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Chemo- and biocatalytic strategies to obtain phenylisoserine, a lateral chain of Taxol by asymmetric reduction

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ABSTRACT

Enriched ethyl 3-benzamido-2-hydroxy-3-phenylpropanoate (protected phenylisoserine), the chiral side chain of Taxol, was obtained via asymmetric reduction with transition metal–diphoshine complexes or with whole cells of non-conventional yeasts. Asymmetric hydrogenation was carried out using different approaches: hydrogenation of the tetra-substituted double bond of (*E*)-1-benzamido-3-ethoxy-3-oxo-1-phenylprop-1-en-2-yl ethyl oxalate **1** with Ir(I)–diphosphine complexes in the presence of TEA, hydrogenation of the carbonyl group of racemic ethyl 3-benzamido-2-oxo-3-phenylpropanoate **2** with Ru(II)–diphoshine complexes in the presence of a Lewis acid and finally a two-step enzymatic transformation of (*E*)-1-benzamido-3-ethoxy-3-oxo-1-phenylprop-1-en-2-yl ethyl oxalate **1** catalyzed by whole cells of yeasts bearing cell-bound esterases and dehydrogenases.

1. Introduction

Drugs that come from plants have always had an important role in the treatment of an enormous array of pathologies, including Cancer. Today the most used antineoplastic drug in therapy is Paclitaxel (Fig. 1), particularly in breast and ovary Cancer and advanced forms of Kaposi's sarcoma.[1,2]

The major differences between the Taxol skeleton and the other 200 members of its family are the presence of a side chain at the C-13 position, esterified by an *N*-benzoyl-phenyl-isoserine group and an oxetanic ring attached to C 4–5 of the cyclohexane ring. Both groups are necessary for the biological activity.

Taxol can be isolated from the bark of *Taxus brevifolia*, a tree that grows slowly, making Taxol production insufficient for world demand. Thus there is a need to develop new strategies for the large-scale synthesis of this drug. Different approaches have been studied: total synthesis,[3–5] production by vegetable crops,[6] preparation from mushrooms,[7] extraction from the leaves of the *Taxus* species,[8] the semi-synthesis from 10-deacetyl baccatine III,[9] and the stereoselective synthesis of the Taxol side chain.[10–14] From these methods, only the last three approaches have become industrially applied, one of which has been patented by Bristol-Myers Squibb.[15,16]

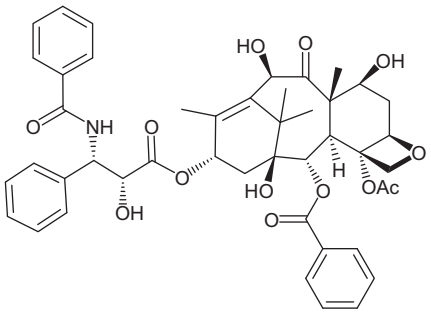

Figure 1. Paclitaxel.

This synthesis provides the esterification of modified 10-deacetyl baccatine III, a tetra substituted diterpene extract from *T. brevifolia* leaves, with the lateral chain (2*R*,3*S*)-*N*-benzoyl-*O*-(1-ethoxyethyl)-3-phenylisoserine. Different strategies have been developed for the synthesis of the lateral chain in an enantiomerically pure form.

The first asymmetric synthesis of the lateral chain was realised by Greene[9] in 1986, utilising a Sharpless epoxidation. Other strategies have been developed by Ojima et al. using β-lactam,[11] and by Sharpless using methyl cinnamate.[12] Another example includes the chemo-enzymatic Kayser approach,[13] which combined a chemical synthesis with biocatalysis reactions using yeasts.

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An enzymatic resolution with *Ps. cepacia* was adopted by Bristol-Myers Squibb from a racemic mixture of azetin-2-one acetate. Bristol-Myers Squibb's catalytic approach involved the reduction and resolution of 2-keto-3-(*N*-benzoylamino)-3-phenylpropionic acid ethyl ester, as reported in the U.S. patient.[16] Herein, we have evaluated different chemo and enzymatic strategies for improving the yields and the selectivity of the conversion of **1** into **3**.

2. Results and discussion

Asymmetric hydrogenation is one of the most applied methods to produce enantiomerically pure amino acids.[17–20] In order to reduce the number of synthetic steps for the production of enantiomerically pure *N*-benzoyl-phenyl-isoserine **3**, the first substrate investigated in the asymmetric reduction was 3-benzoylamino-3-phenyl-(ethyl, 2-oxalyl) propenoic acid ethyl ester **1**, already used as a synthon in Bristol-Myers Squibb's synthesis (Fig. 2).

Generally, tetra-substituted double bonds are difficult to reduce, particularly in the presence of substituents such as –OCOCOOEt and –NHCOPh. Our first attempts were made with [Rh(Me-Duphos)(COD)$^+$ClO$_4^-$], one of the catalysts of choice in the reduction of a tetra-substituted double bond,[21–23] but this approach was unsuccessful. Subsequently, iridium complexes with diphosphines, characterised by an atropoisomeric chirality, were used: the commercially available BINAP[24] and TolBinap[25] and the particular electron rich TetraMe-Bitiop[26] (Fig. 3).

