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Microbial Synthesis of (2R,3S)-(-)-N-benzoyl-3-phenyl isoserine ethyl ester-a taxol side-chain synthon

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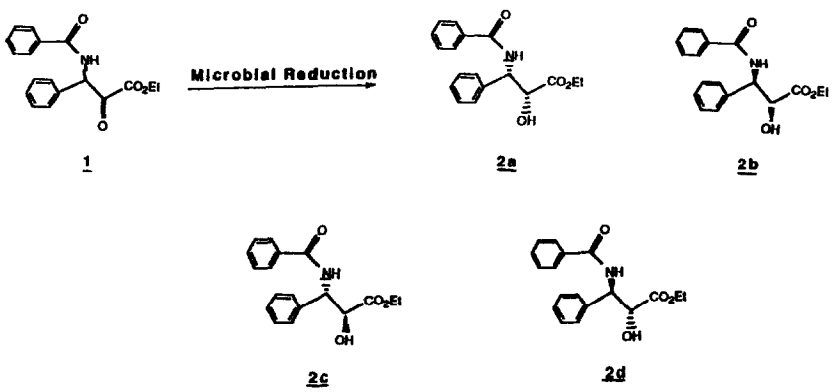
ABSTRACT. The chiral intermediate (2R,3S)-(-)-N-benzoyl-3-phenyl isoserine ethyl ester 2a , a potential taxol 5 side-chain synthon, was prepared by microbial and enzymatic processes. Taxol 5, is an anticancer compound recently approved by FDA for the treatment of ovarian cancer. The stereoselective reduction of racemic 2-keto-3-(N-benzoylamino)-3-phenylpropionic acid ethyl ester 1 to the corresponding alcohol 2 was carried out using microbial cultures. Among microorganisms evaluated, Hansenula polymorpha SC 13865 and Hansenula fabianii SC 13894 effectively reduced compound 1 to the desired syn diastereomer 2a. Reaction yields of >80% and enantiomeric excesses of >98% were observed for these bioreduction process. About 10-20% of anti diastereomers (2c,2d) were produced during bioreduction.

INTRODUCTION

Among the antimitotic agents, taxol (paclitaxel) 5[1,2], a complex, polycyclic diterpene[3], exhibits a unique mode of action on microtubule proteins responsible for the formation of the spindle during cell division. In contrast to other "spindle formation inhibitors" such as vinblastine or colchicine, both of which prevent the assembly of tubuline[4,5], taxol is the only compound known to inhibit the depolymerization process of microtubulin[6]. Because of its biological activity and unusual chemical structure, taxol may represent the prototype of a new series of chemotherapeutic agents. Various types of cancers have been treated with taxol and the results in treatment of ovarian cancer are very promising[2]. In collaboration with the National Cancer Institute, Bristol-Myers Squibb is developing taxol for treatment of various cancers. Taxol was originally isolated from the bark of the yew,

Taxus brevifolia[1] and has also been found in other Taxus species[7,8] in relatively low yield. Alternative methods for production of taxol by cell suspension cultures and by semi-synthetic processes are being evaluated[9-13]. In this report, we describe the enzymatic synthesis of a taxol side-chain intermediate. The stereoselective microbial/enzymatic reduction of 1 [2-keto-3-(N-benzoylamino)-3-phenyl propionic acid ethyl ester] to yield 2a [(2R,3S)-(-)-N-benzoyl-3-phenyl isoserine ethyl ester] is demonstrated . The reduction of 1 could result in the formation of four possible alcohol diastereomers (2a-2d) [scheme 1]. Remarkably, conditions were found under which predominantly only the single isomer 2a was obtained in the microbial reduction.



