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MINI-REVIEW

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Engineering an indene bioconversion process for the production of cis-aminoindanol: a model system for the production of chiral synthons

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Abstract Cis-aminoindanol, a key chiral precursor to the HIV protease inhibitor CRIXIVAN, can be derived from indene oxidation products of (2R) stereochemistry. A number of different microorganisms, notably strains of the genera *Pseudomonas* and *Rhodococcus*, have been isolated that catalyze the oxygenation of indene to indandiol with greater stereospecificity than is achievable through traditional chemical synthesis. The yield and ultimate optical purity of indandiol produced in such biocatalytic processes is influenced by the intrinsic stereospecificity of the oxygenase(s), enantioselective dehydrogenation, and the loss of substrate to alternate, undesirable metabolites. Metabolic engineering of any indene bioconversion system for the commercial-scale production of cis-aminoindanol must account for these influences, as well as pathway fluxes and enzyme regulation, to optimize the formation of oxygenated precursors with useful stereochemistry. As such, the process by which bacterial systems carry out the bioconversion of indene to indandiol serves as a model for biological production of industrially relevant chiral synthons.

Introduction

Harnessing biological systems to serve as living catalysts for the production of key metabolites holds great promise for industrial applications. From the annual production of 30 thousand tons of acrylamide with *Rhodococcus rhodocrous* (Yamada and Kobayashi 1996) and over 13 million tons in fuel grade ethanol production from *Saccharomyces cerevisiae* (Wilke 1999) to the specialized production of antibiotic cephalosporins from *Cephalosporium acremonium* (Demain and Elander 1999), biocatalysts have proven commercially viable. Advances in metabolic and genetic engineering, screening technologies, and strain isolation have led to the identification of biological systems with novel, industrially relevant, catalytic activities. The development of biocatalysts as a complement to traditional chemical catalysts has clear benefits for the directed synthesis of fine chemicals. Bacterial enzymes are known to catalyze chemical reactions with an intrinsic stereospecificity that is not achievable through chemical synthesis, making large-scale bioconversions an attractive alternative to the traditional methods of asymmetric synthesis.

A growing percentage of small molecule therapeutics are chiral compounds where the pharmacological activity is due to a single stereoisomer. Racemates of the active compound may prove biologically inert, or result in different, often undesirable, activities (Ariens 1993). This, coupled with increasing regulatory pressures, has made developing chirally pure therapeutics a virtual necessity (Persidis 1997). In 2000, single-enantiomer chiral drugs represented a US$ 133 billion market and accounted for 40% of all dosage-form drug sales (Stinson 2001). The development of new, selective methods of asymmetric syntheses has become a key economic hurdle in the development of novel pharmaceuticals.

Indinavir sulfate is a potent inhibitor of the HIV-1 protease, a virally encoded protein that mediates the cleavage of viral precursor proteins (Vacca et al. 1994). HIV protease activity is required for the successful assembly of infectious virions (Kohl et al. 1988). Containing five chiral centers with 32 possible stereoisomers, only a single stereo conformation of indinavir sulfate confers the desired therapeutic effect. This enantiomerically pure compound is the active ingredient of the HIV protease inhibitor CRIXIVAN (Merck; Fig. 1). Combined with two HIV reverse transcriptase inhibitors as a much lauded "triple therapy", CRIXIVAN has proven to be a powerful therapeutic. Such therapy clinically reduces viral load to undetectable levels in many HIV-infected patients (Plosker and Noble 1999), thereby arresting the progression of HIV and its resultant pathogenic sequence. Therapeutic dosing of 0.8–1.0 kg/year (Physi-

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cians' Desk Reference 2000) with CRIXIVAN represented US$ 500 million in sales in 2000 (Merck Annual Report 2000).

A key synthon in the production of CRIXIVAN is cis-(1S,2R)-1-aminoindan-2-ol, an indene derivative that contributes two chiral centers to indinavir sulfate (Fig. 1). cis-Aminoindanol is currently produced on a multi-ton scale via a technically demanding chemical synthesis. Scalability and downstream processing requirements led to the development of stereospecific catalysis in a biphasic system for the synthesis of an activated epoxide precursor, 1,2-indan oxide (Hughes et al. 1997; Reider 1997; Senanayake et al. 1995). The activated epoxide is then aminated with acetonitrile by a Ritter-type reaction to form the final aminoindanol (Senanayake et al. 1995; Fig. 2). The resultant product loss due to racemization is unlikely to be significantly reduced through further optimization of this chemical synthesis. For this reason, the suitability of a bioconversion process for the production of cis-aminoindanol has been explored (Buckland et al. 1999a; Chartrain et al. 1997, 2001).

Bacteria-mediated formation of chiral synthons

Bacteria that metabolize aromatic hydrocarbons have garnered much attention because of their ability to degrade xenobiotic compounds in bioremediation applications. In such bacteria, the degradation of aromatic hydrocarbons proceeds through an enzymatic oxidation. These multicomponent enzyme systems catalyze a remarkable range of oxidation reactions (Gibson and Parales 2000; Gibson et al. 1995). Of particular interest is the incorporation of molecular oxygen into the aromatic nucleus to form vicinal arene cis-diols (reviewed in Butler and Mason 1997). Characteristic of these enzyme systems, a Rieske nonheme iron oxygenase serves as an electron receptor preceded by a reductase chain, which transfers electrons from NAD(P)H to this terminal oxygenase. This reduced terminal dioxygenase catalyzes the direct insertion of molecular oxygen into the substrate (Fig. 3). Mononuclear iron is believed to be the site of oxygen activation. Based on recent protein structure determinations, Rieske iron dioxygenases are believed to share a 2-His-1-carboxylate facial triad active site motif (Kauppi et al. 1998). Many monooxygenase enzymes, thought to proceed through an epoxide intermediate, are also included in this enzyme family (Gibson and Parales 2000). More than 300 arene cis-diols have been identified as initial reaction products of these oxygenases and, in many cases, the cis-diols produced are of a high enantiomeric purity (Boyd and Sheldrake 1998; Hudlicky et al. 1999; Resnick and Gibson 1996). The exquisite stereoselectivity of these biocatalytic systems represents a unique source of chiral synthons for the production of fine chemicals. Additionally, biocatalytic systems present the possibility of a more environmentally benign synthesis process, as they could potentially obviate the harsh catalytic conditions required in many chemical syntheses.

