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Microbial Transformation of Ibuprofen by a *Nocardia* Species

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The carboxylic acid functional group of ibuprofen [α-methyl-4-(2-methylpropyl)benzene acetic acid] is reduced to the corresponding alcohol and subsequently esterified to the acetate derivative by cultures of *Nocardia* species strain NRRL 5646. The alcohol and ester microbial transformation products were isolated, and their structures were determined by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. By derivatization of synthetic and microbiologically produced ibuprofen alcohols with *S*(+)-*O*-acetylmandelic acid, nuclear magnetic resonance analysis indicated that the carboxylic acid reductase of *Nocardia* sp. is *R* enantioselective, giving alcohol products with an enantiomeric excess of 61.2%. The *R* enantioselectivity of the carboxylic acid reductase enzyme system was confirmed by using cell extracts together with ATP and NADPH in the reduction of isomeric ibuprofens.

Microorganisms and their enzymes have received much attention and are widely used as a class of biocatalytic reagents in synthetic organic chemistry (5, 8, 10, 14–21, 25). Biocatalysts are valued for their intrinsic abilities to bind organic substrates and to catalyze highly specific and selective reactions under the mildest of reaction conditions. These selectivities and specificities are realized because of highly rigid interactions occurring between the enzyme active site and the substrate molecule. Useful biocatalytic reactions that have been well developed to date include a variety of oxidations, reductions, hydrolyses, and carbon-carbon bond ligations. Enzyme reactions such as those catalyzed by esterases, for example, may be used either hydrolytically or to synthesize esters, depending on the reaction medium, which may be aqueous or organic in composition.

Biocatalytic reactions are particularly useful when they may be used to overcome difficulties encountered in catalysis achieved by the use of traditional chemical approaches. The reduction of carboxylic acids by microorganisms is a relatively new biocatalytic reaction that has not yet been widely examined or exploited. Jezo and Zemek reported the reduction of aromatic acids to their corresponding benzaldehyde derivatives by actinomycetes (9). Kato et al., reported the reduction of benzoate to benzyl alcohol by a *Nocardia* strain (13), and Tsuda et al. described the reduction of 2-aryloxyacetic acids (23) and arylpropionates (24) by *Glomerella* species and *Gloeosporium* species. Carboxylic acid reduction reactions catalyzed by several additional microorganisms have been described recently by Arfmann and Abraham (1).

Ibuprofen is a 2-arylpropionic acid derivative, and it is a potent oral nonsteroidal antiinflammatory, antipyretic, and analgesic agent (11, 22). Pharmacological activities of ibuprofen reside almost exclusively in its *S* enantiomer (7). This is the first report which describes microbial transformations of ibuprofen. We describe the bioconversion of ibuprofen and ibuprofen isomers to the corresponding alcohol and acetate derivatives by *Nocardia* sp. strain NRRL 5646 and the stereoselectivity of these bioconversion reactions.

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MATERIALS AND METHODS

Instrumentation and general procedures. Electron impact gas chromatography-mass spectrometry was achieved with an OV-1 3% column (6 m by 0.25 cm; 80 to 100 mesh) with a helium (He) carrier gas flow of 20 ml/min; the temperatures for the injector column and detector were held at 220, 180, and 300°C, respectively. Chemical ionization mass spectral analyses were obtained by using a Nermag R 1010c instrument linked to a gas chromatograph (Girdel series 32; Girdel, Delsi, France) with He as the carrier gas and a 5% phenylmethylsilicon column (25 m by 0.2 mm) with 0.33-mm film thickness. Nuclear magnetic resonance (NMR) spectra were obtained with Bruker AMX-600-MHz or Bruker WM-360-MHz high-field spectrometers equipped with an IBM Aspect-2000 processor. ^{1}H NMR spectra were recorded in deuteriochloroform at 600.136 or 360.134 MHz, and ^{13}C NMR spectra were recorded at 90.03 MHz with tetramethylsilane as the internal standard. Chemical shift values are reported in parts per million, and coupling constants (*J* values) are given in hertz. Abbreviations for NMR signals are as follows: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; and m, multiplet. Optical rotations at the sodium D-line were determined with ethanol solutions in a 0.1-dm cell. Spectral measurements with cell-free enzyme preparations were obtained with a Shimadzu UV-Visible Recording Spectrophotometer, model 160, in 1-cm quartz cuvettes.