Scheme 1

RESULTS AND DISCUSSION

Various microorganisms were screened for the stereoselective microbial reduction of ketone 1 to the alcohol 2. As shown in the table 1, the reaction yield and stereoselectivity were dependent upon the microorganism used during the reduction of 1 to 2a. Organisms from genus Nocarida, Candida, Rhodococcus, Mortierella, Saccharomyces, and Hansenula predominantly converted ketone 1 to the desired alcohol 2a in high optical purity (>91%), while organisms from genus Pullularia and Trichoderma gave lower optical purity (75-88%) of the desired alcohol 2a. Pichia methanolica ATCC 16623 reduced 1 to 2a in the lowest optical purity (the undesired enantiomer 2b is produced). H. polymorpha SC 13865 and H. fabianii SC 13894 effectively reduced compound 1 to 2a. Reaction yields of >80% and optical purities of >95% were observed for these biotransformations.

TABLE 1: Microbial Reduction of **1** to **2a**

Microorganisms	Reaction yield compound **2a** (%)	Optical purity compound **2a** (%)
Candida guilliermondi ATCC 20318	81	95
Rhodococcus erythropolis ATCC 4277	39	96
Saccharomyces cerevisiae ATCC 24702	35	94
Hansenula polymorpha SC 13865	80	99
Pseudomonas putida ATCC 11172	32	94
Nocardia globerula ATCC 21505	36	92
Mortierella rammanianna ATCC 38191	35	97
Hansenula fabianii SC 13894	85	95
Pichia methanolica ATCC 58403	80	26
Pullularia pullulans ATCC 16623	73	75
Trichoderma polysporium SC 14962	86	77
Rhodococcus sp. ATCC 29675	53	91
Nocardia salmonicolor SC 6310	45	99
Nocardia restricta SC 13107	75	88

Reactions were carried out as described in the Methods. The reaction yield and the optical purity were determined by HPLC.

Cells of H. polymorpha SC 13865 and H. fabianii SC 13894 were grown in a 25L fermentor containing 15L of medium containing glucose as carbon source. During growth in a fermentor, broth samples (1L) were taken at 24, 32, 40 and 48 hour after the inoculating fermentor. Cells were collected by centrifugation, suspended in buffer and reduction of 1 was carried out as described in the experimental sec-

tion. Cells harvested after 40 hours gave 90% and 94% reaction yield for H. polymorpha SC 13865 and H. fabianii SC 13894, respectively (table 2). Lower optical purity (95%) of 2a was obtained with H. fabianii SC 13894 compared with H. polymorpha SC 13865 (99% optical purity).

TABLE 2: Growth of H. polymorpha and H. fabianii in a 15-L fermentor: Reduction of compound 1

Microorganism	Sample time (hour)	anti diastereomers (2c, 2d) (%)	syn diastereomers (2a, 2b) (%)	Optical purity of 2a (%)
Hansenula polymorpha SC 13865	24	28	72	99.6
	32	25	75	98.9
	40	20	80	99.3
	48	20	80	99.3
Hansenula fabianii SC 13894	24	12.7	87.3	93
	32	9.8	90.2	93.3
	40	10.8	89.2	93.6
	48	8.9	91.1	94

Cultures were grown in a 15-L fermentor as described in the Materials and Methods.
Broth samples were taken at designated time and reduction of 1 was carried out by cell-suspensions (20 % w/v,wet cells) of organisms. After 48 hours reaction time, the reaction yield of anti and syn diastereomers and the optical purity of 2a were determined as described in the Materials and Methods

Preparative-scale reductions of ketone 1 to desired alcohol 2a was carried out in a 5L fermentor using cell suspensions of H. polymorpha SC 13865 and H. fabianii SC 13894 in independent experiments as described in the experimental section. In both batches, a reaction yield of 85-90% and an optical purity of >95% were obtained for compound 2a (table 3). From one batch (H. polymorpha SC 13865), 5.2 gram of 2a was isolated in 65% overall yield. The isolated compound gave 99.6% optical purity (figure 1b) and 99.8% GC area % purity. The specific rotation $[\alpha]_{D,}^{25}$ of -21.7 was obtained for 2a in chloroform.

