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ORIGINAL PAPER

Enantioselective Baeyer–Villiger oxidation of bicyclo[3.2.0]hept-2-en-6-one with fungi: optimization of biotransformation and use of TiO_2 as support of cell growth

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Abstract Fungi from Amazonian forest soil (Ecuador) and an Italian factory were screened for Baeyer–Villiger (BV) oxidation of bicyclo [3.2.0]hept-2-en-6-one to 2-oxabicyclo[3.3.0]oct-6-en-3-one (Corey's lactone). Isolates of *Fusarium* sp. and *F. solani* produced the (+)-(1R,5S)-lactone while isolates of *Aspergillus terricola* and *A. amazonicus* afforded the (−)-(1S,5R)-lactone. Highest conversions (85% yield and 70% enantiomeric excess) were obtained with *A. amazonicus* grown in presence of 2.7 mM titanium dioxide.

Keywords Baeyer–Villiger oxidation · bicyclo[3.2.0]hept-2-en-6-one · microbial oxidation · titanium dioxide

Introduction

The Baeyer–Villiger (BV) oxidation of linear or cyclic ketones into their corresponding esters or lactones is a basic reaction of organic chemistry (Renz and Meunier 1999) and its relevance in the cellular metabolic pathways was ascertained about 40 years ago (Forney et al. 1967). Its regioselectivity is governed by electronic factors that cause migration of the more substituted carbon–carbon bond to oxygen, affording the so-called ''expected'' lactone (Fig. 1). The BV oxidation reaction can be achieved in its asymmetric version using either microbial whole cells or by enzymatic biotransformations (Alphand et al. 2003; Mihovilovic et al. 2002).

On the other hand, bicyclic compounds with different functionalities in each ring are suitable for the stereocontrolled synthesis of wide variety of natural products. In particular bicyclo[3.2.0]hept-2-en-6-one, **1**, and the corresponding 2-oxabi-cyclo[3.3.0]oct-6-en-3-one (Corey's lactone), **2**, are used for the synthesis of prostaglandins (Newton and Roberts 1980) and are interesting as precursors of antibiotics (Andrau et al. 1997) while the ''unexpected'' 3-oxabicyclo[3.3.0]oct-6-en-2-one, **3**, is also a valuable chiral synthon (Lebreton et al. 1997; Hudlicky et al. 1983). The use of biocatalytic approach with this interesting substrate afforded in many cases both the regioisomeric lactones (−)-(1S,5R)-**2** and (−)-(1R,5S)-**3** in high enantiomeric excesses (Alphand et al. 1989; Doig et al. 2002) (Fig. 1). On a preparative scale this feature implies a delicate chromatographic separation (Hilker et al. 2004).

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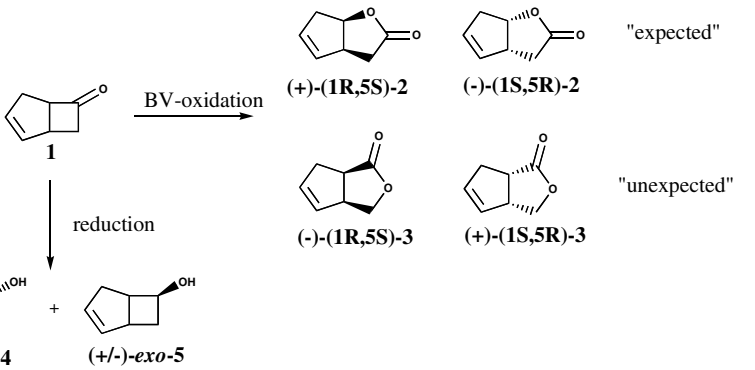

Fig. 1 Biotransformation of bicycloheptenone, 1, with wild type microrganisms

On the other hand, with wild type microbial strains it is possible to obtain various side reactions (Alphand et al. 1990) as lactone hydrolysis and/or ketone reduction to *endo*- and *exo*-alcohol, **4** and **5**.

In this paper we describe the BV-oxidation of bicyclo[3.2.0]hept-2-en-6-one, **1**, with various fungal isolates and the optimization of the biocatalytic conditions in order to obtain high yield and enantiomeric excess of the Corey's lactone and minimize the side reaction products.