Chromatography. Thin-layer chromatography (TLC) was performed on Silica Gel GF_{254} (Merck) layers of 0.5-mm thickness for analysis and 1-mm thickness for preparative layer chromatography. Plates were prepared on glass plates (5 by 20 or 20 by 20 cm) with a Quickfit Industries (London, England) spreader. Plates were air dried and activated at 120°C for 1 h before use. Plates were developed in several solvent systems, and the developed chromatograms were visualized under 254-nm UV light to observe compound fluorescence. Plates were then sprayed with *p*-anisaldehyde visualization reagent (60 ml of methanol, 0.5 ml of H_2SO_4, and 0.5 ml of *p*-anisaldehyde) before being warmed with a heat gun to develop the pink to brown colors.

Column chromatography was carried out with silica gel (Baker 3404; 60 to 120 mesh). Silica gel was oven activated at 120°C for 30 to 60 min. Columns were slurry packed in benzene and eluted with a mixture of benzene-acetic acid (99:1, vol/vol),

while 3-ml fractions were collected with a Retriever II fraction collector at a flow rate of 1 ml/min. The collected fractions were examined by TLC.

High-performance liquid chromatography (HPLC) was performed with a Shimadzu LC-6A pump and a variable-wavelength UV detector recorder set at 254 nm. Samples of 5 μl were resolved on a Versapack C_{18} column (10 μm; 0.41 by 30 cm, inside diameter) preceded by a guard column (0.41 by 1 cm) of the same stationary phase, using acetonitrile-water-acetic acid (55:45:0.3, vol/vol/vol) at a flow rate of 0.7 ml/min. (+)-Naproxen was used as an internal standard for quantitative analysis. The range of standard curves was 0 to 200 mg/liter. With this system, retention volumes for each compound were as follows: (+)-naproxen, 3.8 ml; ibuprofen, 4.6 ml; ibuprofenol, 5.4 ml; and ibuprofenol acetate, 9.3 ml.

Chemicals. Racemic ibuprofen [α-methyl-4-(2-methylpropyl)benzene acetic acid], (+)-naproxen, *S*(+)-*O*-acetylmandelic acid, and dicyclohexylcarbodiimide were purchased from Sigma Chemical Co. *S*(+)-Ibuprofen was obtained from Aldrich Chemical Co. *R*(−)-Ibuprofen was a gift from Ching-Shih Chen, University of Rhode Island (4). The identities and purities of all compounds were confirmed by TLC, ^1H and ^{13}C NMR spectroscopy, and mass spectrometry.

Nocardia sp. strain NRRL 5646 is kept in the University of Iowa College of Pharmacy culture collection and was maintained on Sabouraud dextrose agar or sporulation agar (ATCC no. 5 medium). Medium 5 slants consisted of 0.1% yeast extract, 0.1% beef extract, 0.2% tryptone, 1 mg of $FeSO_4 \cdot 7H_2O$ per liter, 1% glucose, and 1.5% agar. The medium was adjusted to pH 7.2 before it was autoclaved.

Fermentation procedure. Cultures were grown by our standard two-stage fermentation protocol (2) in 25 ml of sterile soybean meal-glucose medium held in stainless-steel-capped, 125-ml DeLong culture flasks. The medium contained (weight/volume) 2% glucose, 0.5% yeast extract, 0.5% soybean meal, 0.5% NaCl, and 0.5% K_2HPO_4 in distilled water and was adjusted to pH 7.0 with 6 N HCl and then autoclaved at 121°C for 15 min. Cultures were incubated with shaking at 250 rpm at 27°C on New Brunswick Scientific G25 Gyrotory shakers. A 10% inoculum derived from 72-h-old stage I cultures was used to initiate stage II cultures, which were incubated for 24 h before receiving 1 mg of ibuprofen per ml as substrate. A stock solution of ibuprofen in dimethylformamide (250 mg/ml) was prepared for addition of the substrate to cultures. Substrate controls consisted of sterile medium incubated under the same conditions but without microorganisms.