A single-stage fermentation/bioreduction process was developed for conversion of compound 1 to compound 2a with cells of H. fabianii SC 13894. Cells were grown in a 5-L fermentor and after 48 hour growth cycle, the bioreduction process was initiated by addition of substrate and glucose and continued for a 48 hour period. A reaction yield of 88% and an optical purity of 95% was obtained for the desired alcohol 2a (table 4). From the 2-L fermentation broth after bioreduction, 2.4 grams of compound 2a was isolated. The isolated compound gave 99.5% optical purity and 99.5% GC area% purity.

TABLE 3: Preparative-scale reduction of 1 by cell-suspensions of Hansenula strains

Microorganisms	Reaction time (hours)	Anti diastereomers (2c, 2d) (%)	Syn diastereomers (2a, 2b) (%)	Optical purity of 2a (%)
Hansenula polymorpha SC 13865	72	20	80	99
Hansenula fabianii SC 13894	48	10	90	94

Cells were suspended in 4-L of 0.1 M phosphate buffer (pH 6) at 20% (w/v) concentration.

Cell-suspensions were supplemented with 2 gl^{-1} of substrate 1 and 35 gl^{-1} of glucose. The bioreduction of 1 was carried out at 28 C, 250 RPM in a 5-L NB Bioflo fermentor.

Cell extracts each of H. polymorpha SC 13865 and H. fabianii SC 13894 were prepared and examined for the reduction of ketone 1. Reaction mixtures in 50 ml of extract contained 0.5 mM NADH and 100 mg of substrate 1. After a 48-hour reaction time, a reaction yield of 80% and an optical purity of 98% were obtained for compound 2a in both batches.

The control of absolute stereochemistry is a key requirement for the synthesis of many pharmaceutical products[17]. For the production of optically active alcohols, the microbial reduction of the inexpensive prochiral ketones is a very promising method. The use of whole cells (yeasts) is of limited success due to the low enantioselectivity of these system[18-21]. Three commercially available alcohol dehydrogenases have been investigated including yeast [22], horse liver [23,24] and Thermoanaerobium brockii [24]. Alcohol dehydrogenase from T. brockii is heat stable and has broad substrate specificity toward aliphatic ketones. Substrates with

bulky side chains (such as acetophenone) are poor substrates. Secondary alcohol dehyrogenase from methylotrophic organisms reduced aliphatic ketones stereoselectively to the corresponding alcohols [25]. Alcohol dehydrogenases from yeast, horse liver, T. brockii, and methylotrophs transfer the pro-R hydride to the carbonyl group to give (S) alcohol, a process described by Prelog's rule [22-25]. Recently, alcohol dehydrogenases from Pseudomonas sp. Strain PED and Lactobacillus kefir were described which catalyze the enantioselective reduction of aromatic, cyclic, and aliphatic ketones to the corresponding chiral alcohols [26,27]. Both enzymes exhibit anti-prelog specificity, transferring the pro-R hydride to form (R) alcohols.

TABLE 4: Reduction of 1 by H. fabianii culture: Single-stage process

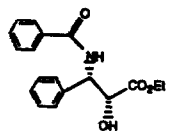
Reaction time (hours)	Anti diastereomers (2c, 2d) gl^{-1}	Syn diastereomers (2a, 2b) gl^{-1}	Optical purity of (2a) (%)
12	0.04	0.3	ND
24	0.09	0.63	ND
36	0.15	0.92	ND
46	0.18	1.18	93
60	0.2	1.5	94
72	0.21	1.7	94.5

Cells were grown in a 25-L fermentor containing 15 L of medium at 28 C, 600 RPM agitation. After 48 hour of growth period, substrate 1 (2 gl^{-1}) and glucose (35 gl^{-1}) were added to the fermentation broth and the bioreduction process was continued.