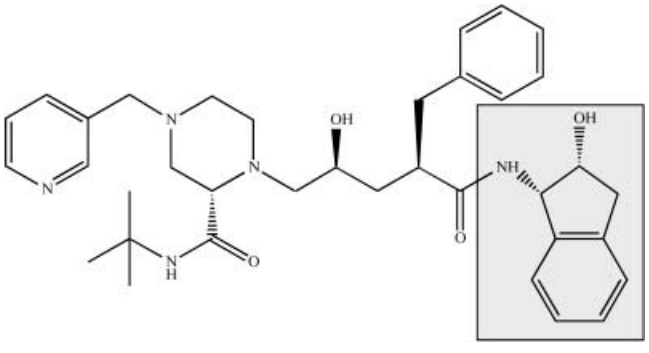


Fig. 1 Chemical structure of CRIXIVAN (indinavir). Cis-(1S,2R)-1-aminoindan-2-ol, an indene derivative contributing two chiral centers, is indicated in *grey*

Buckland et al. (1999b) proposed a hypothetical bioconversion to produce the key chiral synthon, cis-(1S,2R)-1-aminoindan-2-ol (Fig. 4). This scheme of biocatalysis requires a purified enzyme or whole cell preparation capable of converting indene to oxygenation products of (2R) stereochemistry (Buckland et al. 1999a). Although generally more expensive, cell-free enzyme preparations are free of side reactions catalyzed by endogenous enzymes and therefore offer increased productivity. However, the complex and labile nature of the oxygenase enzyme system, coupled with cofactor regeneration

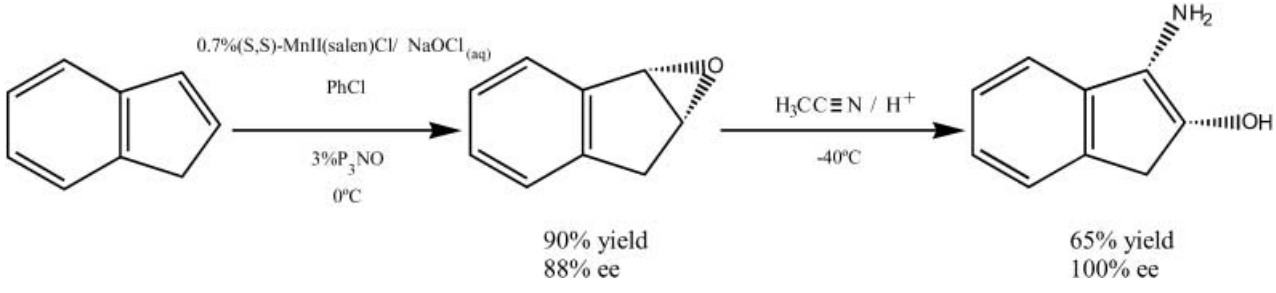
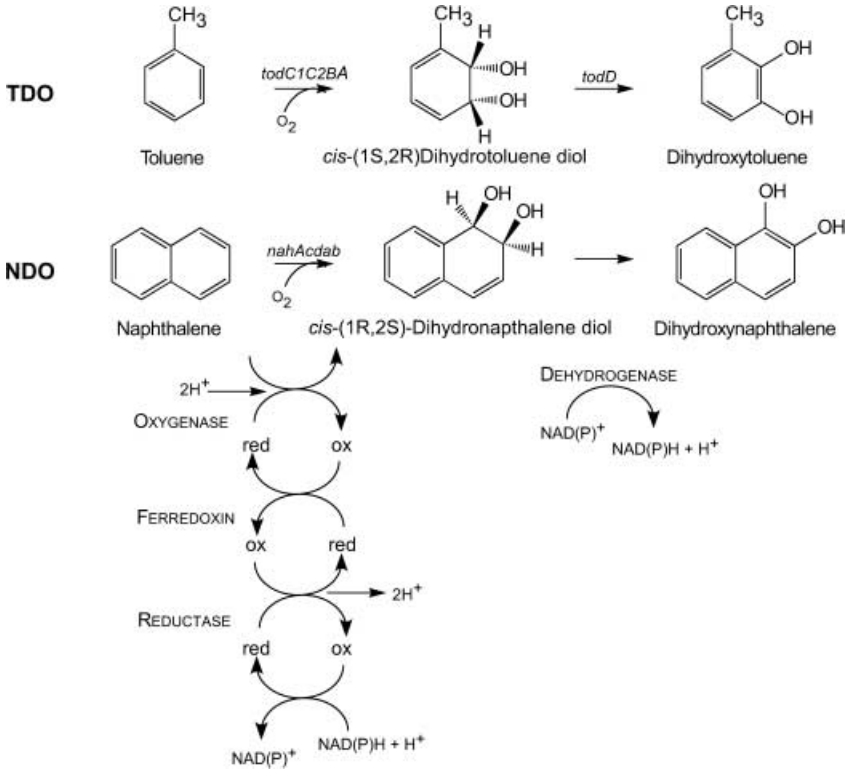


Fig. 2 Chemical synthesis of cis-(1S,2R)-1-aminoindan-2-ol. Indene is oxidized by *NaOCl* to (2R)-indan oxide (88% ee) using a Jacobsen salen catalyst in a biphasic phenyl chloride/aqueous system (Senanayake et al. 1996). The reaction is promoted by the activity of 4-(3-phenylpropyl) pyridine N-oxide *(P_3NO)*. This activated epoxide intermediate is then aminated with acetonitrile under acidic conditions using a Ritter type reaction, maintaining the stereochemistry of the C2 carbon. The resultant yield of the desired aminoindanol from indene is ca. 60%



