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Preparative scale production of 3-substituted catechols using a novel monooxygenase from *Pseudomonas azelaica* HBP 1

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Abstract

Pseudomonas azelaica HBP 1 contains a 2-hydroxybiphenyl 3-monooxygenase (E.C. 1.14.13.44, HbpA) which produces 3-substituted catechols from 2-substituted phenols. The tetrameric enzyme (subunit mass 60 kDa) carries FAD as a prosthetic group, uses NADH as cofactor and is homologous to a number of NADPH dependent flavin containing phenol monooxygenases. HbpA regio-selectively oxidizes 2-substituted phenols and has a broad substrate-range. As *ortho*-substituent it accepts various alkyl-residues, halogen atoms and substituted phenyl-residues. We have used this monooxygenase for the production of 3-substituted catechols, which are useful synthons that are difficult to obtain by chemical means. A recombinant *E. coli* JM101, containing the *hbpA* gene, was used for whole-cell biotransformations. Since the phenols as well as the corresponding catechols produced are toxic to cells, a process with a limited feed of starting-material combined with in situ product recovery was used. 3-Phenyl-, 3-chloro-, 3-bromo-, 3-ethyl-, 3-propyl-, 3-*i*-propyl-, and 3-*sec*-butyl-catechol could thus be produced in gram amounts with space-time yields up to 0.45 g $l^{-1}h^{-1}$. The products were recovered and further purified by recrystallization. Authenticity of all products, some of which had not been described before, was verified by ^{1}H NMR, ^{13}C NMR, IR spectroscopy and GC/MS.

Keywords: *Pseudomonas azelaica*; Catechol; HbpA; 2-Hydroxybiphenyl 3-monooxygenase; In situ recovery

1. Introduction

Catechol derivatives (pyrocatechols or 1,2-dihydroxybenzenes) carrying a substituent on the aromatic ring are a group of physiologically important molecules. Catechols carrying *para* substituents include, for instance, adrenalin (epinephrine or L-3,4-dihydroxy-α-(methylaminomethyl)benzyl alcohol) and the anti Parkinson's disease drug L-DOPA (3,4-dihydroxy-L-phenylalanine). Although *meta*-substituted catechols have received less attention than their *para*-substituted counterparts, these 3-substituted catechol functionalities (or the oxidized analogues: 3-substituted *ortho*-quinones) are present in a large number of natural compounds with adverse or beneficial effects on the human body. Allergic reactions caused by poi-

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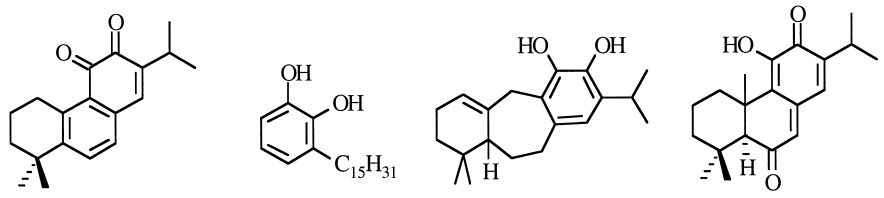

Fig. 1. Examples of physiologically active compounds containing a 3-substituted catechol or *ortho*-quinone functionality.