Substrate-containing cultures were generally sampled by removing 2 ml of the entire culture. Samples were adjusted to pH 2 with 6 N HCl and extracted with an equal volume of ethyl acetate, and the organic and aqueous layers were separated by centrifugation for 1 min in a desktop centrifuge. The organic solvent layer was removed, evaporated to dryness, and reconstituted in 0.5 ml of methanol, and the samples were spotted onto TLC plates for analysis.

Preparative biotransformation of ibuprofen (compound 1) to ibuprofenol (metabolite 2) and ibuprofenol acetate (metabolite 3). The standard two-stage fermentation procedure was used to grow *Nocardia* sp. strain NRRL 5646, and a total of 1 g of racemic ibuprofen was distributed among five 1-liter DeLong flasks, each of which contained 200 ml of medium. The microbial transformation reaction was terminated after 24 h, at which time about half of the added substrate had been consumed, as shown by TLC. The culture was acidified to pH 2 with 6 N HCl and extracted with 3 liters of ethyl acetate. The organic extracts were washed with an equal volume of distilled water, dried over anhydrous sodium sulfate, and concentrated by rotary evaporation to 900 mg of a viscous residue.

Rates of microbial transformations of ibuprofen. The standard two-stage fermentation procedure was used for kinetic experiments in which the rates of ibuprofen conversion and alcohol and acetate formation were determined. Racemic ibuprofen, *S*(+)-ibuprofen, and *R*(−)-ibuprofen were each added to duplicate, 125-ml flasks which contained 25 ml of medium and 1 mg of substrate per ml. Substrate-containing cultures were sampled at various time intervals by removing 1 ml of the entire culture. The internal standard, (+)-naproxen (0.5 mg), was added to the samples. Samples were adjusted to pH 2 with 6 N HCl extracted with 5 ml of ethyl acetate, and the organic and aqueous layers were separated by centrifugation for 1 min in a desktop centrifuge. A 1-ml volume of the organic extract was removed, evaporated to dryness under N_2, and reconstituted in 1 ml of HPLC-grade methanol for quantitative analysis by HPLC. The results of microbial transformations of *R*(−)- and *S*(+)-ibuprofen can be seen in Fig. 3A and B, respectively.

Cell-free enzymatic reduction of ibuprofen to ibuprofenol. Cultures were grown as usual by the fermentation procedure for 24 h. An inducer, sodium benzoate, was directly added to the cultures at 5 mg/ml. Cultures were taken at 48 h, filtered through cheesecloth, and centrifuged at 8,000 × *g* for 10 min. The resulting cell paste (5 g, wet weight) was suspended in 25 ml of cold 50 mM Tris-HCl buffer (pH 7.5), and the suspension was disrupted for 5 min over ice with a Sonifier Cell Disrupter 350 (Branson Sonic Power Co.). Cell debris was removed by centrifugation at 16,000 × *g* for 30 min. Incubation mixtures contained 1.3 mg of protein, 5.0 mM $MgCl_2$, 2.0 mM ATP, 0.12 mM NADPH, 4.8 mM *R*(−)- or *S*(+)-ibuprofen, and 50 mM Tris-HCl (pH 7.5) in a total volume of 1.0 ml. Conversion rates were measured by the decrease in A_{340} per minute at 25°C. Controls consisted of reaction mixtures minus ibuprofen.

Hydrolysis of ibuprofenol acetate to ibuprofenol. A 100-mg (0.43-mmol) sample of the microbial metabolite, ibuprofenol acetate metabolite (3), was dissolved in 5 ml of methanol; then 200 mg of potassium carbonate dissolved in 2 ml of water was added. The reaction was kept overnight until no ester remained and was extracted with 5 ml of chloroform. The organic extracts were dried over anhydrous sodium sulfate and concentrated by rotary evaporation to give 68 mg of viscous oil. The product was identified as ibuprofenol by spectral methods.