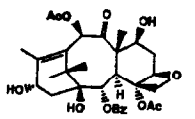
Holland et al. [28] demonstrated the conversion of ethylbenzene and a number of para-substituted derivatives by Mortierella isobellina to the corresponding optically active 1-phenylethanol with enantiomeric excesses between 5 and 40%. In most cases, the R absolute configuration of product predominated. Recently, we have demonstrated the stereoselective microbial reduction of 1-(4-fluorophenyl)-4-[4-(5-fluoro-2-pyrimidinyl)-1-piperazinyl]-1-butanone to either R-(+) and S-(-) alcohols in enantiomeric excesses of >98% [29].

Recently preparation of taxol side-chain precursors by the lipase-catalyzed resolution of racemic cis-1-benzoyl-3-acetoxy-4-phenylazitidin-2-one [30] by enantioselective hydrolysis and esterification of methyl trans-β-phenylglycidate has been demonstrated [31]. However, the resolution method gives maximum theoretical yield of 50%.

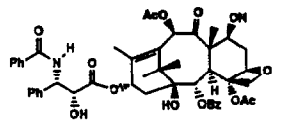
In this report, we have described the stereoselective reduction of ketone 1 to desired alcohol 2a, which is a key chiral intermediate for the semi-synthesis of taxol. This method gave greater than 80% yield. Coupling of alcohol 2a to baccatin III (4), produces taxol [scheme 2]. Taxol 5 is one of the most important anticancer compounds to emerge from the screening of natural products in recent years. Initial progress on developing taxol was slow, because of the difficulty of isolating it from bark of the yew, Taxus brevifolia. It is a most promising antitumor agent recently approved for treatment of ovarian cancer. It also has shown promise for treatment of breast, lung and colon cancer [32,33]. The partial synthesis of taxol from the simpler diterpenoid baccatin III which is derived from the renewable resource of yew leaves, is an attractive alternative [11, 33].



Taxol Side-chain 2a



Baccatin III 4



Taxol 5

Scheme 2

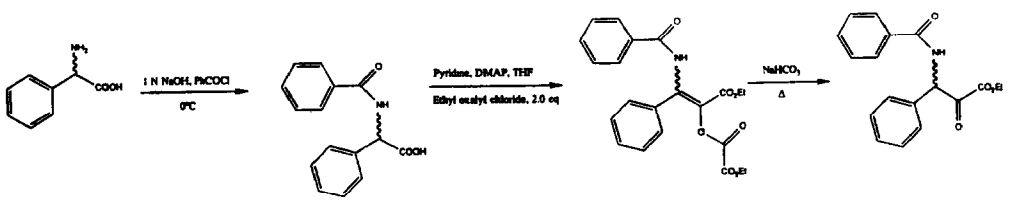
EXPERIMENTAL SECTION

Materials:

Starting substrate 1 and reference compounds 2a-2d were synthesized in the Department of Chemical Process Research, Bristol-Myers Squibb Pharmaceutical Research Institute. The physico-chemical properties including spectral characteristics (^{1}H NMR, ^{13}C NMR, Mass spectra) were in full accord for all these compounds. ^{1}H NMR and ^{13}C NMR spectra were recorded on Brucker AM-300 MHZ or Brucker AM-500 MHz spectrometers using tetramethylsilane as internal standard. Optical rota-

tions were recorded on a Perkin Elmer 241 polarimeter. Column chromatography was performed using silica gel (grade 60, Merck, 230-400 mesh)