Materials and methods

Materials

All chemicals and solvents were from commercial sources and of analytical grade. (+/−)-Bicyclo[3.2.0]hept-2-en-6-one, **1**, (Merck) (+)-(1R,5S)- and (−)-(1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one, **2**, and (−)-(1R,5S)-3-oxabicyclo[3.3.0]oct-6-en-2-one, **3**, (Fluka) were commercially available. The *endo*- and *exo*-bicycloheptenols, **4** and **5**, were prepared and characterized according to the literature (Fantin et al. 1996).

Gas chromatographic analyses were performed using a fused capillary column (Megadex 5, 25 m × 0.25 mm) containing *n*-pentyl-*β*-cyclodextrin on OV 1701: helium as carrier gas (86 kPa); temp. 90–200°C (2.5°C/min). Retention times (in min): (−)-1S,5R-**1** , 8.27; (+)-1R,5S-**1**, 8.72; racemic *endo*-alcohol **4**, 13.20; (+)-1S,5R,6R-*exo*-alcohol **5**, 14,16; (+)-1R,5S,6S-*exo*-alcohol **5**, 14.93; (−)-1R,5S-lactone **3**, 23.16; (+)-1R,5S-lactone **2**, 23.67; (−)-1S,5R-lactone **2**, 24.22; (+)-1S,5R-lactone **3**, 24.61. Saboraud culture medium contained glucose (40 g/l) and peptone (10 g/l). Williams' culture medium was prepared with glucose (20 g/l), $(NH_4)_2SO_4$ (5 g/l), KH_2PO_4 (2 g/l), $CaCl_2$ (0.25 g/l), $MgSO_4 \cdot 7H_2O$ (0.25 g/l), inositol (25 mg/l), H_3BO_3 (1 mg/l), $ZnSO_4$ (1 mg/l), $MnCl_2$ (1 mg/l), $FeCl_2$ (0.5 mg/l), $CuSO_4$ (0.1 mg/l), KI (0.1 mg/l), thiamine (0.3 mg/l), biotin (0.025 mg/l), calcium pantothenate (0.3 mg/l), pyridoxine (0.3 mg/l) and nicotinic acid (0.3 mg/l). The pH was adjusted to 6.5 with 10% (w/v) NaOH. In Williams' modified culture medium glucose was replaced by mannitol (20 g/l). Sabouraud dextrose agar (SDA) was commercially available (Oxoid).

Isolation of microrganisms from environmental samples

Various water samples, collected from Italian chemical factory (I.C.E. -Reggio Emilia) and from Amazonian forest (Ecuador) soils, were used for the isolation of microrganisms. Each sample (1 ml) was diluted with a sterile saline solution (9 ml) and each diluted sample (1 ml) has been streaked on Petri dishes of SDA containing 200 mg chloramphenicol/l. After 24 h of incubation at 28°C different colonies were picked and transferred to fresh SDA to obtain pure cultures: 20 strains were selected from Ecuadorian soil and 17 from the Italian factory soil. The strains were used in the oxidation screening without identification. The determination of genus and species of fungi, that afforded the best results (Table 1), has been made according to the literature (Barnett and Hunter 1998). The fungi isolated from Italian soil samples belong to *Fusarium* genus, while the strains from Ecuadorian

Table 1 Screening of biotransformation of bicyclo[3.2.0]hept-2-en-6-one, **1**

Strain	**1** (%)	(+)-**2** (%)	e.e. (%)	(−)-**2** (%)	e.e. (%)	**3** (%)	**4** (%)	**5** (%)
Fusarium sp.	24	23	82			6	33	14
Fusarium solani	25	27	68			–	28	20
Aspergillus terricola	30			28	72	5	26	11
Aspergillus amazonicus	47			26	90	8	13	6

Fungi cultures were obtained inoculating a loopful of the selected strain in sterilized Williams' broth (10 ml) and the growth was continued for 48 h at 28°C and 150 rpm. The biotransformation was initiated adding a solution (0.1 g/ml in DMF) of bicycloheptenone, **1**, (100 μl) and the incubation was continued for further 48 h. GLC analysis afforded the yields of compounds **2–5** and of the recovered **1**

soil belong to *Aspergillus* genus (Mares et al. 2005).