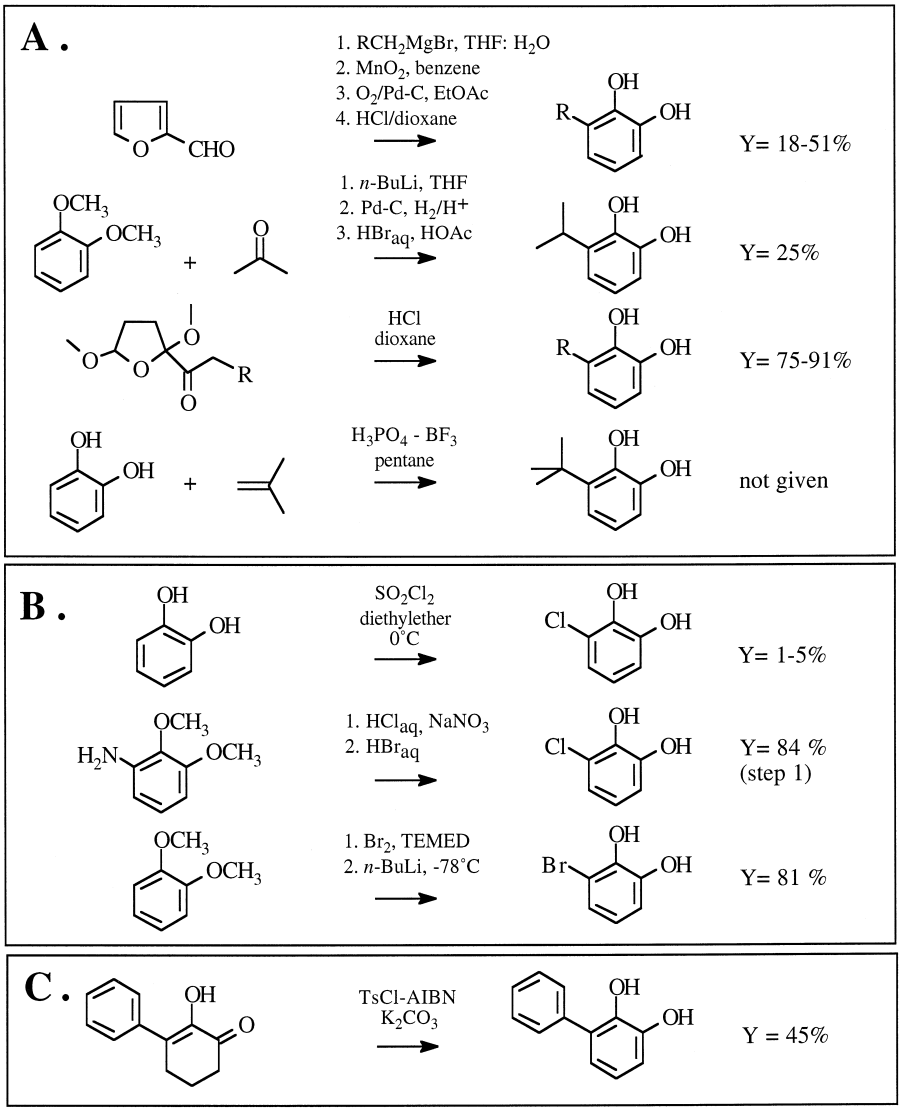

Fig. 2. Chemical routes to 3-substituted catechols: panel A, published syntheses of 3-alkyl catechols [4,17,18] and 3-halocatechols (panel B) [3,19–21] and 3-phenylphenol (panel C) [24].

son ivy have been linked to the allergen urushiol (3-pentadecanylcatechol) [1], but other natural 3-substituted catechols are used as pharmaceuticals. For instance, the natural compounds miltirone, extracted from the roots of *Salvia miltiorhiza* which is used in traditional chinese medicine [2], and taxodione from *Taxodium distichum*, an anti carcinoma drug [3], as well as the synthetic blood pressure lowering drug barbatusol [4], contain these functional groups (Fig. 1).

The marked physiological activity of 3- and 4-substituted catechols has stimulated research towards the synthesis of catechol derivatives for use as potential drugs.

A possible route to 4-substituted catechols proceeds via the regioselective hydroxylation of phenolic compounds, but by organic chemical means, this is tedious. One of the chemical methods for aromatic ring hydroxylation of phenols is the classical Elbs reaction, which makes use of potassium persulfate in alkaline media to introduce oxygen at the *para* position relative to the phenolic hydroxyl moiety leading to 1,4-dihydroxybenzenes [5]. Hydroxylation occurs at the *ortho* position only when the *para* position is blocked and this reaction, resulting in the formation of the desired 4-substituted catechols, occurs at a low yield [5].

A biocatalytic alternative to obtain 4-substituted catechols using polyphenol oxidase has been developed [6], but the applicability of this method is limited by subsequent oxidation of the products by the same enzyme to *ortho*-quinones [7].