Iridium(I) complexes, prepared from [Ir(COD)Cl$_2$]$_2$ and the aforementioned atropoisomeric diphosphines, are very active especially in the reductions of imines.[27–29] However, in this case, they proved ineffective (Table 1, entries 1 and 2); the catalysts were only able to reduce substrate **1** after the addition of a base such as TEA. The reduction in the presence of TEA with an iridium(I) catalyst proceeded with good diastereoselectivity for the *anti*-isomers but in an almost racemic form (Table 1, entries 3–5). Table 1 illustrates the effect of the addition of the base: the reactivity increased when the amount of base was set up at the optimal ratio of 5:1 (base/substrate), but decreased when the ratio was raised to 10:1. TEA base helped with the efficiency of the catalyst by hydrolysis of substrate **1** to give keto-ester **2**.

In Table 2 it can be seen that TEA was necessary for high activity and diastereoselectivity (compare entry 3 to entry 2) but no enantioselectivity. For further insight, a partial characterisation of the Ir/diphosphine/TEA complex was performed. The [Ir(COD)(P–P)Cl] complex, prepared from [Ir(COD)Cl$_2$]$_2$ and (*S*)-BINAP and characterised by two doublets at −2.2 ppm (J = 18.3 Hz) and −11.6 ppm in the ^{31}P NMR spectrum,[30] was able to reduce substrate **2** with modest efficiency (Table 2, entry 3).

The addition of 1 equiv of TEA had no effect on the ^{31}P NMR spectrum and the catalyst was completely inactive. When the amount of TEA was raised to 10 equiv, the ^{31}P NMR was changed and showed two doublets at 1.0 ppm (J = 21.4 Hz) and at 13.0 ppm. Adding a further excess of TEA (12.5 equiv) and heating at 60 °C to mimic the reaction conditions, caused two doublets at 3.6 ppm (J = 17.4 Hz) and −1.2 ppm to appear; this complex was completely inactive in the reduction of **1** (Table 2, entry 1), but very active in the reduction of **2** (Table 2, entry 2).

Since all of the results previously reported showed that iridium complexes were inactive on tetra-substituted double bonds without the addition of TEA, and given that substrate **1** was hydrolyzed into substrate **2**, the reduction of an α-keto ester was investigated with ruthenium diphosphine complexes as described in Table 3.

Ruthenium phosphines complexes, prepared according to standard procedures[31] from [Ru(DMF)$_n$Cl$_2$]$_2$ gave the α-hydroxy-*N*-benzoyl phenyl isoserine **3** in quantitative yield and with good de but with modest to no enantioselectivity (Table 3, entries 1–3). In order to improve the enantiodifferentiation, several Lewis acids ($AlCl_3$, $MnCl_2$, $CrCl_3$, and $FeCl_3$) and HBF_4 were evaluated[32,33] (Table 3, entries 4–15). The investigation of additive effects was studied on complexes derived from atropoisomeric ligands and extended to some chiral phosphorous ligands with sp^3 stereogenic carbon atoms (Fig. 3).

When atropoisomeric diphosphines were used, the addition of the Lewis acids to the catalyst generally decreased the de, which changed from a maximum of 53% (entry 6) to less than 10% (entry 7). On the other hand addition of Lewis acid increased the ee of both diastereoisomers (Table 3, entries 4–11 vs 1–3).

Whatever Lewis acid was added, BINAP furnished a *syn*-diastereodifferentiation, opposite to the other two atropoisomeric diphosphines; the closely related Tolbinap and the more basic TetraMe-BITIOP gave the *anti*-diastereoisomers with good ee (Table 3, entries 4–8 vs 1–3 and 9–11).

Moving from pure atropisomeric ligands to BDPP,[34] characterised by an sp^3 stereogenic carbon atom and to (R,R_{ax})-Isaphos diphosphine bearing both the chiralities,[35] the *anti*-diastereoisomers were predominant following the behavior of Tolbinap and TetraMe-BITIOP (Table 3, entries 12–15).

The results of the asymmetric homogeneous catalysis with transition metal complexes suggested that both hydrolytic and reductive activities were required for the one-pot reduction of substrate **1** into **3** to by-pass a step of the synthesis reported by Bristol-Myers Squibb's patent.

This conversion can be realized in two different pathways: reduction of the alkene-group of **1** to give **4** which after hydrolysis furnishes **3**, or hydrolysis of **1** to form ketone **2** which is then reduced to **1** (Scheme 1).

We attempted to perform the two-step transformation in one pot. Different yeasts known for the simultaneous occurrence of cell-associated esterase and carbonyl reductase activities[36–38] were evaluated for the biotransformation of **1** (Table 4).