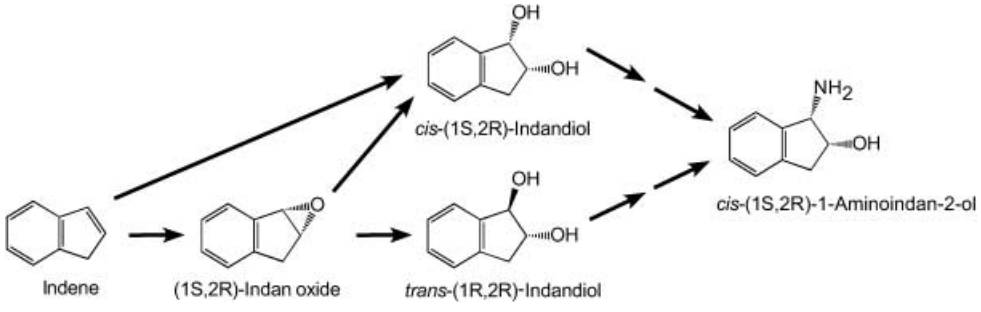


Fig. 3 Canonical pathways for the oxidation of aromatic hydrocarbons in bacteria. In bacterial oxygenation of aromatic hydrocarbons, an electron transport chain transfers electrons from NAD(P)H through a flavoprotein (*reductase*) and a [2Fe-2S] protein (*ferredoxin*) to a terminal Rieske non-heme iron dioxygenase (*oxygenase*). This reduced terminal oxygenase catalyzes the direct insertion of molecular oxygen into the aromatic nucleus to yield a dihydrodiol in which the hydroxyl groups have a cis-relative stereochemistry. Toluene dioxygenase (*TDO*) from *Pseudomonas putida* F1 catalyzes the enantioselective oxidation of toluene to cis-(1S,2R)-dihydrotoluene diol (Zylstra and Gibson 1989). Naphthalene dioxygenase (*NDO*) from *P. putida* 9816 similarly catalyzes the oxidation of naphthalene to cis-(1R,2S)-dihydronaphthalene diol (Ensley et al. 1982)

Fig. 4 Hypothetical bioconversion process for the production of cis-(1S,2R)-1-aminoindan-2-ol. Cis-aminoindanol can be chemically synthesized directly from cis- or trans-indandiol of (2R) configuration. Ideally, the stereospecific addition of an amine, catalyzed by a bacterial transaminase, would be incorporated into this biocatalytic network allowing the conversion of indene to cis-aminoindanol at high yields in a single-stage fermentation

requirements, necessitates the development of a whole cell bioconversion system for harnessing the stereospecific oxygenation activity of bacterial catalysts.

The possibility of engineering the proposed bioconversion process for the production of cis-aminoindanol is encouraging as biological mechanisms capable of converting indene to the desired oxygenation products have been characterized.

Bacterial oxidation of indene

The enzymatic conversion of indene to both cis- and trans-(1,2)-indandiol was originally reported in mammals by Brooks and Young (1956). They hypothesized an epoxy indan intermediate that was later confirmed in a similar mammalian system (Francis et al. 1975). More recent studies of aromatic metabolism in microbes have identified a number of naphthalene- and toluene-degrading isolates capable of oxygenating indene, often in a stereospecific manner.

Filamentous fungi have been identified that can convert indene to indan oxide and cis-indandiol by the action of a haloperoxidase followed by a pH-dependent resolution (Chartrain et al. 1997). The vast majority of research, however, has focused on bacterial systems. The bacterial oxygenation of indene was originally observed in Gram-negative pseudomonads, a genus characterized by the ability to metabolize a wide range of aromatic substrates (Gibson et al. 1993). *Pseudomonas putida* F1 was shown to transform both toluene (Gibson et al. 1970) and indene (Wackett et al. 1988) to their respective cis-dihydrodiols. In addition, Wackett et al. (1988) observed the benzylic monooxygenation product of indene, 1-indenol. These reactions are catalyzed by a three-

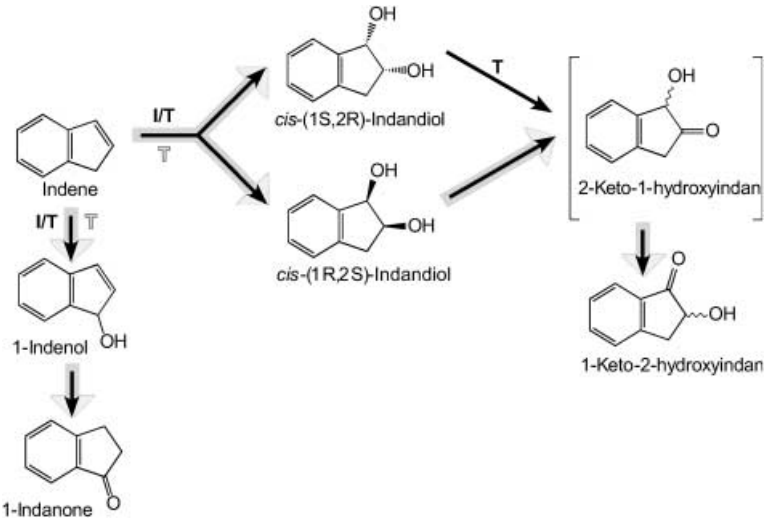


Fig. 5 Indene bioconversion by *P. putida* F1 and *Rhodococcus* sp. B264-1. Both strains oxygenate indene to the major products cis-(1S,2R)-indandiol and (1R)-indenol (Chartrain et al. 1998; Wackett et al. 1988). *P. putida* F1 (*grey arrows*) requires toluene (*T*) for the induction of indene oxidation. B264-1 (*black arrows*) will oxygenate indene with either toluene or indene (*I*) as the sole inducer. Both enantiomers of cis-indandiol are produced, but the cis-(1R,2S)-indandiol is selectively dehydrogenated. B264-1 also has a toluene-induced dehydrogenase activity that specifically degrades cis-(1S,2R)-indandiol. The monooxygenation product, 1-indenol, is an alternate oxidation product of the dioxygenase. Recombinant *Escherichia coli* strains constitutively expressing the tolC1C2BAD portion of the *P. putida* TDO operon oxygenate indene similarly to F1 without a requirement for toluene

component toluene dioxygenase enzyme system (TDO) (Wackett et al. 1988; Fig. 3; Fig. 5) consistent with a Rieske non-heme iron type dioxygenase. TDO was shown to convert indene to cis-(1S,2R)-indandiol with an enantiomeric excess (ee) of 30%, which increased to greater than 80% upon the selective dehydrogenation of the cis-(1R,2S)-indandiol, and the essentially pure (1R)-indenol, in a toluene-dependent manner (Boyd and Sheldrake 1998; Brand et al. 1992; Wackett et al. 1988). A *P. putida* mutant, lacking (2S)-cis-glycol dehydrogenase activity, grown in the presence of toluene, shows similar cis-indandiol formation, but produces (1S)-indenol with an ee of 26% (Connors et al. 1997). Due to the absence of toluene-derived monooxygenase products, Connors et al. (1997) extended the hypothesis that the monooxygenation activity responsible for the 1-indenol product is the result of an “improper fit” of indene in the active site of the dioxygenase.