Although a number of reports have appeared that describe the synthesis of 3-alkyl, 3-aryl or 3-halo substituted catechols (Fig. 2), efficient chemical routes to 3-substituted catechols have yet to be discovered. The production of 3-sub-

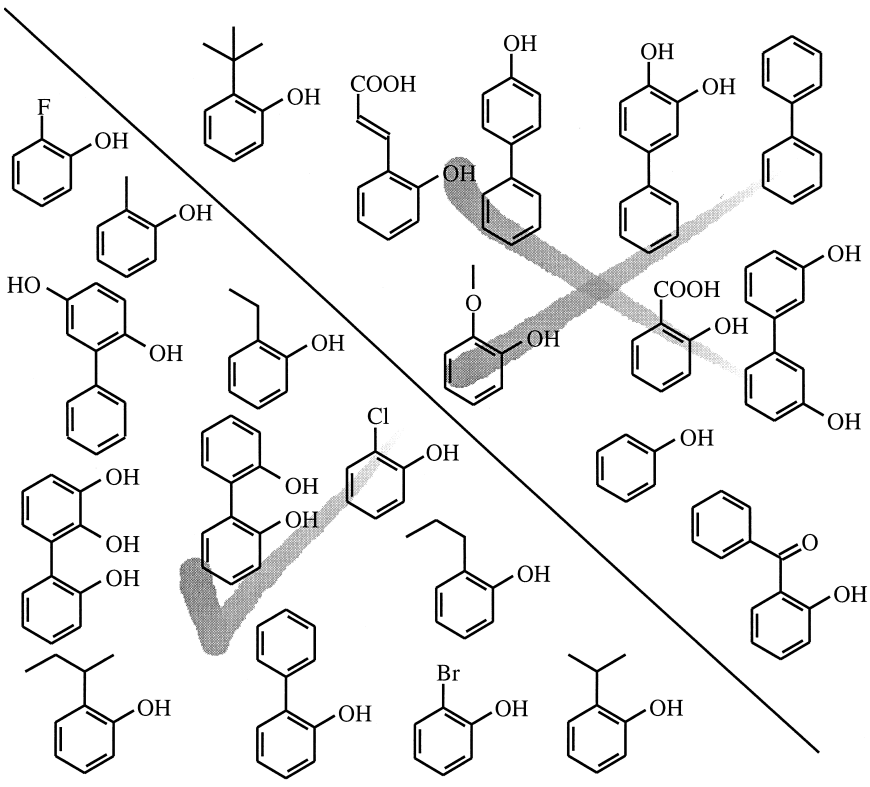

Fig. 3. Compounds that have been tested as potential substrates of 2-hydroxybiphenyl 3-monooxygenase [12,13,16,22,23]. Oxidized substrates are indicated by a tick mark, untouched compounds are marked with a cross, separated by a diagonal line.

stituted catechols by chemical methods is hampered by low yields, agressive and expensive reagents or difficult to obtain starting materials. Furthermore, a general route to produce 3-alkyl, 3-aryl and 3-halocatechols is not available.

Biocatalytic routes to 3-substituted catechols, which are based on the subsequent action of an aromatic ring dioxygenase and a *cis*-diol dehydrogenase in whole cells of *Pseudomonas* or *Rhodococcus* species, have been proposed [8–11]. This method is promising for the synthesis of 3-halocatechols from halogenated benzenes, since the product is not affected by catechol cleaving enzymes in the microorganisms used [8]. The synthesis of 3-alkylcatechols using these biocatalysts is problematic because these compounds are readily cleaved by catechol dioxygenases [9,10]. To overcome this limitation, Warhurst et al. have used 3-fluorocatechol added to approximately equimolar amounts relative to starting material to inhibit catechol-2,3-dioxygenase from *R. rhodochrous* NCIMB 13259, thus allowing the production of 3-ethylcatechol [11].

We propose a new biocatalytic route to 3-substituted catechols based on 2-hydroxybiphenyl 3-monoxygenase (HbpA) from *P. azelaica* HBP1 [12]. The enzyme catalyzes the regioselective hydroxylation of phenols that carry alkyl-, aryl- or halo-substituents at the 2-position, resulting in the formation of the corresponding 3-substituted catechols (Fig. 3). By using a recombinant strain, devoid of catechol degrading activity, which expressed the *hbpA* gene, we have developed an efficient and general route to produce 3-substituted catechols from 2-substituted phenols, which are readily available starting materials.