The *syn*- and *anti*-stereoisomers were obtained depending on the yeast used; high diastereoselectivity was sometimes observed. In particular, *Pachysolen tannophilus* CBS 4044 (entry 7) and *Torulopsis molischiana* CBS 837 (entry 9) gave only *anti*-**3** with modest enantioselectivities, while *Lindnera fabiani* (entry 13) gave only *syn*-**3** with notable enantioselectivity. Other yeasts (entries 10–12) gave poor diastereoselectivity in the formation of *syn*-**3**, but high enantioselectivity. In most cases, the pharmacologically relevant steroisomer (2*R*,3*S*)-*syn*-**3** was produced with an ee ranging

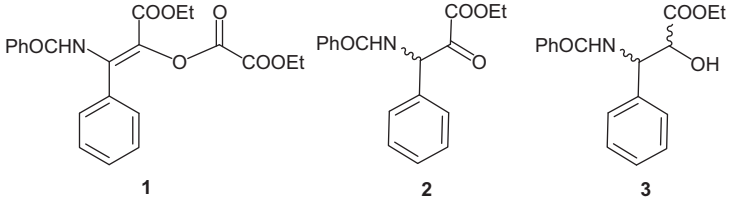


Figure 2. Substrates and products used and obtained in asymmetric hydrogenation.

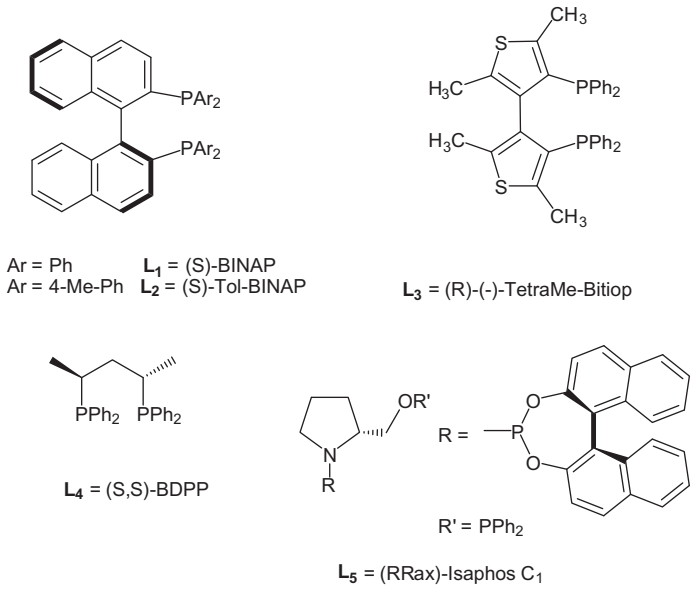
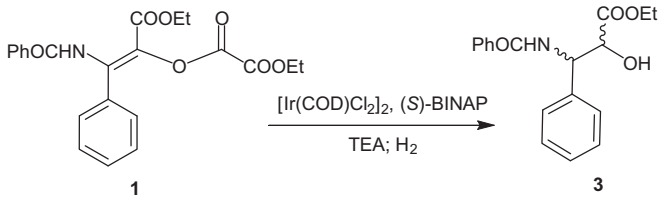


Figure 3. Chiral chelating diphosphines used in the asymmetric hydrogenation of **2**.

Table 1
Asymmetric reduction of **1** with $[Ir(COD)Cl_2]_2$, (*S*)-BINAP and different amounts of TEA



Entry	TEA/substrate	Conversion (%)	*syn*:*anti* (%)	ee *anti* (%)
1	—	0	—	—
2	0.5:1	0	—	—
3	1:1	30	0:100	0
4	5:1	100	14:86	0
5	10:1	73	12:88	0

Reaction conditions: Solvent: toluene; reaction time 24 h except for entry 4 (5 h); substrate/[Ir] = 50:1; substrate concentration = 0.012 mmol/mL; *T* = room temperature; *P* = 30 atm H_2. The ee and ed values were determined by chiral HPLC with a Daicel Chiralpak AD column (80:20 = hexane–isopropanol, flow = 1.0 mL/min).

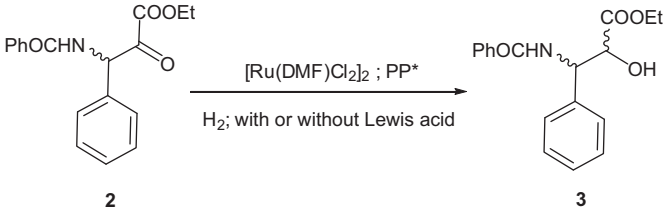
Table 2
Asymmetric reduction of **1** and **2** with $[Ir(COD)Cl_2]_2$, (*S*)-BINAP and TEA complex

Entry	Substrate	Conversion (%)	*syn*:*anti*	ee *anti* (%)
1	**1**	0	—	—
2	**2**	100	15:85	0
3[a]	**2**	20	10:90	0

Reaction conditions: Solvent: toluene; reaction time 5 h; substrate/[Ir] = 50:1; substrate concentration = 0.012 mmol/mL; *T* = room temperature for substrate **1** and *T* = 60 °C for substrate **2**; *P* = 30 atm H_2. The ee and ed values were determined by chiral HPLC with a Daicel Chiralpak AD column (80:20 = hexane–isopropanol, flow = 1.0 mL/min).