Preparation of the 2-keto-3-(N-benzoylamino)-3-phenylpropionic acid, ethyl ester 1.- To (+) Phenylglycine (9 g, 60 mmol) in aqueous NaOH (1N, 180 ml) at 0°C, benzoyl chloride (7.73 ml, 66 mmol) was added dropwise and stirred for 1 hr. The resulting solution was washed with ethyl acetate (20 ml x 2), then neutralized with 6 N HCl and again extracted with ethyl acetate (60 ml x 2). The combined ethyl acetate layer was washed with brine, dried over anhydrous $MgSO_4$ and concentrated. Benzoyl phenylglycine was crystallized from ethyl acetate/hexane to produce a white solid (10.65 g, 70 % yield). To a stirred solution of benzoyl phenylglycine (6.12 g, 24 mmole) in anhydrous THF, 4-dimethyl aminopyridine (100 mg, 0.82 mmole) was added. To the above solution, ethyl oxalyl chloride (5.35 ml, 48 mmole) was added dropwise to initiate gentle refluxing. The mixture was refluxed for 3.5 hr. To the cooled reaction mixture, water (48 ml) was added and stirred for 0.5 hr. The resulting organic layer was separated and the aqueous layer was extracted with ethyl acetate (36 ml x 2). The combined organic layer was washed with brine (30 ml x 1), dried over anhydrous Na_2SO_4, filtered, concentrated and crystallized from ethyl acetate/hexane mixture to give 4.68 g of enol ester (63% yield). To the suspensions of enol ester (3 g, 7.3 mmole) in 10 ml of ethanol, anhydrous sodium bicarbonate (0.4 g, 4.75 mmole) was added, then refluxed for 0.5 hr. The reaction mixture was then filtered and the filtrate was concentrated and purified by flash column chromatography to give 2.5 g of 1 (95% yield) : mp 80-83°C ; ^{1}H NMR ($CDCl_3$, 300 MHz) δ1.22 (t, 3H), 4.22 (m, 2H), 6.45 (d, J= 4 Hz, 1H), 7.25-7.51 (m, 8H), 7.76-7.79 (d, J= 9 Hz, 2H); ^{13}C NMR ($CDCl_3$, 75.46 MHz) δ 188.8, 159.4, 133.2, 133.0, 131.8, 129.2, 129.0, 128.8, 128.5, 127.0, 62.8, 60.2, 13.7; mass spectrum, m/z 312 (M+H), 226, 210; Anal. Calcd. for $C_{18}H_{17}NO_4$: C, 67.60; H 5.65, N, 4.38; Found: C, 67.59; H, 5.41; N, 4.38.



Scheme 3

Syn-(2R,3S)-(-)-N-Benzoyl-3-phenylisoserine ethyl ester 2a-

Prepared as described earlier for methyl ester [14]. mp 164-165°C; $[\alpha]_D^{25}$ = -21.7 (C1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 1.26 (t, 3H), 3.26 (brs, 1H), 4.25 (m, 2H), 4.26 (d, J= 2 Hz, 1 H), 5.74 (dd, J= 2 HZ, 9 Hz, 1 H), 6.98 (d, J= 9 Hz, 1 H), 7.22-7.6 (m, 10 H), 7.7-7.82 (d, J= 9 Hz, 2 H); 13C NMR ($CDCl_3$, 75.4 MHz) δ 172.7, 138.5, 131.5, 129.2, 128.9, 128.6, 127.5, 126.9, 73.1, 62.4, 54.6, 113.9; Mass spectrum, m/z 314.2 (M+H); Anal. calcd. C, 69.0; H, 6.11, N, 4.47; Found C, 68.83; H, 6.11; N, 4.4.

Syn-(2S, 3R)-(+)-N-Benzoyl-3-phenylisoserine ethyl ester 2b-

Prepared from cis-3S-Hydroxy-4-phenylazetidine-2-one as described earlier[15]. mp 162-165°C, $[\alpha]_D^{25}$ = +22.1 (C1, $CHCl_3$); ^1H NMR and ^{13}C NMR are identical to compound 2a; Mass spectrum, m/z 314 (M+H); Anal Calcd. C, 69.0; H, 6.11; N, 4.47; Found C, 69.01; H, 6.05; N, 4.51.