Reduction of racemic ibuprofen (compound 1a) to racemic ibuprofenol (compound 2a) with $LiAlH_4$. A total of 200 mg (0.48 mmol) of compound 1a dissolved in 2 ml of anhydrous tetrahydrofuran was reduced with $LiAlH_4$, as previously described (3). Preparative TLC (hexane-ethyl acetate, 3:1) gave a major UV (254 nm) quenching band at R_f 0.35 which was scraped and eluted with ethyl acetate to give 44 mg of compound 2a after evaporation. The ^1H NMR and mass spectrometry data for compound 2a were in agreement with those for ibuprofenol.

Reduction of *S*(+)-ibuprofen (compound 1b) to *S*(−)-ibuprofenol (compound 2b) with $LiAlH_4$. The procedures of reaction and purification were the same as in the reduction of compound 1a to 2a with $LiAlH_4$. The major band at R_f 0.35 by preparative TLC was scraped and eluted with ethyl acetate to give 45 mg of enantiomer ibuprofenol 2b after evaporation. The ^1H NMR and mass spectrometry data for compound 2b were in agreement with those obtained for ibuprofenol.

Preparation of the *S*(+)-*O*-acetylmandelate ester of ibuprofenol. The *S*(+)-*O*-acetylmandelic acid ester of microbiologically produced ibuprofenol was prepared as previously described by our laboratory (6). Preparative TLC (hexane-ethyl

acetate, 3:1) was used to isolate the major UV (254 nm) quenching band at R_f 0.6 for spectral and physical analyses. *S*(+)-*O*-Acetylmandelate derivatization of synthetic compounds 2a and 2b and analyses of compounds 4a and 4b were done for comparison purposes. The optical rotations, $[\alpha]^{25}$, for compounds 4a and 4b were measured as +55.1° and 60.1° [c 4.0, ethanol], respectively. Mass and NMR spectral properties of synthetic and microbially produced alcohol *O*-acetylmandelates were nearly identical. The resolved singlet signals for the *O*-acetyl methyl groups for compounds 4a and 4b are shown in Fig. 2A and B, respectively.

RESULTS

Microbial transformation of racemic ibuprofen. Methods commonly used in our laboratory for the screening of microorganisms were employed to determine the ability of *Nocardia* sp. strain NRRL 5646 to biotransform ibuprofen into new products. TLC was used initially as the method for analysis, and no attempts were made at this point to optimize ibuprofen metabolite yields. With benzene-acetic acid (5:2, vol/vol) as the developing solvent, ibuprofen gives R_f 0.8. Three major spots at R_f 0.9, 0.8, and 0.7 were observed in extracts taken after 24 and 72 h of incubation, and an additional spot appeared when cultures were incubated for 120 h. No similar running products were observed in chromatograms of extracts from cultures containing no ibuprofen.

A preparative-scale incubation of *Nocardia* sp. with 1 g of racemic ibuprofen gave three products which were extracted and purified by silica gel column chromatography (100 g of silica gel, 3 by 35 cm) by elution with benzene-acetic acid (99:1, vol/vol) for structure analysis. A total of 338 mg of recovered compound 1, 283 mg of metabolite 2, and 92 mg of metabolite 3 were characterized as described below.

Schemes of ibuprofen, metabolites, and derivatives are given in Fig. 1.

Identification of ibuprofen metabolites. Recovered compound 1 was identical to ibuprofen by ^1H and ^{13}C NMR and mass spectrometry. However, it displayed a specific rotation, $[\alpha]^{25}$, of +28.3° [c 4.0, ethanol] versus +55.1° for authentic *S*(+)-ibuprofen.

