–112 °C from ether:ethanol[20]); IR: 3377 cm^{-1} (NH, OH); ^1H NMR: δ 7.30 (m, 2, ArH), 6.97 (m, 3, ArH), 4.55 (m, 1, OCH), 4.12 (dd, 1, J = 10.3 and 5.0 Hz, OCH_2), 4.04 (dd, 1, J = 10.3 and 5.7 Hz, OCH_2), 3.56 (heptet, 1, J = 6.5 Hz, CH), 3.43 (dd, 1, J = 12.7 and 2.8 Hz, NCH_2), 3.19 (dd, 1, J = 12.7 and 9.5 Hz, NCH_2), 3.00 (br, s, 2, OH, NH), 1.50 (d, 3, J = 6.5 Hz, CH_3), 1.48 (d, 3, J = 6.5 Hz, CH_3).

Anal.–High resolution EIMS: calc. for $C_{12}H_{19}NO_2$:209.1416. Found 209.1418.

Prenalterol (2) was similarly prepared from 4-benzyloxyphenol. The HCl salt was recrystallized from acetonitrile to give a white solid, mp 165 °C (reported 167–168 °C from ethanol:ethyl acetate[21]); IR: 3263 cm^{-1} (NH, OH); ^1H NMR: δ 6.78 (m, 4, ArH), 3.93 (m, 3, OCH_2CH), 3.05–2.62 (m, 6, CH_2NHCH, OH), 1.04 (d, 6, J = 6.4 Hz, CH_3); CIMS (NH_3): m/z 226 ($M + H^+$).

Anal.–High resolution EIMS: calc. for $C_{12}H_{19}NO_3$ 225.1365. Found 225.1365.

Fermentation Procedures—*Uptake Studies—Cunninghamella echinulata* UAMH 4145 was maintained on oatmeal agar slants at 4 °C. Spores from the slants were used to inoculate Czapek Dox broth (25 mL) which was then incubated at 28 °C with gyratory shaking (250 rpm). After 72 h of incubation, 1-mL inocula were transferred to 125-mL Erlenmeyer flasks containing Czapek Dox broth (25 mL). Cultures were incubated for 48 h and 11.6, 5.8, and 2.9 mg of the hydrochloride of 1 (equivalent to 10, 5, and 2.5 mg of free base) were added to separate flasks. Aliquots (25 μL) from each flask were analyzed by GC at 12-h intervals for 8 d. Culture controls were prepared by incubating the micro-organism in the absence of substrate, and substrate controls were prepared by incubating 1 in sterile media.

Preparative-Scale Incubations—The microbial cultures were prepared as described above except that a 20-mL inoculum was added to each of four 1-L Erlenmeyer flasks containing 500 mL of Czapek Dox broth. After incubation for 48 h at 28 °C and 250 rpm, 117 mg of the hydrochloride of 1 (equivalent to 100 mg of free base) was added to each flask and the incubation was continued for 8 d. The incubates were then filtered and each mycelial cake was washed with distilled water (2 × 100 mL). The filtrates were adjusted to pH 12 with Na_2CO_3 and extracted with 2 × 200 mL of ether:dichloromethane (3:2). The aqueous phase was adjusted to pH 9.9 with $NaHCO_3$ and extracted with 3 × 200 mL of the same solvent mixture. The organic phases were combined, dried over $MgSO_4$, and the solvent was evaporated under reduced pressure. The residue was recrystallized from acetonitrile to give 0.31 g (72%) of (±)-prenalterol as a white solid, mp 155–156 °C. The hydrochloride salt was also recrystallized from acetonitrile and gave a melting point of 166 °C (reported 167–168 °C from ethanol:ethyl acetate[21]).

Results and Discussion

Prenalterol has been analyzed in biological samples using HPLC[22,23] and electron-capture GC.[24] The method utilized in this study is similar to a reported GC–MS procedure[25] except that trifluoroacetic anhydride was used for derivatization followed by nitrogen–phosphorus detection. The retention times of derivatized 1, 2, and internal standard were 5.09, 6.14, and 8.34 min, respectively. On GC–MS comparison with similarly derivatized authentic standards, the components were identified as *N,O*-bis(trifluoroacetylated)1, *N,O,O*-tris-(trifluoroacetyl)prenalterol, and *N,O*-bis(trifluoroacetyl)propranolol. Typical calibration curves could be described by: y = 0.715x − 0.004 for 1 (r^2 = 0.997); and y = 0.421x − 0.049 for prenalterol (r^2 = 0.984).