Anti-(2S,3S)-(-)-N-Benzoyl-3-phenylisoserine ethyl ester 2c-

To a solution of 2a (2 g, 6.38 mmole) in pyridine at 0°C, methane sulfonyl chloride (0.52 ml, 6.7 mmole) was added dropwise and stirred at 0°C for 1.5 hr and then at 65-70°C for 18 hr. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (80 ml) and 33.3% saturated $CuSO_4$ solution (80 ml). The aqueous layer was extracted with ethyl acetate (40 ml x 1). The combined organic extract was washed with brine (80 ml x 1), dried over anhydrous Na_2SO_4, filtered and azetroped with heptane (20 ml x 2) to give crude cis oxazoline as a solid (1.88 g). The crude compound was crystallized from a ethyl acetate/hexane mixture to give 1.34 g of cis oxazoline. To a solution of cis oxazoline (0.79 g, 2.67 mmole) in methanol (57 ml), 1N HCl (57 ml) was added while stirring. After 10 min, THF (57 ml) was added and stirred for 2.5 hr. The pH of the solution was then adjusted to 9.0 with $NaHCO_3$ (120 ml) and the mixture was allowed to stir at room temperature for 18 hr. To the reaction mixture, ethyl acetate (200 ml) was added and the aqueous phase was separated and extracted with ethyl acetate (100 ml x 1). The combined organic phase was then washed with brine (150 ml x 1) , dried over anhydrous Na_2SO_4, filtered and concentrated. The crude solid was dissolved in hot methanol (15 ml), then cooled to 4°C. The precipitated solid was filtered and washed with

methanol to give 0.67 g of 2c, mp 160-161°C; $[\alpha]_D^{25}$ = -40.3 (C1, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 1.26 (t, 3H), 3.26 (bs, 1H), 4.06 (m, 2H), 4.62 (brs, 1H), 5.62 (dd, J=2Hz, 1H), 7.15-7.60 (m, 10H), 7.8-7.84 (d, J= 9 Hz, 2 H). ^{13}C NMR ($CDCl_3$, 125.7 MHz), 174.2, 165.1, 137.5, 132.1, 130.5, 128.6, 128.4, 127.6, 125.8, 73.1, 62.8, 54.8, 13.6; Mass spectrum, m/z 314 (M+H); Anal. Calcd. C, 69.0; H, 6.11; N, 4.47; Found C, 68.91; H, 6.07; N, 4.60.

Anti-(2R,3R-(+)-N-Benzoyl-3-phenyl isoserine ethyl ester 2d-

Prepared by the enzymatic reduction of compound 1 by β-hydroxy butyrate dehydrogenase (Sigma Chemicals St. Louis, MO). The reaction mixture in 250 ml of 50 mM phosphate buffer contained 1 gram of compound 1, 200 units of enzyme, and 250 mg of NADH. The reaction was carried out at 25°C and 200 RPM on a rotary shaker for 24 hours. At the end of bioreduction, 250 ml of reaction mixture containing product 2d was extracted with 500 ml of ethyl acetate. The ethyl acetate layer was separated by centrifugation, washed with brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to obtain 0.9 grams of crude 2d. Product was further purified by flash column chromatography and recrystallized from acetonitrile to obtain 0.7 g (70% yield) of 2d, mp 163-165°C, $[\alpha]_D^{25}$ = + 41.0 (C1, $CHCl_3$) ^1H NMR and ^{13}C NMR are identical to compound 2c.

Microorganisms

Microorganisms (Table 1) were obtained from the culture collection of the Bristol-Myers Squibb Pharmaceutical Research Institute and from the American Type Culture Collection, Rockville, MD. Microbial cultures were stored at -90°C in vials.

Growth of Microorganisms

For screening purposes, one vial of each organism was used to inoculate 100 ml of medium A containing 1% yeast extract, 1% malt extracts, 2% glucose, and 0.3% peptone. The medium was adjusted to pH 6.8 before sterilization. Cultures were grown at 28°C and 280 RPM for 48 hours on a rotary shaker. Cultures were harvested by centrifugation at 18,000 x g for 15 minutes, washed with 100 mM potassium phosphate buffer (pH 6.0) and then used for reduction studies.