[a] Reaction was performed with $[Ir(COD)Cl_2]_2$, (*S*)-BINAP without TEA in 24 h.

Table 3
Chiral chelating diphosphines used in the asymmetric hydrogenation of **2**



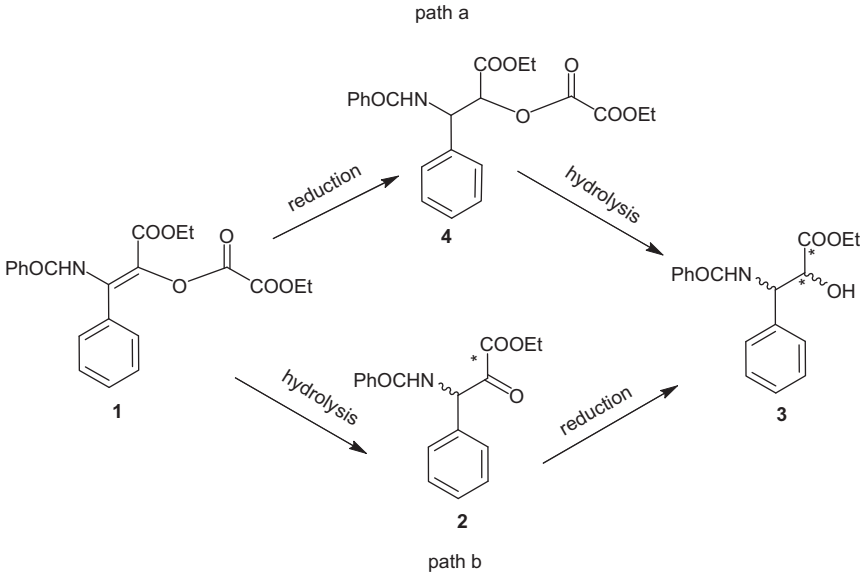
Entry	Lewis acid	Ligand	ed (%)	ee *syn*(%)	ee *anti* (%)
1	—	L_1	62 *anti*	0	9 (2*S*,3*S*)
2	—	L_2	76 *anti*	30 (2*R*,3*S*)	32 (2*R*,3*R*)
3	—	L_3	58 *anti*	15 (2*S*,3*R*)	19 (2*S*,3*S*)
4	$FeCl_3$	L_1	30 *syn*	38 (2*S*,3*R*)	60 (2*S*,3*S*)
5	HBF_4	L_1	20 *syn*	51 (2*S*,3*R*)	53 (2*S*,3*S*)
6	$CrCl_3$	L_1	53 *syn*	57 (2*S*,3*R*)	55 (2*S*,3*S*)
7	$MnCl_2$	L_1	9 *syn*	52 (2*S*,3*R*)	19 (2*S*,3*S*)
8	$AlCl_3$	L_1	25 *syn*	31 (2*S*,3*R*)	63 (2*S*,3*S*)
9	$FeCl_3$	L_2	24 *anti*	76 (2*S*,3*R*)	56 (2*S*,3*S*)
10	$FeCl_3$	L_3	21 *anti*	52 (2*R*,3*S*)	39 (2*R*,3*R*)
11	$CrCl_3$	L_3	47 *anti*	40 (2*R*,3*S*)	7 (2*S*,3*R*)
12	$FeCl_3$	L_4	12 *anti*	26 (2*S*,3*R*)	1 (2*S*,3*S*)
13	$CrCl_3$	L_4	24 *anti*	9 (2*S*,3*R*)	0
14	$FeCl_3$	L_5	8 *anti*	25 (2*S*,3*R*)	45 (2*S*,3*S*)
15	$CrCl_3$	L_5	0	50 (2*S*,3*R*)	34 (2*S*,3*S*)

Reaction conditions: Solvent: EtOH–CH_2Cl_2 = 50:50; reaction time 48 h; conversion >99%; substrate/$[RuCl_2(DMF)_n(PP^*)]$ = 50:1; catalyst/additive = 1:10, substrate concentration = 0.012 M; *T* = 60 °C; *P* = 30 atm H_2. The ee and de values were determined by chiral HPLC with a Daicel Chiralpak AD (80:20 = hexane–isopropanol, flow = 1.0 mL/min).

from 59% up to 99%, in accordance with Prelog's rule. The best performing yeast in terms of diastereoselectivity was *L. fabiani*, previously used for the stereoselective reduction of **2** into **3**.

Although the reduction of tetra-substituted double bonds by yeasts is not common, to discard this possibility, the reduction of the double bond (substrate **1**) by hydrogenation with Pd/C in the heterogeneous phase was performed. Preliminary studies with Pd/C as the catalyst showed a low molar conversion. The addition of NH_3 via ammonium formate allowed the total conversion of the substrate (Fig. 4).[39,40]

The strong dependence of the solvent used has been highlighted: in the presence of aprotic solvents (i.e. AcOEt) the desired oxalyl ester **4** was obtained, while when protic solvents (i.e. MeOH



Scheme 1. Different pathways to obtain **3** from **1**.