Metabolite 2 was a viscous oil which gave the following physical properties: $[\alpha]^{25}$, +6.2° [c 4.5, ethanol]; high-resolution electron impact mass spectrum, *m/z* 192.1513 for $C_{13}H_{20}O$ (calculated, 192.1514); low-resolution electron impact mass spectrum, *m/z* (percent relative abundance) 193 (5%, M^+ +1), 192 (20%, M^+), 161 (100, M^+ —CH_2OH, 119 (40%, M^+ —C_4H_8OH), and 91 (20%, C_7H_7). Both ^1H and ^{13}C NMR spectra at 360 and 90.03 MHz, respectively, were in good agreement with the structure of ibuprofenol (metabolite 2) previously described by Castell et al. (3), and with that of ibuprofenol prepared by chemical reduction with $LiAlH_4$.

Metabolite 3 was also a viscous oil that gave the following physical properties: $[\alpha]^{25}$, +4.5° [c 4.5, ethanol]; high-resolution fast atom bombardment (Na) mass spectrum, *m/z* 257.1519 for $C_{15}H_{22}O_2Na$ (calculated, 257.1518); low-resolution chemical ionization mass spectrum (NH_3), *m/z* (percent relative abundance) 252 (5.3%, MH $^+$ + NH_3), 174 (60%, MH^+ —$C_2H_5O_2$), 131 (100%, $C_{10}H_{11}$), 91 (22%, C_7H_7); NMR 360 MHz ($CDCl_3$) δ_H, 1.17 [6H, d, J = 6.5 Hz, —CH($C\underline{H}_3$)$_2$], 1.56 (3H, d, J = 7 Hz, >CHC$\underline{H}_3$), 2.12 [1H, m, —C$\underline{H}$(CH_3)$_2$], 2.27 (3H, s, —COC$\underline{H}_3$), 2.71 [2H, d, J = 7 Hz, —C$\underline{H}_2$CH(CH_3)$_2$], 3.32 (1H, m, >C$\underline{H}$CH_3), 4.34 to 4.48 (2H, m, —C$\underline{H}_2$OH), 7.40 (4H, m, aromatic $\underline{H}$); ^{13}C NMR 90.03 MHz ($CDCl_3$) δ_C, 18.02, 22.33, 30.14, 38.45, 44.98, 69.46, 126.86, 129.09, 139.86, 140.23, and 170.85. These data were in agreement with the structure of metabolite 3 as ibuprofenol acetate $C_{15}H_{22}O_2$.

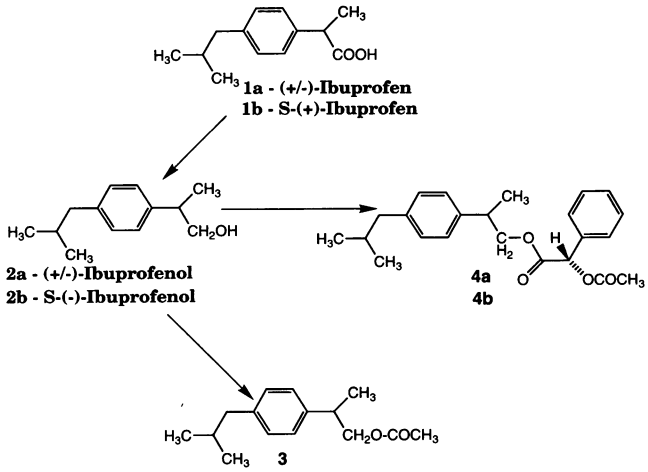

FIG. 1. Schemes of ibuprofen, metabolites, and derivatives. 1a, racemic ibuprofen; 1b, *S*(+)-ibuprofen; 2a, racemic ibuprofenol; 2b, *S*(−)-ibuprofenol; 3, ibuprofenol acetate; 4a, *S*(+)-*O*-acetylmandelate ester of compound 2a; 4b, *S*(+)-*O*-acetylmandelate ester of compound 2b.

Ibuprofenol obtained by hydrolysis of metabolite 3 was identical to metabolite 2 described above, and it gave a specific rotation of +2.0° [c 1.0, EtOH].