2. Results and discussion

2.1. The enzyme: 2-hydroxybiphenyl 3-monooxygenase

2-Hydroxybiphenyl 3-monooxygenase (E.C. 1.14.13.44) was isolated from *P. azelaica* HBP1 [13]. The enzyme is a homotetrameric flavoprotein (subunit molecular mass 60 kDa), which carries FAD as a prosthetic group and it preferentially uses NADH as cofactor. Amino-terminal sequence analysis of the first 74 residues and comparison to the SWISSPROT database revealed that the enzyme is homologous to a number of NADPH dependent flavin containing phenol monooxygenases [13]. Nevertheless, 2-hydroxybiphenyl 3-monooxygenase was not capable of hydroxylating phenol to catechol, but did accept an exceptionally wide range of 2-substituted phenols as substrates instead (Fig. 3). Since the enzyme is soluble, is active as a single component, can readily be purified, is stable and utilizes NADH as a cofactor, it could also be suited for cell free applications.

2.2. The biocatalyst: E. coli JM101 (pHBP461)

We chose to develop a specialized, whole cell biocatalyst containing the active 2-hydroxybiphenyl 3-monooxygenase. The use of whole cells ensured the renewed synthesis of enzyme and regeneration of cofactor during the process. Rather than using the wild-type *P. azelaica* HBP1 strain, which would rapidly degrade the desired catechols, since it contains two catechol-2,3-dioxygenases [23] and probably catechol-1,2-dioxygenase activity as well, we chose *E. coli* as a production strain. Furthermore, 2-hydroxybiphenyl 3-monooxygenase activity in *P. azelaica* HBP1 is induced by 2-phenylphenol (2-hydroxybiphenyl) and a number of other 2-substituted phenols, but not by all substrates [14]. To overcome this, the control elements that govern *hbpA* expression in *P. azelaica* were replaced by the *lac* promoter on plasmid pHBP461 (previously called pIV61 [13]).

E. coli JM101, carrying the *hbpA* gene on this multi-copy plasmid under *lac* promoter control, provided a biocatalyst that could not consume the products that made and expressed the *hbpA* gene without the need to add a 2-substituted phenol other than the starting compound as inducer. Also, the amount of enzyme pro-

Table 1
Production and in situ recovery of 3-substituted catechols produced by *E. coli* (pHBP461)

Product	Educt fed [g]	Product adsorbed [g]	Conversion [mol%][a]
3-Phenylcatechol	7.8	8.1	94
3-Ethylcatechol	2.0	~ 2.1[b]	~ 95[b]
3-Propylcatechol	0.8	0.6	71
3-*i*-Propylcatechol	2.6	2.2	77
3-*sec*-Butylcatechol	1.8	1.7	85
3-Chlorocatechol	1.1	0.9	71
3-Bromocatechol	3.1	2.1	71

[a] Based on the amount of product adsorbed to the XAD-4 resin, eluted with acidic methanol.
[b] Oil, no reliable extinction coefficient could be determined.

duced by the recombinant strain was 3–5 fold higher than in *P. azelaica*.

2.3. The process: fed-batch biotransformation

Phenols are toxic to microorganisms. To be able to efficiently convert 2-substituted phenols to their respective catechols, the concentration of these educts has to be kept below a level that endangers cell viability. We have observed that 2-substituted phenols are toxic to cells at levels as low as 100 mg/l, so we have developed a fed batch strategy to keep the educt concentration below that level [15]. A culture of *E. coli* JM101 (pHBP461) was grown in a 3 l bioreactor on mineral medium containing glucose as a carbon source. Overnight, the cells reached a final cell density of approximately 4 g/l cell dry weight. Subsequently, glycerol was administered to the culture, which abolishes catabolite