Reduction of 1 by Cell Suspensions

Cells of various cultures were suspended separately in 100 mM potassium phosphate buffer (pH 6.0) at 20% (w/v, wet cells) cell concentration and supplemented with 2.0 mg/ml of 1 and 35 mg/ml of glucose. Reduction was conducted at 28°C and 280 RPM. Periodically, samples of 1 ml were taken and extracted with four volumes of ethyl acetate. After centrifugation, the separated ethyl acetate phase was collected and dried with a nitrogen stream. The oily residue obtained was dissolved in 2 ml of mobile phase (hexane: isopropanol, 5:95,v/v), filtered through a 0.2 μm LID/X filter and analyzed by HPLC.

Two-stage Process:

Growth of Hansenula strains in a Fermentor

H. polymorpha SC13865 and H. fabianii SC 13894 were grown in 25-L fermentors containing 15 L of medium A. Growth consisted of two inoculum development stages and fermentation. Inoculum development consisted of F1 and F2 stages. In the F1 stage, a frozen vial of each culture was inoculated independently in to 100 ml of medium A. The growth was carried out in 500-ml flasks at 28°C and 280 RPM for 48 hours. In the F2 stage, 100 ml of F1 stage culture of each organism was inoculated independently into 1.5 L of medium A in a 4-L flask and incubated at 28°C and 180 RPM for 24 hours. Fermentors containing 15 L of medium A were inoculated with 1.5 L of F2 stage inoculum and grown at 28°C and 500 RPM with 10 LPM (liter per minute) aeration. During fermentation, cells were periodically harvested by centrifugation from 200 ml of culture broth. Cell suspensions (20% w/v, wet cells) were prepared in 100 mM potassium phosphate buffer (pH 6.0) and supplemented with 2.0 mg /ml of compound 1 and 35 mg/ml of glucose. The reaction was conducted at 28°C and 280 RPM on a shaker. Periodically samples were removed, extracted with four volumes of ethyl acetate and separated organic phase was evaporated under gentle stream of nitrogen. The oily residue was dissoled in mobile phase, filtered through a 0.2 μm LID/X filter and analyzed for the reduction of 1 to 2 by HPLC for the reaction yield and the optical purity of 2a. Cells were harvested at an optimum activity period with the aid of a Cepa centrifuge and the wet cell pastes were collected and stored at 60°C until further use.

Reduction of Compound 1 in a Fermentor

Cells (H. polymorpha SC 13865 and H. fabianii SC 13894) from the above batches were used to conduct the reduction of compound 1 in a 5-L fermentor in an inde-

pendent experiment. Cell suspensions (20 % w/v, wet cells) in 4 L of potassium phosphate buffer (pH 6.0) were supplemented with 8 grams of compound 1 and 140 grams of glucose. The reduction was conducted at 28°C and 250 RPM. Periodically samples were removed and extracted with four volumes of ethylacetate. After centrifugation, the organic layer was collected and dried with a nitrogen stream. The oily residue was dissolved in mobile phase, filtered through a 0.2 μm LID/X filter and analyzed by HPLC to determine the % conversion of 1 to 2a. The optical purity of 2a was determined by chiral HPLC. At the end of bioreduction, 4 L of broth (H. polymorpha SC 13865) containing product 2a (7.2 grams) was extracted with 8 L of ethyl acetate. The ethyl acetate layer was separated by centrifugation and washed with 4 L of 7% sodium chloride solution. The separated organic phase was dried with anhydrous sodium sulfate and evaporated under reduced pressure to obtain 6.4 grams of pale yellow solid. Product 2a (5.2 grams) was crystallized from acetonitrile with >99% chemical purity and >99.8% optical purity.