Preparation of working stock

The slant of the strains were treated with sterilized Tween 80 (5 ml) and saline solution (4 ml). The homogenized suspension (10 ml) was added to a sterilized solution (100 ml) of 20% (v/v) glycerol in water, homogenized, transferred in cryovials (1 ml) and maintained in liquid air. The subsequent experiments were carried out using a suspension (1 ml) obtained diluting the content of a cryovial in 10 ml of sterilized culture medium.

Effect of culture medium and growth time on the biotransformation

Biotransformations were carried out as above using Saboraud, Williams' and modified Williams' broth. Modified Williams' broth afforded best results with *Fusarium* strains instead Sabourad appeared the better media for *Aspergillus* (Table 2).

BV-specific activity, defined as the ratio between the initial velocity and the biomass amount (given in mmol/h per g of cells), was determined at different growth times. For each strain, an appropriate number of cultures (50 ml of the selected medium) was incubated in 250 ml flask at 28°C and 150 rpm. After 48 h, a culture was used for the dried weight determination (filtration and drying in oven at 105°C for 12 h) and a second one for measurement of the initial velocity after adding ketone, **1**, (500 μl of a solution 0.1 g/ml in DMF) and monitoring the biotransformation at 18 and 26 h. The linear regression's slop (variation of lactone concentration vs. time) was assumed as the initial velocity of the reaction. The remaining cultures were used for the BV-specific activity determination after 3, 4, 5 and 6 days growth, respectively. The results have been

Table 2 Biotransformations of **1** in different culture media

Strain	Culture medium	**1** (%)	(+)-**2** (%)	e.e. (%)	(−)-**2** (%)	e.e. (%)	**3** (%)	**4** (%)	**5** (%)
Fusarium sp.	A	28	49	51			6	15	4
	B	–	30	4				65	7
	C	–	80	34				15	1
Fusarium solani	A	30	43	49			–	12	7
	B	–	86	38				–	8
	C	–	85	17				–	15
Aspergillus terricola	A	15			44	74	5	28	7
	B	15			64	48		9	6
	C	–			93	12		–	–
Aspergillus amazonicus	A	13			45	68	8	30	6
	B	49			28	97		17	4
	C	20			59	45		12	5

The biotransformations were carried out as above using Saboraud (A), Williams' (B) and modified Williams' (C) culture media. *Fusarium* biotransformations were carried out adding the substrate, **1**, after 4 days growth and monitoring the results after 6 days incubation at 28°C, while *Aspergillus* biotransformations were carried out after 3 days growth and monitoring the results after 2 days incubation. GLC analysis afforded the yields of compounds **2–5** and of the recovered **1**

reported in the Fig. 2 while the optimized biotranformations summarized in Table 3.

Determination of BV-specific activity of *A. amazonicus* using TiO_2 as support of cell growth

The determination was made as above using 30 *A. amazonicus* cultures (50 ml in 250 ml flasks): 10 were the blank experiment, 10 were added of TiO_2 (0.3 g/l, 2.7 mM) and 10 were added with TiO_2 (0.6 g/l, 5.4 mM). The flasks with TiO_2 were maintained in the dark. Dried weight and BV-initial velocity were monitored at 2, 3, 4, and 5 days. Biomass amount was not affected by the presence of TiO_2. The BV-specific activities were reported in Fig. 3 while the optimized biotranformation was described in Table 3.

Preparative scale BV-oxidations with *Fusarium* sp. and *A. amazonicus*

Fusarium sp. and *A. amazonicus* cultures (50 ml) obtained as described in Table 3 were used to inoculate 500 ml (in 2 l flask) of the appropriate culture medium (modified Williams' for *F.* sp. and Sabourad containing 2.7 mM TiO_2 for *A. amazonicus*). After 96 h and 72 h incubation, respectively, at 28°C bicycloheptenone, **1**, (0.5 g, 4.6 mmol) in DMF (5 ml) was added. Incubation was continued for 48 h and then the cultures were extracted with diethyl ether (2×300 ml). The organic phases were dried over sodium sulphate and concentrated. Chromatography on silica gel (cyclohexaneethylacetate, 80:20 (v/v) as eluent) afforded lactone (+)-**2** (0.43 g, 3.45 mmol) in 75% yield (ee 73%) with *Fusarium* sp. On the other *A. amazonicus* catalysed reaction gave lactone (−)-**2** (0.47 g, 3.7 mmol) in 82 % yield (ee 70%).