Table 4
Preparation of 3 by the bioreduction of **1** with different yeasts

Entry	Yeast	de (%)	ee *syn* (%)	ee *anti* (%)
1	*Kluyveromyces marxianus* CBS 1553	22 *anti*	>99 (2*R*,3*S*)	41 (2*S*,3*S*)
2	*Kluyveromyces marxianus var. lactis* CL69	20 *anti*	80 (2*R*,3*S*)	10 (2*S*,3*S*)
3	*Pichia etchellsii* MIM	42 *anti*	59 (2*R*,3*S*)	47 (2*R*,3*R*)
4	*Pichia glucozyma* CBS 5766	30 *anti*	9 (2*S*,3*R*)	42 (2*S*,3*S*)
5	*Pichia pastoris* CBS 2612	40 *anti*	20 (2*S*,3*R*)	43 (2*S*,3*S*)
6	*Pichia henricii* CBS 5765	66 *anti*	>99 (2*R*,3*S*)	52 (2*R*,3*R*)
7	*Pachysolen tannophilus* CBS 4044	>99 *anti*	0	48 (2*S*,3*S*)
8	*Torulopsis magnoliae* MIM 42	74 *anti*	69 (2*R*,3*S*)	38 (2*R*,3*R*)
9	*Torulopsis molischiana* CBS 837	>99 *anti*	0	10 (2*S*,3*S*)
10	*Saccharomyces cerevisiae* Zeus	16 *syn*	>99 (2*R*,3*S*)	43 (2*S*,3*S*)
11	*Torulopsis castelli* MIM 1705	22 *syn*	>99 (2*R*,3*S*)	68 (2*S*,3*S*)
12	*Sporobolomyces salmonicolor* MIM	26 *syn*	>99 (2*R*,3*S*)	59 (2*S*,3*S*)
13	*Lindnera fabiani* CBS 5640	>99 *syn*	82 (2*R*,3*S*)	—

The ee and de were determined after complete conversion of **1** in **3** obtained in 72 h.

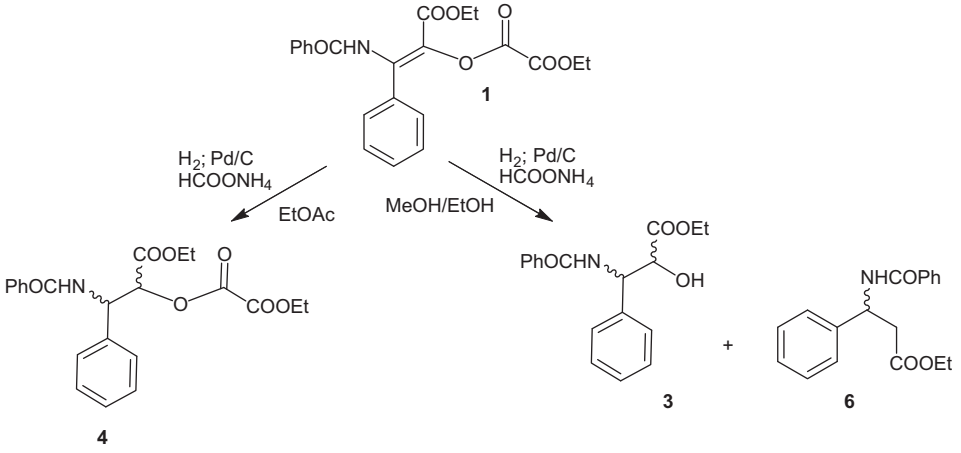
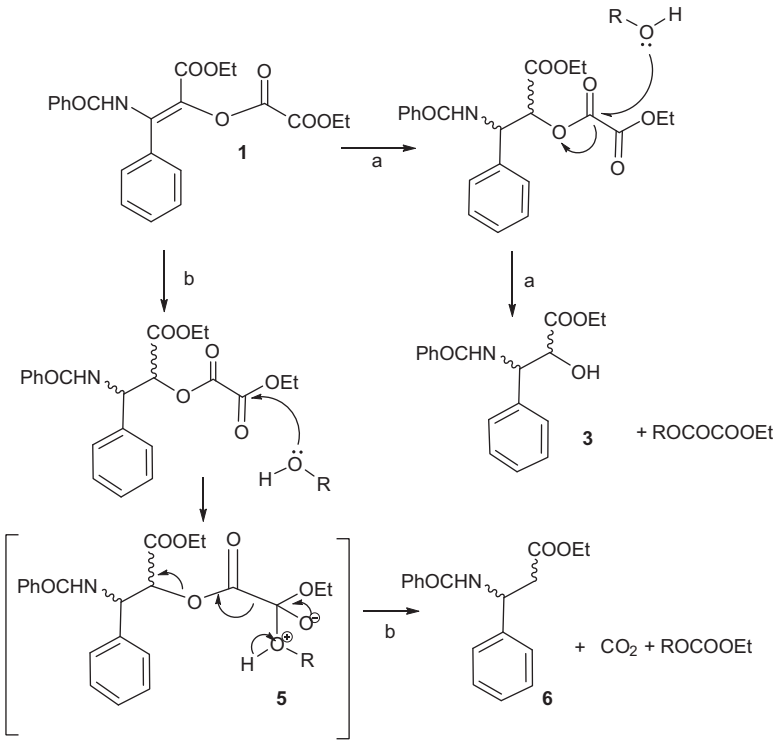


Figure 4. Reduction of **2** with Pd/C and $HCOONH_2$.

or EtOH) were used, a mixture of **3** and 3-benzamido-3-phenylpropanoate **6** was obtained.