Single-stage Fermentation and Bioreduction

Growth of H. fabianii SC 13894 was conducted in a 5-L fermentor (contained 4 L of medium) at 28°C and 300 RPM with 3 LPM aeration for 48 hour as described earliar. At the end of the fermentation cycle (48 hour), the bioreduction process was initiated by the addition of substrate (2 gl^{-1}) and glucose (35 gl^{-1}). The bioreduction process was completed in 48 hours. At the end of the bioreduction, 2 liter of broth containing product 2a (3.4 gram) was extracted with 4 liter of ethyl acetate. The ethyl acetate layer was separated by centrifugation and washed with 2 liter of 7% sodium chloride solution. The organic phase was separated, dried with anhydrous sodium sulfate and evaporated under reduced pressure to obtain 3.2 grams of pale yellow solid. Product 2a (2.4 grams) was crystallized from acetonitrile to >98% chemical purity and >99.5% optical purity.

Preparation of Cell Extracts

Cultures of H. polymorpha SC 13865 and H. fabianii SC 13894 were grown in a 25-liter fermentor as described earlier. Cell suspensions (10% w/v, wet cells) in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM dithiothreitol and 10 % glycerol (buffer A) were disintegrated by Microfluidizer M-110F (Microfluidics, Inc. Newton, Mass) at 12,000 psi pressure at 4°C. The lysates were centrifuged at 20,000 x g for 30 minutes at 4°C, and the clear supernatant collected was referred to as cell extracts.

Reduction of compound 1 by cell extracts

Cell extracts from H. polymorpha SC 13865 and H. fabianii SC 13894 were evaluated for the reduction of 1. The reaction mixture contained 0.5 mM NADH, and 100 mg of 1 in 50 ml of cell extracts. The reactions were conducted at 28°C, 250 RPM on a shaker. Control experiment was carried out under similar conditions except, NADH was not added to the reaction mixture. Protein was determined by Bradford's method[16].

Analytical Methods

Samples (5 ml) from the reaction mixture were extracted with two volumes of ethyl acetate and the separated organic phase was filtered through a 0.2 μm LID/X filter. Collected samples were analyzed for substrate and product concentration with a Hewlett Packard 1070 HPLC. A Phenomenex Cyanopropyl column (150 x 4.6 mm, 5 μ) was used. The mobile phase was consisted of 5% isopropanol in hexane. The flow rate was 230 nm. The retention times for substrate, syn diastereomer (2a, 2b) and anti diastereomer (2c,2d) of product were 26.8 min, 20.4 min, and 22.2 min, respectively.

The separation of the two enantiomers of the syn and anti diastereomers was achieved by HPLC using dual columns connected in a series. The first column was a Pirkle column (DNBPG, dinitrophenylglycine, 250 x 4.6 mm, 5 μ) and the second column was chiralcel OB (250 x 4.6 mm, 5 μ). The mobile phase was consisted of 25: 2.5: 2.5: 70 of isopropanol: n-butanol, methanol:hexane. The flow rate was 0.5 ml/min and the detector wavelength was 230 nm. The retention times for the two enantiomers of the syn diastereomer of 2 were 20.4 min and 23.4 min, respectively. The retention times for the two enantiomers of the anti diastereomer of 2 were 21.3 min and 27.2 min, respectively (figure 1 a).

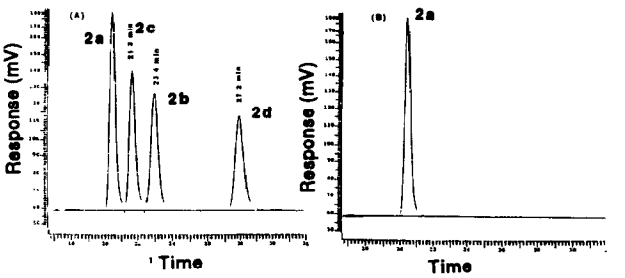


Figure 1: (A) HPLC resolution of the four diastereomers of alcohol **2**
(B) HPLC chromatography of microbially made 2a.

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