Pseudomonas sp. NCIB 9816-4 was shown to oxidize indene in a naphthalene-inducible manner via the multicomponent naphthalene dioxygenase system (NDO) to cis-(1R,2S)-indandiol (ca. 90% ee) and (1S)-indenol (ca. 94% ee) (Gibson et al. 1995; Fig. 3). Although induction of an oxygenase activity is dependent upon the substrate for which it is named, the TDO and NDO enzyme systems characterized in *Pseudomonas* both initiate the oxygenation of toluene and naphthalene to form cis-(1S,2R)-dihydrotoluene diol (Gibson et al. 1970; Kobal et al. 1973) and cis-(1R,2S)-dihydronaphthalene diol (Jeffrey et al. 1975; Jerina et al. 1971), respectively. Interestingly, these enzymes appear to catalyze the oxygenation of indene to favor products of different stereochemistry. This seems to suggest inherent differences in the alignment of indene in the active site of these enzymes. A similar enantiomeric bias is observed in the oxidation of 1,2-dihydronaphthalene (Torok et al. 1995). Torok et al. (1995) attributed this to the twisted configuration of 1,2-dihydronaphthalene about the plane – in contrast to the planar naphthalene molecule – postulating a 180° shift in orientation in the active site of TDO relative to that of NDO. A similar argument might be extended to explain the differential oxygenation of indene by these enzymes, as the five-membered ring substituent of indene shows similar twist about the plane. Perhaps such twist may account for the enantiomeric bias and lower intrinsic stereospecificity of these enzymes with respect to indene.

The recent determination of the NDO protein structure from NCIB 9816-4 (Kauppi et al. 1998) coupled with analysis of the active site motif believed to characterize Rieske non-heme iron dioxygenases (Que 2000) provides an insight into the chemical nature of their catalytic diversity. In this active site structure, two histidines and one carboxylate ligand occupy one face of the iron(II) coordination sphere (Hegg and Que 1997). This allows the metal center to bind O_2 and a variety of substrates on the opposite face. Such a juxtaposition of reactants may serve to facilitate catalysis and provide an intrinsic flexibility to the active site structure. Additionally, Que (2000) proposes that the electronic properties of the trans ligand may modulate the chemical reactivity of the bound O_2. Such changes in reactivity mediated by the neutral His or anionic carboxylate could allow distinct enzymatic transformations of the same substrate. Perhaps this, coupled with the flexibility of the active site structure, is the basis for the versatility of these enzymes.

Gram-positive rhodococci have been shown to have similar abilities to oxygenate indene. This genus has

been the focus of much biotechnological attention, stemming from the diverse range of reactions, including novel activities catalyzed by their enzymes (Bell et al. 1998; Larkin et al. 1998). Of particular interest is the presence of parallel, non-redundant, enzyme systems for the metabolism of the same substrate (Asturias et al. 1995). A naphthalene metabolizing strain, *Rhodococcus* sp. NCIMB 12308, oxidizes indene to cis-(1S,2R)-indandiol (80% ee) and the monooxygenase product (1S)-indenol (>97% ee). Unlike *Pseudomonas*, this strain also appears to possess a distinct monooxygenase activity evidenced by the production of trans-(1,2)-indandiol via an epoxide intermediate (Allen et al. 1997). Naphthalene-induced *Rhodococcus* sp. 1BN similarly oxidizes indene to cis-(1S,2R)-indandiol (75% ee) with evidence of monooxygenase activity (Andreoni et al. 2000).

Rhodococci appear to metabolize aromatic hydrocarbons in a manner that is homologous to, yet functionally distinct from, that characterized in pseudomonads. In contrast to pseudomonads, where the catabolic plasmids responsible for the degradation of aromatic hydrocarbons appear to be highly conserved (Fritsche and Hofrichter 2000; Stuart-Keil et al. 1998), aromatic degradation pathways characterized in rhodococci suggest more diverse evolutionary origins. Large, linear extrachromosomal elements, and their recombinant derivatives, are believed to mediate the horizontal transfer of many degradative phenotypes in this genus (Larkin et al. 1998; Uz et al. 2000). Often flanked by ORFs with homology to integrases and transposases (Poelarends et al. 2000), the presence of "silent" genes and partial pathways for aromatic metabolism have been extensively observed in *Rhodococcus* (Irvine et al. 2000; Kulakov et al. 1998; Kulakova et al. 1995; Larkin et al. 1998), with novel degradative phenotypes emerging as a function of recombination (Kulakova et al. 1996). Several distinct mechanisms for non-homologous recombination have been implicated in *Rhodococcus* (Kasweck and Little 1982; Larkin et al. 1998), perhaps mediating the eclectic assembly of these functional systems.

Feasibility of large-scale indene biocatalysis

Encouraged by bacterial isolates capable of catalyzing the oxygenation of indene to products of (2R) stereochemistry in high enantiomeric excess, several bacterial systems were analyzed to determine the feasibility of an industrial-scale process for the production of cis-aminoindanol.