Stereochemical purity of ibuprofenol (metabolite 2). The stereochemistry of ibuprofenol (metabolite 2) obtained after microbial reduction of racemic compound 1 was determined by analysis of the *S*(+)-*O*-acetylmandelate derivative 4, which gave the following physical properties: $[\alpha]^{25}$, +65.2°[c 4.5, ethanol]; high-resolution fast atom bombardment mass spectrum, *m/z* 369.2073 for $C_{23}H_{29}O_4$ $(M + H)^+$ (calculated, 369.2066); low resolution chemical ionization mass spectrum, *m/z* (percent relative abundance) 386 (3%, M +1 + NH_3), 309, 263, 174, 131, and 91; ^1H NMR ($CDCl_3$) δ_H, 0.88 [6H, d, J = 6.5, —CH($C\underline{H}_3$)$_2$], 1.18 (3H, d, J = 7 Hz, >CHC$\underline{H}_3$), 1.85 [1H, m, —C$\underline{H}$(CH_3)$_2$], 2.158 [3H, s, —COC$\underline{H}_3$ for the *R*(+)-alcohol enantiomer, 80.5% (see Fig. 2C)], 2.167 [3H, s, —COC$\underline{H}_3$ for the *S*(−)-alcohol enantiomer, 19.5% (see Fig. 2C)], 2.42 (2H, d, J = 7.2, >C$\underline{H}_2$), 2.99 (1H, m, >C$\underline{H}$CH_3), 4.08 to 4.26 (2H, m, —C$\underline{H}_2$O—), 5.89 (1H, s, >C$\underline{H}$COO—), 7.02 (4H, m, aromatic $\underline{H}$), 7.32 to 7.41 (5H, m, aromatic $\underline{H}$); ^{13}C NMR ($CDCl_3$) δ_C, 14.20, 17.73, 20.65, 22.40, 30.19, 38.40, 45.05, 60.36, 70.47, 74.49, 126.89, 127.56, 128.64, 129.12, 133.82, 139.94, 168.68, and 170.19.

For comparison, racemic ibuprofenol (optical rotation, $[\alpha]^{25}$ = 0°; c 1.0 in ethanol) from (+/−)-ibuprofen and *S*(−)-ibuprofenol (optical rotation, $[\alpha]^{25}$ = −11.3°; c 0.5 in ethanol) obtained from *S*(+)-ibuprofen by $LiAlH_4$ reduction (3) were also derivatized and analyzed by NMR (Fig. 2A and B, respectively). In the 600-MHz spectra of the *S*(+)-*O*-acetylmandelates (Fig. 2), the signal at 2.158 ppm represents the *O*-acetyl methyl group of the *R*(+)-ibuprofenol enantiomer, while the signal at 2.167 ppm represents the *O*-acetyl methyl group of the *S*(−)-ibuprofenol enantiomer. Since the signals for acetyl methyl groups were well resolved, it was possible to use these to ascertain the enantiomeric purities of racemic ibuprofenol (Fig. 2A), *S*(−)-ibuprofenol (Fig. 2B), and ibuprofenol obtained from the microbial reduction reaction (Fig. 2C). On the basis of this spectral evidence, the microorganism produced *R*(+)-ibuprofenol with a measured enantiomeric

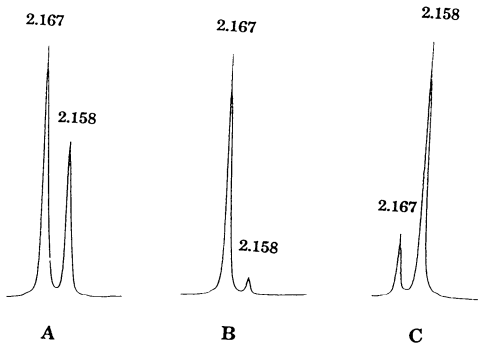

FIG. 2. ^{1}H NMR signals 600 MHz, for the acetyl esters of *S*(+)-*O*-acetylmandelates of racemic ibuprofenol (A), *S*(−)-ibuprofenol (B), and *R*(+)-ibuprofenol (C) from microbial transformation of racemic ibuprofen.

excess of 61.2%. Ibuprofenol obtained by hydrolysis of ibuprofenol acetate gave a specific rotation of +2.0°, indicating an enantiomeric excess of 58.9%, similar to that for the underivatized ibuprofenol.