Results and discussion

Screening of fungi for BV oxidation

Among the fungal isolates from ICE factory soil and Amazonian forest soil only four strains gave interesting results towards BV oxidation of bicycloheptenone, **1** (Table 1), affording the regioisomeric lactones, **2** and **3**, together with the

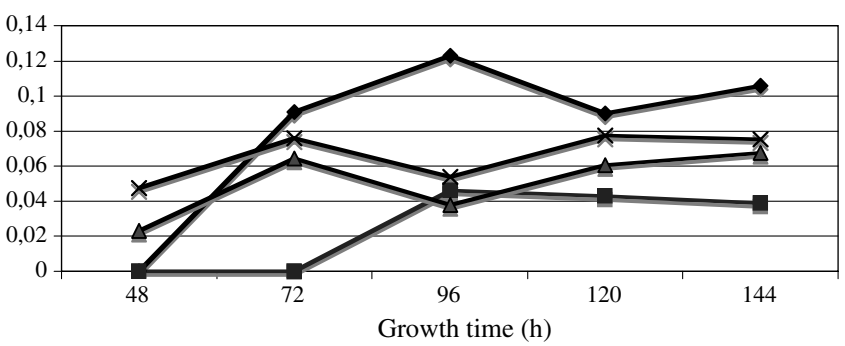

Fig. 2 BV-specific activity (SA) of fungi. ♦ *Fusarium* sp.; ■ *F. solani*; ▲ *A. terricola*; × *A. amazonicus*

Table 3 Optimized BV-oxidation of bicycloheptenone **1**

Strain	**1** (%)	(+)-**2** (%)	e.e. (%)	(−)-**2** (%)	e.e. (%)	**4** and **5** (%)
Fusarium sp.	3	78	73			2
Fusarium solani	1	86	70			2
Aspergillus terricola	8			62	64	23
Aspergillus amazonicus	2			68	61	11
Aspergillus amazonicus (TiO_2)	–			85	70	3

Fusarium biotranformations were carried out inoculating the substrate (1 g/l) after 96 h growth in modified Williams' culture medium. *Aspergillus* biotranformations were carried out inoculating the substrate (1 g/l) after 72 h growth in Saboraud culture medium. All the biotransformations were stopped after 48 h incubation at 28°C. A further experiment has been made with *A. amazonicus* in the presence of 2.7 mM TiO_2. GLC afforded the yields of compounds **2–5** and of the recovered **1**

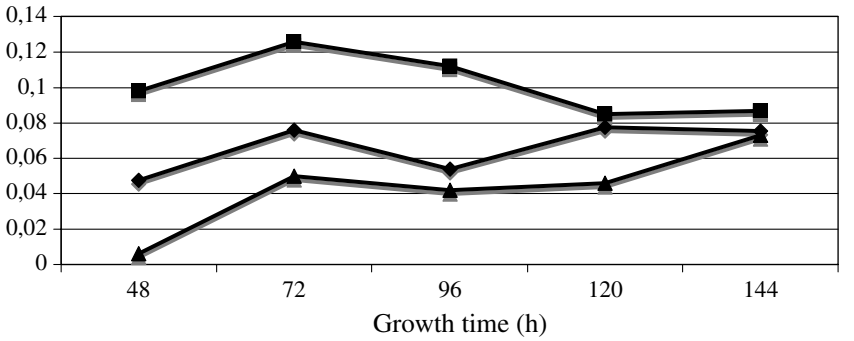

Fig. 3 TiO_2 effect on *A. amazonicus* BV-specific activity (SA) ♦ SA without TiO_2; ■ SA with 2.7 mM TiO_2; SA with 5.4 mM TiO_2

diastereomeric alcohols, **4** and **5** (Fig. 1). This preliminary approach showed the presence of the lactone **2**, Corey's lactone, as main product with the prevalence of the (+)-enantiomer with *Fusarium* strains and the (−)-enantiomer with *Aspergillus* strains. At this stage the amount of the unreacted ketone was still high and side reaction products (i.e. *exo*- and *endo*-alcohol **4** and **5**) were present.