Pseudomonads

Buckland et al. (1999b) investigated the adaptability of the extensively characterized *P. putida* F1 system to an industrially relevant indene bioconversion yielding cis-aminoindanol precursors (Buckland et al. 1999a; Connors 1998). As discussed, *P. putida* F1 expresses TDO in a toluene-dependent manner, catalyzing the oxidation of indene to the desired cis-(1S,2R)-indandiol product. A silicone-oil-based, biphasic system was used to deliver the hydrophobic substrates on a 23 l and 70 l fermentation scale. Using this system, cis-indandiol was produced with the ee of the desired (1S,2R) enantiomer gradually increasing from ca. 40% to greater than 98% with a dehydrogenase-dependent bioresolution phase (Fig. 5). In contrast to the intrinsic stereospecific oxidation of toluene, the high ee of indene oxidation products is believed to result from selective metabolism of the (1R,2S) enantiomer to ketohydroxyindan and, as such, the increase in ee of the desired cis-(1S,2R)-indandiol was accompanied by a proportional drop in total cis-indandiol concentration (Buckland et al. 1999b). Although clearly not an optimal system, ca. 1 kg of enantiomerically pure cis-(1S,2R)-indandiol was extracted for analysis via a four-step isolation procedure from this bioprocess, a portion of which was carried through to indinavir sulfate (Buckland et al. 1999b).

Due to the safety issues involved in the scale-up of a process involving volatile organics, and concerns of TDO substrate competition, a toluene-independent system for indene oxidation was desired. Mutants of *P. putida* that behaved in a toluene-independent manner had been previously identified, but the resultant TDO activity was of poor stereospecificity (Connors et al. 1997). Buckland et al. (1999b) were able to isolate a mutant stain of *P. putida* F1 that increased cis-(1S,2R)-indandiol titers by 20-fold over the parent strain, independent of toluene induction. It was determined, however, that end-product inhibition of TDO activity prevented bioconversions from achieving indandiol titers greater than 2 g/l. These end-product inhibitions negatively impact the productivity of this system and thus sharply limit its suitability for large-scale bioconversions.

Recombinant *Escherichia coli*

There is precedence in the literature for the functional expression of *Pseudomonas*-derived TDO in an *E. coli* background (Brand et al. 1992; Zylstra and Gibson 1989). The feasibility of producing a recombinant *E. coli* strain expressing *P. putida* TDO was investigated by Buckland et al. (1999b) as an alternate route to toluene-independent activity (Connors 1998). A recombinant *E. coli* construct harboring the tolC1C2BA from *P. putida* (Fig. 3) showed constitutive activity and oxidized indene to cis-(1S,2R)-indandiol (30% ee) at a final titer of 1 g/l. The addition of the tolD dehydrogenase gene to this expression construct (Fig. 3) led to a sharp increase in the ee of cis-(1S,2R)-indandiol (>99% ee) and a corresponding proportional decrease in indandiol titer (650 mg/l) confirming that the stereospecificity of TDO dioxygenase, with respect to indene, is intrinsic and the selective metabolism of the cis-(1R,2S)-indandiol enantiomer was responsible for the time-dependent increase of ee observed in this system (Buckland et al. 1999b; Fig. 5).

Further optimization of this recombinant *E. coli* TDO system led to a single stage bioconversion process with final cis-(1S,2R)-indandiol titers of 1.2 g/l at an enantiomeric excess of greater than 99% (Reddy et al. 1999). The recombinant *E. coli* strain proved to be as susceptible to the toxic effects of indene and its bioconversion products as the corresponding *P. putida* strain (Reddy et al. 1999). This recombinant strain, however, is a powerful tool for the development of a modified enzyme system. The metabolism and regulation of *E. coli* systems are well characterized. Additionally, a variety of genetic tools are in place to manipulate the catalytic properties of TDO by protein modification in this strain. Indeed, this recombinant *E. coli* TDO was subjected to a series of directed evolution cycles using error prone PCR, resulting in a TDO mutant with a 40% increase in cis-indandiol yield and a 3-fold reduction in the formation of the 1-indenol byproduct (Zhang et al. 2000).

Rhodococci

In addition to investigating the suitability of characterized indene oxygenating strains, Chartrain et al. (1998) began broad microbial screening to identify bacteria better suited to large-scale bioconversion. Specifically, strains were identified that were able to both tolerate high concentrations of indene and its metabolites and stereospecifically oxygenate indene into indandiols of (2R) stereochemistry. Many of the strains identified in this manner were determined to be rhodococci, a genus known to metabolize a wide range of aromatic substrates (Finnerty 1992). *Rhodococcus* cell surfaces are characteristically hydrophobic due to the aliphatic chains of mycolic acid present in their cell walls (Bell et al. 1998). This property, combined with their demonstrated solvent tolerance, suggest that rhodococci are uniquely suited to the bioconversion of poorly soluble organic substrates. In biphasic systems, rhodococci partition to the interface, perhaps facilitating the degradation of hydrophobic substrates by allowing cells to adhere to the organic/aqueous interfaces and increase local substrate concentrations (Neu 1996). Additionally, rhodococci are known to produce surfactants that reduce interfacial tension between phases, potentially aiding the movement of hydrophobic molecules into the cell (Fiechter 1992).

Rhodococcus strains were isolated that supported indandiol product titers as high as 4 g/l with high early ee relative to that of *P. putida*, perhaps indicative of an indene oxidation system of higher intrinsic specificity. These isolates were significantly more resistant to the toxic effects of indene than both *P. putida* and *E. coli* in a two-phase culture system using silicon oil as an indene carrier. Although rates of bioconversion and product yields were generally lower, the oxidation of indene appeared to be more stereospecific (Chartrain et al. 1998). Additionally, indene was oxidized in the absence of inducer. Two of these isolates, *Rhodococcus* sp. B264-1 (Merck designation MB5655) and *Rhodococcus* sp. I24 (Merck designation MA7205), were further investigated for their suitability as biocatalysts based on the initial performance and stereospecificity of preliminary indene bioconversions (Chartrain et al. 1998).