Differences in the rates of microbial carboxylic acid reduction of racemic and *S*(+)- and *R*(−)-ibuprofens were examined. The results obtained with *S*(+)- and *R*(−)-ibuprofens are shown in Fig. 3A and B, respectively. With whole cultures, mass balances obtained during the reduction and acetylation reactions for racemic (results not shown) and *R*(−)-ibuprofens (Fig. 3A) were nearly 100%, while that for *S*(+)-ibuprofen (Fig. 3B) was only about 60%. After peak levels of products were formed, there was a gradual decline in levels of ibuprofen and metabolites 2 and 3, and no new metabolites were detected. The rate of *R*(−)-ibuprofen whole-cell biotransformation was nearly twice that obtained with either racemic or *S*(+)-ibuprofen. An initial lag of about 10 h was observed in the reduction of *S*(+)-ibuprofen before rapid conversion of substrate to products occurred. There was no apparent lag in the reduction of the enantiomeric *R*(−)-ibuprofen isomer. No *S*(−)-ibuprofenol acetate was formed during whole-cell biotransformations of *S*(+)-ibuprofen (Fig. 3B).

Active and stable cell extracts were prepared by using sodium benzoate-induced *Nocardia* cells harvested at 48 h. Reductions of ibuprofen with cell extracts of *Nocardia* sp. strain 5646 required NADPH and ATP but not coenzyme A. Conditions used for reduction were saturating with respect to ibuprofen, NADPH, and ATP. With crude cell-free enzyme preparations, relative rates of reduction of *S*(+)- and *R*(−)-ibuprofens were measured as 0.028 and 0.115 absorbance units/min, respectively, in 1.3 mg of protein.

DISCUSSION

The microbiological reduction of carboxylic acids is an unusual and potentially useful biocatalytic reaction which has not yet been widely exploited. The enzymatic reaction offers significant advantages over existing methods used in chemical reductions of carboxylic acids or their derivatives. Unlike many substrates subjected to biocatalytic reactions, carboxylic acids are generally water soluble, rendering them of potentially broad application to this class of enzyme. The carboxylic acid reduction reaction appears to bear the usual desirable features of functional group specificity, and it functions well under mild reaction conditions.

We initially discovered the abilities of *Nocardia* sp. strain

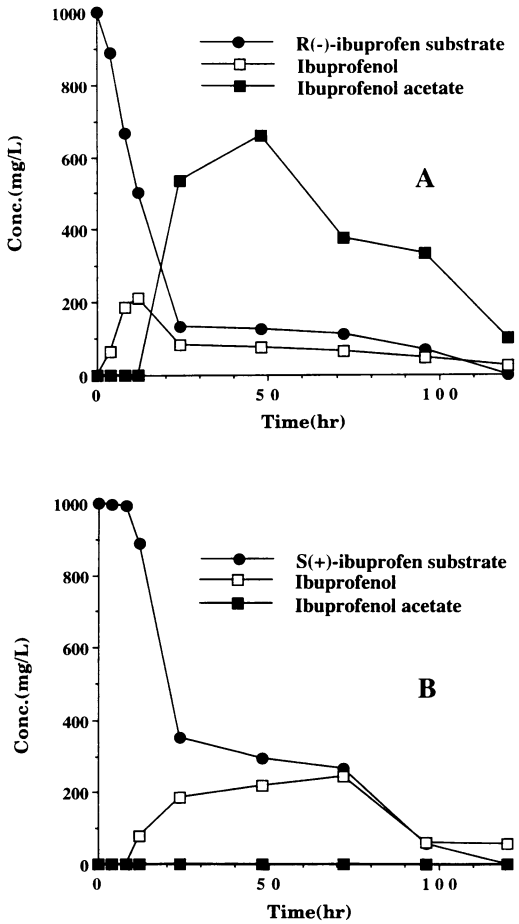

FIG. 3. Time course for the whole-cell biotransformation of ibuprofen to ibuprofenol and ibuprofenol acetate by *Nocardia* sp. strain NRRL 5646. (A) Results with *R*(−)-ibuprofen; (B) results with *S*(+)-ibuprofen.