In Figure 1, the concentration–time curves (n = 5) of 1 and 2 for three substrate concentrations are depicted. The kinetics of prenalterol formation by *C. echinulata* were typically linear at low concentrations, but nonlinear at higher concentrations. With initial concentrations of 100 and 200 mg/L, the disappearance of substrate from the broth proceeds according to first-order kinetics. As expected, the plot of ARF was log–linear and superimposable with the concentration–time plot of the substrate (Figure 1). This indicates that the biotransformation of 1 is mainly, if not exclusively, through the formation of prenalterol. The yields of >85% (Table I) further support this suggestion. However, as shown in Figure 1, with a substrate concentration of 400 mg/L, the kinetic pattern changes considerably. The ARF plot had two distinct phases: an initial slow rate of prenalterol formation, followed by a log–linear phase with a slope similar to that estimated from the lower concentration curves (Table I). Thus, with substrate concentrations >200 mg/L, the kinetics of prenalterol formation and substrate uptake appear to be nonlinear with a K_m value of 427.8 μg/mL and a V_{max} value of 232.3 μg/mL/d. This suggests that the *C. echinulata*-mediated *para*-hydroxylation of 1 to prenalterol proceeds through a single-capacity limited pathway.[18] The initial substrate concentrations of 100 and 200 mg/L were lower than the observed V_{max}, and therefore, in these instances, the biotransformation proceeds with apparent first-order kinetics.

The limited capacity of the hydroxylating enzymes of *C. echinulata* is also evident in the 400-mg/L concentration–time plot (Figure 1). The initial slower disappearance of the substrate was preceded by a rapid phase indicating very quick uptake by the microorganism. Further evidence for rapid uptake was obtained when the incubates were examined following addition of substrate; after 24 h of incubation, the media:cell distribution ratio was found to be 0.483 ± 0.082.

From the preceding discussion it is apparent that for an efficient preparation of prenalterol, the initial substrate concentration must be at a V_{max} of 232.3 mg/L.

Previous studies with β-adrenoceptor antagonists suggested that *C. echinulata* was a useful model for mammalian metabolism; specifically, *para*-hydroxylation of a phenyl ring.[17] It was anticipated, therefore, that 1 would similarly undergo hydroxylation at the position *para* to the oxypropanolamine side chain. Substrate 1 was easily prepared from phenol, in two synthetic steps, using relatively inexpensive reagents. Regioselective microbial hydroxylation completed the synthesis. The same reagents (epichlorhydrin and isopropylamine) were utilized for a total chemical synthesis of

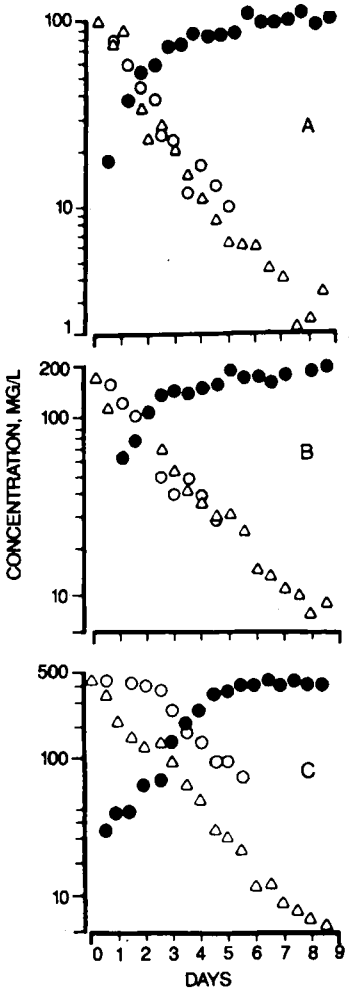

Figure 1—*Concentration–time curves obtained from substrate concentrations of 100 mg/L* (A), *200 mg/L* (B), *and 400 mg/L* (C). *Key:* (△) *substrate;* (●) *prenalterol;* (○) *amount of prenalterol remaining to be formed.*

Table I—Selected Kinetic Parameters in the Preparation of (±)-Prenalterol by *Cunninghamella echinulata*

Substrate (1) Concentration, mg/L	k_1 [a]	k_2 [b]	Prenalterol Yield, %
100	0.509	0.645	90.6
200	0.388	0.407	96.3
400	0.543	0.438	86.4

[a] Substrate disappearance rate constant. [b] Prenalterol formation rate constant.

prenalterol. In this case, however, four synthetic steps are required if hydroquinone is the starting material. One phenolic function must be initially protected (e.g., by *O*-benzylation) in order to construct the oxypropanolamine chain on the opposite side of the ring. The last deprotecting step (hydrogenolysis) affords prenalterol.

Since enzymes are chiral catalysts, microbial transformations frequently show a high degree of regio- and stereoselectivity.[26-28] In view of the relatively high yield of racemic prenalterol (Table I), it is evident that although hydroxylation of the aromatic ring is regioselective, the micro-organism metabolizes (+)- and (−)-1 to the same extent. A possible explanation for this observation is that the chiral center is relatively far removed from the site of hydroxylation and consequently the enzyme is not able to discriminate between the two enantiomers.[29] Utilization of (−)-1 as the substrate should give (−)-prenalterol and, consequently, a potentially attractive alternative to exclusive chemical synthesis.

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