B264-1 was isolated on toluene as a sole carbon source. Final cis-(1S,2R)-indandiol titers of greater than 4 g/l produced by this strain represented a 2-fold increase over the *P. putida* process (Connors et al. 1997). Initial characterization of this strain identified the indene oxidation products cis-(1S,2R)-indandiol (99% ee) and 1-indenol, as well as several downstream degradation products, suggesting a toluene type dioxygenase activity (Fig. 5). Further investigation indicates that both cis-indandiol enantiomers are produced and later resolved via a selective dehydrogenation of the cis-(1R,2S)-indandiol (Buckland et al. 1999a; Chartrain et al. 1998). This is similar to the oxygenation of indene characterized in *P. putida* F1 (Brand et al. 1992; Wackett et al. 1988; Fig. 5). When the bioconversion was carried out in the presence of toluene, however, both enantiomers of cis-indandiol were degraded at similar rates in B264-1 (Chartrain et al. 1998; Fig. 5). This suggested the presence of at least two distinct cis-indandiol dehydrogenase activities, one of which is toluene-responsive.

Recently, Chartrain et al. (2000) identified a nitrosoguanidine mutant of B264-1 blocked in cis-glycol dehydrogenase activity (assigned Merck designation MA7249). The accumulation of cis-dihydrotoluene diol in this strain is consistent with the loss of the toluene-dependent dehydrogenase activity. This mutant also produced racemic cis-indandiol in a toluene-independent manner. Interestingly, unlike the B264-1 parental strain that selectively dehydrogenates cis-(1R,2S)-indandiol, MA7249 selectively dehydrogenated the opposite enantiomer, cis-(1S,2R)-indandiol, in a toluene-independent manner (Chartrain et al. 2000). Dehydrogenation of cis-indandiol in the presence of toluene was not investigated. One explanation of this mutant phenotype postulates that the presence of the cis-glycol dehydrogenase activity in the B264-1 parent is responsible for the constitutive cis-(1R,2S)-indandiol dehydrogenase activity. As the remaining dehydrogenase activity of B264-1 selectively degrades cis-(1S,2R)-indandiol, this attribution is consistent with the observed indene oxidation products of the cis-glycol dehydrogenase blocked mutant. This model, though, would require that the cis-(1S,2R)-indandiol dehydrogenase activity also be relieved of its apparent toluene dependence. The basis of this phenomenon has not been characterized in MA7249. However, considering that it is the toluene dependent cis-dihydrotoluene diol dehydrogenase activity that is blocked in MA7249, the loss of the corresponding toluene dependent cis-(1S,2R)-indandiol dehydrogenase activity seems more intuitive. If this is indeed the dehydrogenation activity blocked in this mutant, how can the apparent change in substrate specificity of the remaining dehydrogenase activity be explained? There is precedence for the substrate preference of a cis-indandiol acting dehydrogenase changing as a function of cis-indandiol concentration. Allen et al. (1995) described such a phenomenon in the indene

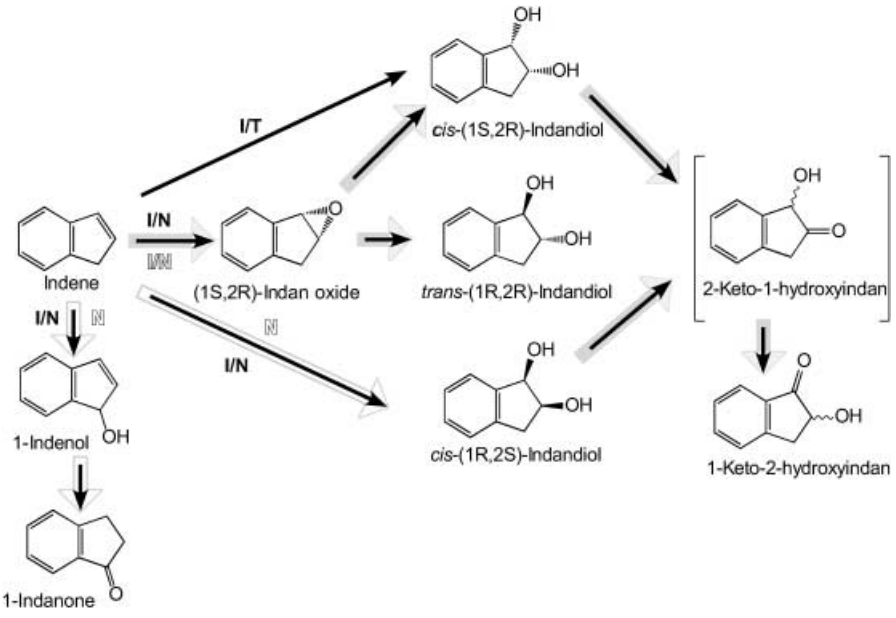


Fig. 6 Indene bioconversion network in *Rhodococcus* sp. I24 and its derivative KY1. In I24 (*black arrows*) and KY1 (*grey arrows*), different subsets of the indene bioconversion network are induced in the presence of toluene (*T*), naphthalene (*N*), or indene (*I*). The predominant indene oxygenation product is trans-(1R,2R)-indandiol. The naphthalene-inducible dioxygenase also catalyzes the formation of 1-indenol (Treadway et al. 1999), as may the toluene-inducible dioxygenase. This activity, as well as a naphthalene-inducible dioxygenase activity that catalyzes the production of cis-(1R,2S)-indandiol, is present but greatly reduced in the KY-1 strain (indicated by *white arrows*). Toluene-grown I24 exhibits an additional dioxygenase activity, producing cis-(1S,2R)-indandiol that is absent in the KY1 strain (Yanagimachi et al. 2001). When indene is the sole inducer, indene oxidation proceeds mainly through a distinct, epoxide-forming monooxygenase activity to (1S,2R)-indan oxide that non-enzymatically resolves to cis-(1S,2R)- and trans-indandiol. The cis-indandiols produced in both strains are further metabolized to ketohydroxyindan (Buckland et al. 1999b)

oxygenating strain, *P. putida* NCIMB 8859. Naphthalene-induced NCIMB 8859 cultures oxidized indene to cis-(1R,2S)-indandiol (85–90% ee) with substrate concentrations of ca. 0.5–1.0 mg/ml. The addition of racemic cis-indandiol at lower concentrations (ca. 0.2–0.4 mg/ml) resulted in the asymmetric degradation of the opposite enantiomer, resulting in a greater than 98% enantiomeric excess of cis-(1S,2R)-indandiol (Allen et al. 1995). Perhaps the difference in ee observed between the original characterization of B264-1 (Chartrain et al. 1998) and MA7249 may be due to a similar concentration-dependant phenomenon. Additional investigation is clearly needed to characterize the nature of this mutant phenotype.