The formation of **3** and **6** can be explained by the proposed mechanisms in Scheme 2. Compound **4** is formed by hydrogenation of **1** and can undergo subsequent alcoholysis to produce **3** (path a); alternatively alcoholysis can occur on the ethyl ester present on the oxalyl residue, followed by decarboxylation and the formation of **3** and the ethyl carbonate (path b).



Scheme 2. Possible mechanism for the production of **3** and **6**.

The biotransformations are carried out in a buffer so that the production of **3** could be justified but the total absence of **6** shows that the hypothesis mentioned above is the only one acceptable.

The biotransformation with *Sporobolomyces salmonicolor* was optimized for improving the diastereoselectivity. Co-substrate type and concentration, co-solvent type and concentration, substrate concentration, and pH were chosen as control variables. Yields and diastereoselectivities after 48 h were used as response targets. Optimization was carried out by employing the Multisimplex® 2.0 software.[41] *S. salmonicolor* completely converted **1** into (2*R*,3*S*)-*syn*-**3** within 48 h with a de >70% and an ee of 98% using DMSO (2%) as the co-solvent, an imidazole buffer 0.1 M pH 7.8, xylose as the co-substrate (50 g/L) and 4.2 g/L of substrate.

3. Conclusion

Different approaches for the preparation of phenylisoserine, the lateral chain of Taxol, were investigated by homogeneous asymmetric catalysis using Ir(I)- and Ru(II)–phosphine complexes and by an enzymatic approach with non-conventional yeasts. While iridium complexes gave good diastereoselectivities, the enantioselectivities were poor. Lewis acids added to ruthenium-diphosphines complexes in a 1:10 ratio gave good enantioselectivities. The best results (up to 76% ee) were obtained using $FeCl_3$ as the Lewis acid.

Yeasts, with cell-associated esterase and carbonyl reductase activities, allowed for the two-step, one-pot preparation of the synthon (2*R*,3*S*)-*N*-benzoyl-3-phenyl isoserine ethyl ester with high yields, furnishing higher yields and a shorter route than the previously reported chemoenzymatic synthesis of **3**.

4. Experimental

Catalytic reactions were performed in a 100 mL glass autoclave equipped with a magnetic stirrer. Unless otherwise stated, materials were obtained from commercial source and used without further purification. ^{1}H NMR spectra were recorded on a Bruker DRX Avance 300 MHz equipped with a non-reverse probe and on a Bruker DRX Avance 400 MHz. HPLC analysis: Merck-Hitachi L-7100 equipped with Detector UV-6000LP and Diacel Chiralpak AD. MS analyses were performed by using a Thermo Finnigan (MA, USA) LCQ Advantage system MS spectrometer with an electrospray ionisation source and an 'Ion Trap' mass analyzer. The MS spectra were obtained by the direct infusion of a sample solution in a mixture MeOH/H_2O/AcOH 10:89:1 under ionization, ESI positive. Literature data were used for the assignment of the absolute configuration of the isomers of *N*-benzoyl phenyl isoserine.[42,16]

4.1. Analytical methods

Analysis was performed on HPLC Merck-Hitachi L-7100 with UV-6000LP Thermo Finnigan and CHIRALPACK AD (4.6 mm × 250 mm). The analysis of **1** and its products was performed with a solvent system consisting of hexane and *i*PrOH in a ratio of 80:20. The flow-rate was 1.0 mL min^{-1}; injection volume was 20 μL. GC–MS spectra were recorded on Thermo Finnigan MD 800 equipped with GC Trace (SE 52 column: length 25 m/ϕ int. 0.32 mm, film 0.4–0.45 μm) and MS analyses were performed by using a Thermo Finnigan (MA, USA). The column temperature was kept at 150 °C and increased by 8 °C min^{-1} up to 270 °C.

4.2. Synthesis of 1-benzamido-3-ethoxy-3-oxo-1-phenylpropan-2-yl ethyl oxalate 1

This compound was prepared in accordance with a literature procedure.[16] ^{1}H NMR($CDCl_3$): δ = 1.30 (t, CH_3, J = 7.3 Hz); 1.32 (t, CH_3, J = 7.0 Hz); 4.29 (q,CH_2, J = 7.3 Hz); 4.33 (q, CH_2, J = 7.3 Hz); 7.44–7.68 (m, aromatic); 7.95 (d, aromatic, J = 6.4 Hz); 11.52 (s, NH); ^{13}C NMR ($CDCl_3$): δ = 14.3 (CH_3), 14.4 (CH_3), 62.1 (CH_2), 64.1 (CH_2), 128.4–134.0 (CH, aromatic), 133.0 (C=C), 133.44 (C=C), 156.7 (C=O, ester), 156.8 (C=O, ester), 162.5 (C=O, ester), 165.8 (C=O, amide); IR (film) 3274, 2995, 1972, 1906, 1781, 1740, 1699,

1685, 1622 cm^{-1}; HRMS of $C_{22}H_{21}NO_7$ (*m*/*z*): calcd 411.23, found 434.1 (M^++Na).