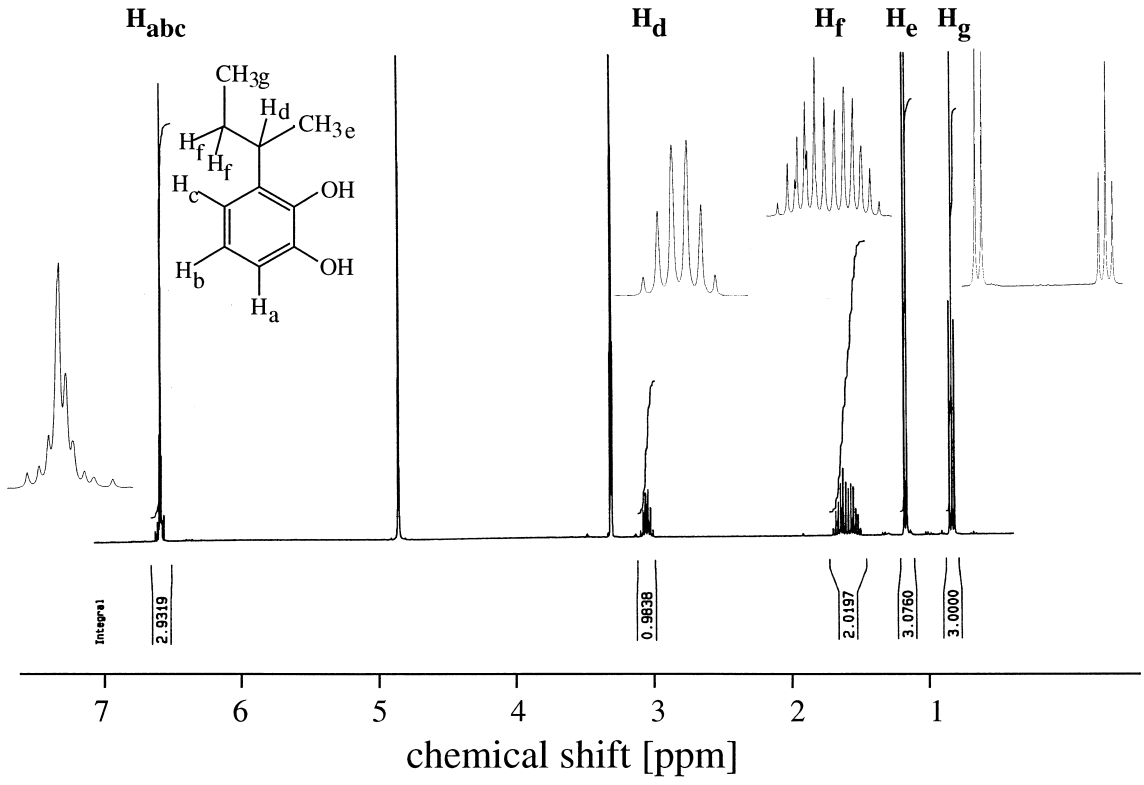

Fig. 4. 400 MHz ^{1}H -NMR spectrum of 3-*sec*-butylcatechol.

repression of the *lac* promoter and results in elevated levels of the monooxygenase. After the cells resumed growth, a stock solution containing 10% (v/v) 2-substituted phenol and 33% (v/v) glycerol dissolved in methanol was fed to the culture. The pump rate was adjusted such that the feed rate of 2-substituted phenols was kept below the rate of their biotransformation, thus avoiding the accumulation of 2-substituted phenols to concentrations above their toxic threshold value [15].

2.4. The process: in situ product recovery

We have noticed that merely controlling the feed of 2-substituted phenols did not result in stable production of the corresponding catechols. Cell viability dropped at increasing product concentrations and the formation of brown coloured catechol polymers was seen. Furthermore, we had observed in assays with the purified enzyme, that high concentrations of various 3-substituted catechols caused uncoupling of NADH conversion and product formation by the enzyme, leading to the formation of hydrogen peroxide [13].