The second strain investigated, *Rhodococcus* sp. I24, was able to tolerate higher concentrations of indene than the earlier characterized strains. I24 was able to use either naphthalene or toluene as a sole carbon source and oxidize indene to desirable (2R) enantiomers of indandiol, in addition to several undesirable byproducts (Chartrain et al. 1999; Fig. 6). Racemic ketohydroxyindan was derived from the cis-indandiols while the other undesirable products, indenol and indanone, were derived directly from indene. Although a subset of the indene oxygenating activities appeared to be dependent on the presence of toluene, naphthalene [trans-(1R,2R)-indandiol] was produced from indene at an ee of greater than 98% in an induction-independent manner (Buckland et al. 1999b; Chartrain et al. 1998). Based on product profiling under various induction conditions, a model of indene bioconversion was proposed for I24 consisting of a network of enzymes capable of oxygenating indene to both cis- and trans-indandiols (Buckland et al. 1999b; Chartrain et al. 1998; Fig. 6). It is not uncommon to find similar catabolic operons preceded by entirely different regulatory mechanisms (de Lorenzo and Perez-Martin 1996). The range of induction mechanisms seen in *Rhodococcus* may reflect the diverse evolutionary origins of these degradative pathways. The proposed bioconversion network was similar to the pathways of naphthalene and toluene degradation characterized in *Pseudomonas* (Gibson and Subramanian 1984). However, unlike previously characterized aromatic degradation pathways, removal of ketohydroxyindan does not appear to proceed through a ring-cleaving type dioxygenase. Because of the favorable strain characteristics and seemingly higher stereoselectivity of the enzymes involved in indene oxygenation, I24 was chosen as a model organism to develop a process platform for the optimization of bioconversion and strain performance (Buckland et al. 1999b). With this goal, targeted genetic and metabolic engineering tools were developed for this purpose (Stafford et al. 2001; Treadway et al. 1999; Yanagimachi et al. 2001).

Dissecting a model indene bioconversion

Tools were developed to allow genetic manipulations of the poorly characterized *Rhodococcus* sp. I24. These tools allowed the characterization of a novel naphthalene

type dioxygenase, NID, that phylogenetically clusters independently from previously described naphthalene dioxygenases in *Pseudomonas* (Gibson et al. 1995). The NID system catalyzes the oxidation of indene to cis-(1R,2S)-indandiol (Treadway et al. 1999).

Several large extrachromosomal elements were observed in I24 (Treadway et al. 1999) and characterized both genetically and phenotypically (O'Brien et al. MS submitted). Sequence data and functional analysis of transconjugant strains suggest that the indene oxygenation activities are localized on two distinct elements of size ca. 50 and 340 kb (O'Brien et al. MS submitted). The localization of biodegradative phenotypes to transmissible large extrachromosomal elements has been recognized in *Rhodococcus* (Meinhardt et al. 1997; Poelarends et al. 2000; Stuart-Keil et al. 1998). These data, complemented by induction studies using reporter gene fusions (O'Brien et al. MS submitted), supports the attribution of the naphthalene inducible dioxygenase enzyme activity to the nid gene cluster. The nid cluster has been localized to the ca. 50 kb transmissible extrachromosomal element. The tid gene cluster, responsible for the toluene inducible dioxygenase (TID) activity, has been identified and mapped to the larger ca. 340 kb element (O'Brien et al. MS submitted; Fig. 6). An additional gene cluster residing on the ca. 50 kb element, designated nim, has also been identified. Ongoing phenotypic studies and induction data implicate the nim cluster in the naphthalene-inducible indan oxide forming monooxygenase activity (O'Brien et al., MS submitted; Fig. 6). Mapping the genetic organization of the different gene systems mediating the metabolism of aromatic hydrocarbons in this strain will provide a basis for understanding and ultimately manipulating indene bioconversion in this species.

A novel chemostat system was developed for flux analysis investigations of I24 using a gas phase delivery of indene, allowing for more quantitative analysis than afforded by the two-phase fermentation system traditionally used (Yanagimachi et al. 2001). Extended chemostat cultivation of I24 in the presence of aromatic inducers led to the emergence of *Rhodococcus* sp. KY1, a stable mutant of I24 with altered indene metabolism (Stafford et al. 2001). In KY1, 1-indenol and 1-indanone were no longer produced in indene bioconversion when indene was the sole inducer. Under these conditions, the primary oxidation products of KY-1 are trans- and cis-indandiol, indan oxide and ketohydroxyindan. Additionally, KY1 has lost the toluene inducible oxygenase activity, as well as the ability to utilize toluene as a sole carbon source (Stafford et al. 2001; Fig. 6). Consistent with this observation, it has been determined that KY1 is missing the ca. 340 kb extrachromosomal element found in the parental I24 strain where the tid gene cluster, responsible for the toluene inducible dioxygenase activity, resides (O'Brien et al. MS submitted). The growth rate advantage of spontaneous mutants in continuous culture that have suffered a full or partial deletion of a toluene catabolic plasmid has been observed in *P. putida* grown on aromatic substrates (Duetz et al. 1991; Williams et al. 1988). Perhaps the observed growth rate disadvantage of wild type degraders is due to toxic or inhibitory effects of intermediates in aromatic metabolism.

Biomass and metabolite concentration analysis of KY1 in a number of steady-state chemostat studies, under different dilution rates and indene feed concentrations, indicated a correlation between biomass and subsequent indene metabolite titers (Stafford et al. 2001). These data suggest that, if indene feed concentrations are low, final titers of the targeted indene oxygenation products may be significantly improved in fed-batch bioconversions. This strategy also minimizes the obstacle of growth attenuation due to substrate toxicity (Stafford et al. 2001).