The morphological observations did not allow the determination of species for one of the strains of *Fusarium* while the other was species *solani* (Barnett and Hunter 1998). On the other hand, the presence of characteristic conidiophores indicated that the *Aspergillus* were *terricola* and *amazonicus* (Mares et al. 2005).

Optimization of culture conditions for biotransformation

The microbial BV-oxidations were carried out in different culture media (Table 2). *Fusarium* and *Aspergillus* were cultured in Saboraud's medium, in a synthetic medium (i.e. Williams') and in the modified Williams' (glucose was substituted with mannitol). The modified Williams medium was the best choice for *Fusarium* species (higher yields but lower enantiomeric excesses) while for *Aspergillus* Saboraud's medium was the best compromise between yields and enantiomeric excesses. On the other hand, in order to determine the lowest growth time, the specific activity of BV-reaction was determined (Fig. 2). *Fusarium* sp. displayed the maximum of BV-specific activity at 96 h growth and then slowly decreased. The highest *F. solani* BV-specific activity was achieved at 96 h growth and was maintained at this until 144 h. On the basis of these results the BV-oxidations of bicycloheptenone, **1**, were carried out adding the substrate after 96 h growth and the incubation was stopped after a further 48 h at 28°C. These conditions afforded the (+)-(1R,5S)-lactone, **2**, in 78% (*F.* sp.) and 86% (*F. solani*) yields (ee 73% and 70%, respectively) (Table 3).

On the other hand, *A. terricola* and *A. amazonicus* had a maximum of BV-specific activity after 72 h growth. This value decreased rapidly at 96 h and then increased at 120 h. These results suggested to add the bicycloheptenone, **1**, after 72 h growth and to stop the biotransformation after 48 h at 28°C. In these conditions (−)-(1R,5S)-lactone, **2**, was obtained in 62% (*A. terricola*) and 68% (*A. amazonicus*) yields (ee 64% and 61%, respectively) (Table 3).

TiO_2 effect on BV-specific activity

Since *A. amazonicus* grew forming very close pellets, we have investigated the possibility of modify this feature in order to improve the BV-oxidation. Various attempts were made modifying the speed of the reciprocatory shaker, using glass bed of different size but the results were poor. The choice of titanium dioxide as support for the growth was due to its well-known biocompatibility (Polonchuck et al. 2000) and low water solubility that formed a fine suspension. Moreover, in the dark, titanium dioxide has no appreciable toxicity for the cells (Blake et al. 1999). In fact the growth of *A. amazonicus*, determined as g dry wt/l, in presence of 2.7 mM and 5.4 mM TiO_2 in the dark compared with the growth without support was substantially the

same. The presence of TiO_2 did not affect the amount of biomass but probably have changed its thickness and porosity. This aspect is under investigation. On the other hand, the comparison of the BV-activity of *A. amazonicus* with or without TiO_2 showed that 2.7 mM TiO_2 increased the activity, while 5.4 mM TiO_2 concentration decreased it. The BV-oxidation, carried out using 2.7 mM TiO_2 as support of growth, afforded improved yields (85%) of (−)-(1S,5R)-2-oxabicyclo[3.3.0]oct-6-en-3-one, **2**, with good enantiomeric excess (70%) (Table 3).

The *Fusarium* sp.- and *A. amazonicus*-catalysed biotransformations were performed also on preparative scale, using 500 ml volume cultures and 9.2 mM substrate, **1**, following the conditions specified in Table 3, (*A. amazonicus* was cultivated in presence of 2.7 mM TiO_2). *F.* sp. catalysed BV-oxidation of **1** affording (+)-**2** lactone in 75% yield (ee 73%) while *A. amazonicus* afforded (−)-**2** lactone in 82% yield (ee 70%).

Conclusions

In conclusion the screening of BV-oxidation with wild type microrganisms has made possible the characterization of a new strain of *Aspergillus* (i.e. *amazonicus*) coming from Amazonian forest soil that has given good results in the synthesis of the ''Corey's'' lactone after a simple preliminary optimization work, this avoiding the separation of the regioisomer **2** and **3** that is a quite complicated process and letting foresee interesting applications.

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