An in situ recovery procedure for catechols was developed to avoid the problems mentioned above. By coupling the bioreactor to a column with a settling compartment on top, filled with a fluidized bed of the solid adsorbent Amberlite™ XAD-4 and by pumping the medium through this recovery module, we could efficiently extract both residual 2-subtituted phenols and 3-substituted catechols from the medium [15]. The recovery loop enabled us to keep the biocatalyst viable and active for prolonged periods of time and product instability, as indicated by browning of the medium, could largely be avoided. This procedure was used to produce gram amounts of the following products: 3-phenyl-,

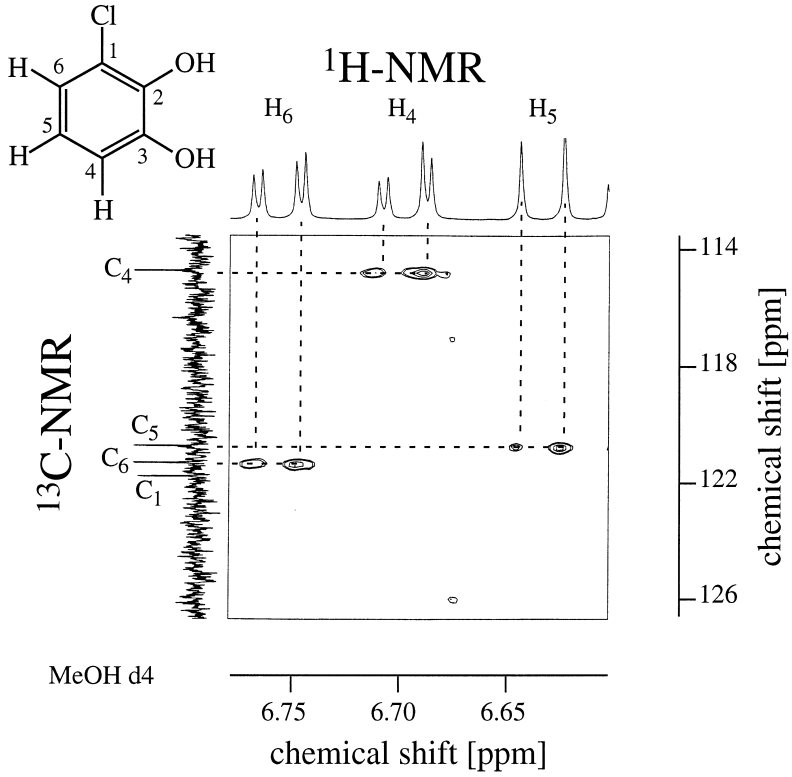

Fig. 5. $^1H - ^{13}C$ shift correlation spectrum of 3-chlorocatechol.

3-chloro-, 3-bromo-, 3-ethyl-, 3-propyl-, 3-*i*-propyl-, and 3-*sec*-butyl-catechol (Table 1) [16].

2.5. Purification and characterization of the products

The adsorbed material was eluted from the XAD resin by acidic methanol elution and was further purified by recrystallization from *n*-hexane [16]. Subsequent purification for melting point determination and ^{1}H NMR or ^{13}C NMR analysis was done by vacuum sublimation.

The authenticity of all produced catechols was verified by GC/MS analysis of trimethylsilyl (TMS) derivatives, IR spectroscopy, ^{1}H NMR and ^{13}C NMR analysis. Melting point determinations were done for all 3-substituted catechols and compared to literature data where available. We could not determine a melting point for 3-ethylcatechol since it is an oil [9]. For 3-*sec*-butylcatechol no reference data were available. To our knowledge, this is the first report describing the synthesis of 3-*sec*-butylcatechol and we therefore present the ^{1}H NMR spectrum in Fig. 4. The ^{1}H NMR spectrum and the $^{1}H-^{13}C$ shift correlation NMR spectrum of 3-chlorocatechol, shows that the protons at carbon 4, 5 and 6 are coupled which excludes that hydroxylation of the ring occured at another carbon than adjacent to the hydroxyl moiety of 2-chlorophenol (Fig. 5).

3. Conclusions

The described biocatalytic production of 3-substituted catechols by regioselective hydroxylation of 2-substituted phenols, catalyzed by cells that contain active 2-hydroxybiphenyl 3-monooxygenase provides a new route to these compounds. The biocatalyst has a number of competitive advantages over the available chemical catalysts. It displays absolute regioselectivity and allows the use of phenols as starting compounds, which are inexpensive and readily available, in a one step reaction. Furthermore, the biocatalytic route is generally applicable to produce 3-alkyl, 3-aryl, and 3-halocatechols since the enzyme catalyzed reaction is virtually insensitive to directional substituent effects.

Acknowledgements

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