4.3. Synthesis of ethyl 3-benzamido-2-oxo-3-phenylpropanoate 2

This compound was prepared in accordance with a literature procedure.[16] ^1H NMR ($CDCl_3$): δ = 1.35 (t, CH_3, J = 7.0 Hz); 4.23 (q, CH_2, J = 5.5 Hz); 6.44 (d, CH, J = 6.6 Hz); 7.24 (br, NH), 7.26–7.54 (m, aromatic); 7.79 (d, aromatic, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$): δ = 13.6 (CH_3), 14.0 (CH_3), 45.6 (CH_2), 60.6 (CH), 127.4–134.6 (CH aromatic), 159.9 (C=O, ester), 166.8 (C=O, carbonyl), 189.3 (C=O, amide). HRMS of $C_{18}H_{17}NO_4$ (*m*/*z*): calcd 311.12, found 334.2 (M^++Na).

4.4. General procedure for the asymmetric hydrogenation

In a Schlenk tube under argon, the substrate was added to the catalyst, iridium or ruthenium complex, followed by 20 ml of solvent, with or without an appropriate amount of Lewis base (TEA) or acid. The solution was stirred for 15 min at room temperature and then transferred with a cannula in an autoclave. The stainless steel autoclave (200 mL), equipped with temperature control and magnetic stirrer, was purged five times with hydrogen. After the transfer of the reaction mixture, the autoclave was pressurized. At the end the autoclave was vented and the mixture was analyzed by NMR spectra and HPLC.

4.4.1. Preparation of Ir(COD)[(*S*)-BINAP]Cl

A solution of $[Ir(COD)Cl_2]_2$ (12 mg, 0.0236 mmol) and (*S*)-BINAP (29.6 mg, 0.0475 mmol) in 8 mL of toluene was stirred at room temperature for 4 h. The solution was concentrated under reduced pressure and the yellow-orange solid obtained was washed with hexane. The complex was dissolved in 0.7 mL of toluene/$CDCl_3$ 7:3 solution in a NMR tube, increasing the amount of TEA added to the complex solution and analyzed by ^{31}P NMR.

^{31}P NMR of Ir(COD)[(*S*)-BINAP]Cl ($CDCl_3$): δ = −2.2 (d, J = 18.3 Hz), −11.6 (d).

^{31}P NMR of Ir(COD)[(*S*)-BINAP]Cl with 10 equiv of TEA ($CDCl_3$): δ = 1.0 (d, J = 21.4 Hz), 13.0 (d).

^{31}P NMR of Ir(COD)[(*S*)-BINAP]Cl with 12.5 equiv of and heating at 60 °C ($CDCl_3$): δ = 3.6 (d, J = 17.4 Hz), −1.2 (d).

4.4.2. Ethyl 3-benzamido-2-hydroxy-3-phenylpropanoate 3

syn-**3**: ^1H NMR ($CDCl_3$): δ = 1.28 (t, CH_3, J = 7.0 Hz); 4.28 (q, CH_2, J = 5.0 Hz); 4.63 (d, CH, J = 2.2 Hz); 5.75 (dd, CH, J = 1.8, J = 6.9 Hz); 7.0 (d, NH, J = 8.0); 7.23–7.59 (m, aromatic); 7.80 (d, aromatic, J = 2.2 Hz). ^{13}C NMR ($CDCl_3$): δ = 14.1 (CH_3), 53.3 (CH), 61.8 (CH_2), 71.4 (CH), 125.4–133.7 (CH aromatic), 169 (C=O, ester), 173.1 (C=O, amide).

anti-**3**: ^1H NMR ($CDCl_3$): δ = 1.25 (t, CH_3, J = 7.0); 4.14 (q, CH_2, J = 5.0 Hz); 4.68 (d, CH, J = 2.2 Hz); 5.60 (dd, CH, J = 1.8, J = 6.9 Hz); 7.16 (d, NH, J = 8.0); 7.23–7.59 (m, aromatic); 7.83 (d, aromatic, J = 2.2 Hz). ^{13}C NMR ($CDCl_3$): δ = 13.9 (CH_3), 53.3 (CH), 61.2 (CH_2), 71.8 (CH), 125.4–133.6 (CH, aromatic), 168.6 (C=O, ester), 173.1 (C=O, amide).

IR (film) 3348, 3063, 3033, 2980, 2962, 2904, 1960, 1985, 1780, 1718, 1644, 1109 cm^{-1} $C_{18}H_{19}NO_4$ Exact Mass: 313.13, found 336.2 (M^++Na).