Yanagimachi et al. (2001) undertook a rigorous metabolic analysis of indene bioconversion in the KY1 strain. Detailed flux analysis of the indene bioconversion, using both radiolabeled induction studies and macroscopic metabolite balancing, was determined at several steady states using different dilution rates and indene feed concentrations. This analysis identified the major route of indene oxidation in KY1 as the naphthalene-inducible monooxygenase, with at least 94% of indene oxidized to indan oxide in all states analyzed (Yanagimachi et al. 2001). Tracer studies indicate that indan oxide is non-enzymatically resolved to cis-(1S,2R)-indandiol and trans-(1R,2R)-indandiol, both of which are suitable precursors to the desired cis-aminoindanol synthon. cis-(1S,2R)-Indandiol, however, is further metabolized to ketohydroxyindan, reducing the amount of indene metabolized to desired product by 25% (Yanagimachi et al. 2001). Despite the loss of the cis-indandiol to ketohydroxyindan, KY1 final titer estimates of 8.7 g/l (Stafford et al. 2001) are sufficient to warrant experimental investigations of an industry-scale bioconversion process.

Considerations for optimizing indene bioconversion

Detailed analysis of the improved indene bioconversion properties of KY1, the stable chemostat-derived mutant of I24, identify a number of strategies for targeted process improvements. The cis-(1S,2R)-indandiol dehydrogenase activity limits the maximum yield of the desired (2R)-indandiol product to 60%, due to the non-enzymatic hydrolysis under the culture conditions examined. Several strategies can be pursued to increase product yields. Certainly, blocking the dehydrogenase activity responsible for this loss of product would increase yields. An alternate strategy would involve the preferential resolution of the indan oxide to the trans-indandiol by a heterologous epoxide hydrolase. A limonene-1,2-epoxide hydrolase from *R. erythropolis* DCL14 has been characterized that shows significant hydrolase activity using indan oxide as a substrate (Barbirato et al. 1998; van der Werf et al. 1998, 1999). This epoxide hydrolase was introduced as an expression construct in KY1. The resulting enzyme mediated resolution of the indan oxide and

resulted in improved yields of trans-(1R,2R)-indandiol in this system (Stafford et al. 2002). Stafford et al. (2002) saw additional improvement in yields of trans-(1R,2R)-indandiol in KY1 expressing epoxide hydrolase when the pH of the bioconversion was adjusted to more alkaline conditions (pH >8).

In the KY1 network, the epoxide forming monooxygenase activity itself provides a target of additional intervention. As the key enzyme in this bioconversion network, the overexpression of the enzyme responsible for this activity may further increase the flux to indan oxide resulting in increased product titers. The effect of end-product inhibitions, as discussed previously, needs to be considered in any attempt to increase productivity of a strain. Buckland et al. (1999b) presented evidence indicating that trans-(1R,2R)-indandiol and ketohydroxyindan may serve to inhibit monooxygenase activity, therefore increasing titers are unlikely to be achieved without addressing this issue. The selective removal of end-products from the bioconversion, perhaps by using a selective resin such as the SP-207 resin used in the isolation of cis-indandiols in *P. putida* indene bioconversions (Buckland et al. 1999b), is one potential strategy to avoid product inhibition.

The addition of a transaminase-type enzyme may allow the conversion of indene to cis-aminoindanol in a single strain, as envisioned in the idealized biocatalytic pathway (Fig. 4). As well as providing a commercially desirable single stage process, the metabolism of products implicated in toxic and inhibitory effects may serve to further improve the overall characteristics of the bioconversion system.

The genomic sequence of the I24 model strain has recently been determined, enabling more detailed genetic analysis. Transcriptional profiling is being investigated to elucidate the apparent growth-dependent expression of indene oxygenation genes, a behavior common to the regulation of catabolic pathways for the degradation of recalcitrant aromatic compounds (Cases and de Lorenzo 2001), as well as global regulation patterns of this strain and its derivatives during indene bioconversion.

Targeted engineering of biocatalysts

Traditionally, the innate characteristics of extant enzymatic activities and biocatalytic strains have dictated the conditions available for the development of a large-scale bioconversion processes. The increasing number of techniques in the genetic and metabolic engineering tool box now affords a generalized strategy for the improvement of a bioconversion process that involves not just manipulating existing strains and activities, but the targeted modification of these enzymes and pathways to engineer a biological catalyst well-suited for the process for which it is intended.

In addition to characterizing pathways and identifying key points of genetic and metabolic control, directed evolution techniques can be employed to optimize an existing activity under process relevant conditions. Iterative random mutagenesis techniques combined with the random recombination of the genes of protein variants with favorable mutations (DNA “shuffling”) create unbiased chimeras of the input genes (Coco et al. 2001). This enables the selection of recombinants carrying multiple mutations beneficial to the target process. DNA shuffling of mutations in the same enzyme, or subunit swapping of heterologous enzymes, has been used to successfully engineer aromatic oxygenases with expanded substrate specificities (Furukawa 2000; Suyama et al. 1996) and improved or unique catalytic activities (Cherry 2000). Regulation characteristics, such as induction requirements and end-product inhibition, have also been targeted on the protein level to improve biocatalyst performance (Ramos et al. 1986, 1987). Burton et al. (2002) offer a timely review on the techniques available for the engineering of enzyme catalysts and the technological barriers remaining that need to be removed before their full potential can be realized.

The increasing volume of genomic data available, coupled with expanding collections of microbial isolates, increases the industrial relevance of biocatalysts both by expanding the catalog of native catalytic activities and by increasing the pool of diversity available for bioprospecting and DNA shuffling technologies. Additionally, transcriptional data coupled with large-scale analysis of protein abundance and enzyme activity is beginning to tease apart the complexity of metabolic networks and elucidate the mechanisms that predict superior strain performance under process relevant conditions.

Selecting an oxygenase with appropriate stereospecificity, overcoming feedback inhibition of this activity, and preventing undesirable dehydrogenation of oxygenation products are all challenges that must be met before biocatalysis of indandiol formation can be incorporated into a commercial process. The goal of single process aminoindanol production entails the additional discovery of appropriate transaminases, where again the stereoselectivity of the enzyme will have a strong influence on the viability of the bioprocess.

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