NRRL 5646 to catalyze the reduction of benzoic acid derivatives. Ibuprofen was deemed to be an attractive substrate candidate for investigation because of its overall importance as a widely used drug, the lack of any previous microbial transformation studies with this compound, and the opportunity to examine the possible stereoselective properties of the carboxylic acid reductase reaction.

With *Nocardia* sp. strain NRRL 5646, two major metabolites of racemic ibuprofen were formed, isolated, and identified as ibuprofenol (metabolite 2) and the corresponding acetate derivative (metabolite 3) by spectral methods. The preparative-scale reaction conducted with (+/−)-ibuprofen was stopped at 24 h when TLC analysis indicated that approximately 50% of the substrate had been converted into other products. Unreacted and recovered ibuprofen was largely *S*(+)-ibuprofen with an enantiomeric excess of 47.9% by optical rotation measurement. This finding indicates that *Nocardia* sp. strain NRRL 5646 contains an R(−)-selective carboxylic acid reductase enzyme system with (+/−)-ibuprofen.

The enantiomeric excess of *R*(+)-ibuprofenol obtained from the biotransformation of racemic ibuprofen was determined to be 61.2% by NMR spectral analysis of the *S*(+)-*O*-acetylmandelate ester. This method affords a powerful and simple probe

in assigning the stereochemistries of secondary alcohols. The derivatization reaction is quantitative in nature, and we have used this approach to ascertain the stereochemical purities of other microbiologically produced secondary alcohols (6, 16). Ibuprofenol obtained by saponification of the acetate ester was of similar enantiomeric composition. These results complement those obtained with recovered and unreacted ibuprofen, and they confirm that the nature of the carboxylic acid reductase enzyme system is *R*(−)-ibuprofen specific.

The rates of biotransformation of ibuprofens were evaluated with whole cultures and HPLC analysis of the resulting products. The reduction of *R*(−)-ibuprofen occurred without an apparent lag (Fig. 3A), and the rate of *R*(−)-ibuprofen reduction was estimated to be twice that for *S*(+)-ibuprofen. There was an apparent lag of about 10 h prior to the reduction of *S*(+)-ibuprofen by *Nocardia* sp. (Fig. 3B). This observation suggests the possibility that *Nocardia* sp. strain NRRL 5646 contains an inducible carboxylic reductase enzyme system for the *S*(+)-ibuprofen isomer similar to that described by Kato et al. (12) for benzoate reduction. In these analytical experiments, mass balances accounting for substrate used and metabolites produced were high between 50 and 70 h for *R*(−)- and racemic ibuprofens. However, after 100 h of incubation, no new products were observed and the levels of ibuprofen and metabolites 2 and 3 declined, indicating that further degradation of the metabolites occurs. The lack of an ibuprofenol acetate as a metabolite with *S*(+)-ibuprofen incubations (Fig. 3B) was surprising.

Carboxylic acid reductases are complex, multicomponent enzyme systems which require the initial activation of carboxylic acids via formation of AMP and possibly coenzyme A intermediates. The reduction of the activated carboxylic acid intermediate occurs stepwise to give aldehyde and then alcohol products (9, 12). It was possible to establish a relatively stable and active crude, cell-free carboxylic acid reductase system that required ATP and NADPH for the reduction reaction. The enzyme system is inducible with benzoate, and properties of the carboxylic acid reductase enzyme system of *Nocardia* sp. strain NRRL 5646 are similar to those described by Kato et al. for benzoate reduction in *Nocardia asteroides* (12). The cell-free preparation was used to demonstrate that the rate of reduction of *R*(−)-ibuprofen was four times faster than that for *S*(+)-ibuprofen. These results confirm the *R*(−)-enantioselective properties of the carboxylic acid reductase enzyme system of *Nocardia* sp. strain 5646. We are presently working to establish the properties and scope of the carboxylic acid reductase enzyme system of nocardiae.

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