4.5. Microorganisms: culture conditions

Strains from official collections or from our collection (Microbiologia Industriale Milano) were routinely maintained on a malt extract (8 g L^{-1}, agar 15 g L^{-1}, pH 5.5). In order to obtain cells for the biocatalytic activity tests, the microorganisms were cultured in 500 mL Erlenmeyer flasks containing 80 mL of the medium and incubated for 48 h at 28 °C on a reciprocal shaker (100 spm). The yeasts were grown on a malt extract with 5 g L^{-1} Difco yeast extract. Fresh cells from submerged cultures were centrifuged (5000 rpm per 10′) and washed with 0.1 M phosphate buffer pH 7 prior to use.

4.6. Bioreduction conditions

General procedure for the screening: reductions were carried out in 10 mL screw-capped test tubes with a reaction volume of 3 mL with cells (20 g L^{-1}, dry weight) suspended in 0.1 M phosphate buffer pH 7 containing 5% of glucose and 4 g L^{-1} of 3-benzoylamino-3-phenyl-(ethyl, 2-oxalyl) propenoic acid ethyl ester **1**. The reactions were carried out at 30 °C under magnetic stirring. Samples (200 μL) were taken at intervals, extracted with an equal volume of ethyl acetate and the organic layer was dried under Na_2SO_4, filtered, and then evaporated. The extracts were analyzed by thin-layer chromatography (CH_2Cl_2–di*iso*propylether = 2:8) and by chiral HPLC and GC–MS.

4.7. Optimisation of the biotransformation carried out with *Sporobolomyces salmonicolor* MIM

The conditions of the sequential experimental trials were selected employing the Multisimplex® 2.0 software (F. H. Walters, L. R. Parker, S. L. Morgan, S. N. Deming, (1991) In *Sequential Simplex Optimization*, Boca Raton: CRC Press). The control variables were pH (5.5 < pH > 8), co-solvent (DMSO, CH_3CN 1–2% v/v), substrate **1** concentration (0.5–5 g L^{-1}) and co-substrate (glucose, xylose 30–70 g L^{-1}). The three response variables were chosen for the optimization: the molar conversion after 48 h, diastereomeric, and enantiomeric excess. The biotransformations were carried out in 25 mL flasks under magnetic stirring; optimization was performed using 5 mL of total volume.

4.8. Reduction by Pd/C with ammonium formate

In a stainless steel autoclave (20 ml), equipped with temperature control and a magnetic stirrer, was purged five times with hydrogen, a solution of **1** in ethyl acetate with 5% of Pd/C after which 1 equiv of ammonium formate was transferred. The autoclave was pressurized at 10 atm and kept at room temperature. The reaction was monitorated by GC–MS analysis. The mixture was filtered on Celite and the solvent was evaporated in vacuo. A mixture of **4** and **6** was obtained. The products were isolated by preparative TLC on silica gel using an eluent CH_2Cl_2–di*iso*propylether = 2:8.

4.8.1. Ethyl 3-benzamido-2-((ethoxycarbonyl)oxy)-3-phenylpropanoate 4

^1H NMR ($CDCl_3$) δ = 1.26 (t, CH_3, J = 7.1 Hz), 1.39 (t, CH_3, J = 7.0 Hz), 4.28 (q, CH_2, J = 5.0 Hz), 4.35 (q, CH_2, J = 5.2 Hz), 5.47 (d, CH, J = 2.6 Hz), 5.98 (dd, CH, J_1 = 2.6, J_2 = 9.5 Hz), 7.14 (d, NH, J = 9.1 Hz), 7.27–7.58 (m, aromatic), 7.77 (d, aromatic, J = 1.5 Hz). ^{13}C NMR ($CDCl_3$): δ = 13.6 (CH_3), 14.1 (CH_3), 53.3 (CH), 61.8 (CH), 63.2 (CH_2); 63.7 (CH_2); 127.4–134.7 (CH, aromatic); 160.3 (C=O, ester), 162.8 (C=O, ester) 168.4 (C=O, carbonyl); 171.2 (C=O, amide). HRMS of $C_{22}H_{23}NO_7$ (*m*/*z*): calcd 413.15, found 436.1 (M^++Na).

4.8.2. Ethyl 3-benzamido-3-phenylpropanoate 6

^1H NMR ($CDCl_3$) δ = 1.17 (t, CH_3, J = 7.0 Hz), 2.98 (t, CH_2, J = 3.0 Hz), 4.10 (q, CH_2, J = 4.6 Hz), 5.63 (m, CH), 7.29–7.52 (m, aromatic), 7.83 (d, aromatic, J = 1.5 Hz). ^{13}C NMR ($CDCl_3$): δ = 14.3 (CH_3), 40.1 (CH_2), 50.0 (CH), 61.1 (CH_2), 126.5–139.8 (CH, aromatic); 160.0 (C=O, ester), 171.8 (C=O, amide). HRMS of $C_{18}H_{19}NO_3$ (*m*/*z*): calcd 297.14, found 192.2 (M^+−C_6H_5CO).

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