Pergamon

Tetrahedron 54 (1998) 5781–5788

TETRAHEDRON

Baeyer-Villiger Oxidation by *Pseudomonas putida* NCIMB 10007 in Organic Solvents: the Influence on the Regioselectivity.

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Received 17 February 1998; revised 12 March 1998; accepted 19 March 1998

Abstract: Microbiological Baeyer-Villiger oxidation of (±)-Norbornanone by whole-cells of *Pseudomonas putida* NCIMB 10007 have been performed in the presence of organic solvents. Thus, two phase systems consisting of water and different water-immiscible organic solvents, and monophasic organic systems have been examined and referred to an aqueous medium. The effect of the solvent used on the regioselectivity of Baeyer-Villiger oxidation have also been discussed. © 1998 Elsevier Science Ltd. All rights reserved.

Enzyme-catalysed Baeyer-Villiger oxidation has been put forward as one of the best options to achieve enantioselective synthesis of lactones. Although interesting results have been obtained with metal-catalysed oxidations,[1] biotransformation continues to be a field of tremendous activity.

Enantioselective characteristics of the Baeyer-Villiger oxidation have been extensively reported and, even nowadays, many efforts are being employed in clarifying its rules. It has been found that the results obtained in this enzymatic reaction are strongly related to the type of enzyme involved, as well as to the structural features of the substrate to be transformed.[2,3]

Initial work of Alphand *et al.* in Marseille[4,5] and Levitt *et al.* in Exeter[6] using whole-cells of *Acinetobacter calcoaceticus* NCIB 9871 and *Acinetobacter* TD 63 exploited the enantiomeric potential of this biotransformation over monocyclic and bicyclic substrates where one enantiomer yielded the corresponding lactone in high enantiomeric excess and the other enantiomer remained unreacted.

Later, Königsberger *et al.*[7] described another kind of enantioselective lactonization using whole-cells of *Acinetobacter calcoaceticus* NCIB 9871 showing a reduction-oxidation pair over bicyclic ketones.

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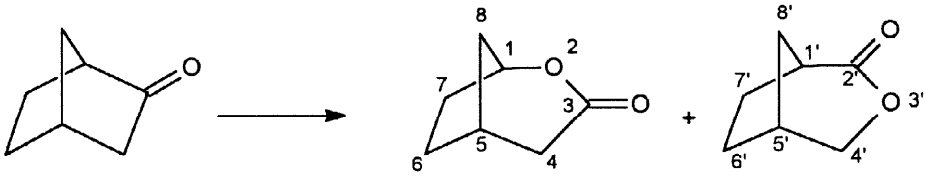
PII: S0040-4020(98)00266-X

Recently, the enantioselective feature of this biotransformation has been expanded towards the regioselectivity in the oxygen atom insertion, obtaining two regioisomeric lactones in optically pure form from the bicyclic racemic ketone [3.2.0] hept-2-en-6-one. This regioselectivity is also closely related to the enzyme involved, enabling the obtention of enantiocomplementary results using the isolated MO1 monooxygenase from *Pseudomonas putida* NCIMB 10007[8] against those obtained with *Acinetobacter* TD63,[9,10] *Acinetobacter calcoaceticus* NCIMB 9871,[10,11] *Pseudomonas putida* AS1,[11] *Acinetobacter* junii,[11] *Rhodococcus coprophilus* WT1[11] and *Rhodococcus fasciens* WT13.[11] When monooxygenase MO2 isolated from *Pseudomonas putida* NCIMB 10007, which is not selective for bicyclic substrates, is used, a mixture of the corresponding lactones with low enantiomeric excess - thus in a different ratio - is obtained.[8]

These results show, besides the enormous potential of these biotransformations as a tool for enantioselective synthesis, the possibility of applying this methodology to obtain interesting regioselective enriched lactones.

In this sense, Sandey *et al.*[12] described the biotransformation of racemic norbornanone **1** with whole-cells of *Pseudomonas* sp. NCIMB 9872 to give two racemic regioisomeric lactones **2** and **3** (Scheme 1). This microbiological alternative has been useful for the synthesis of prostanoid analogs due to its capability of increasing the regioisomeric ratio **2/3** to the desired lactone **2**: 38:1 as opposed to 9:1 obtained from the chemical transformation approach.[13]



(±) 1 (±) 2 (±) 3

Scheme 1.

Later research over the same substrate with *Acinetobacter* TD 63 and *Acinetobacter* NCIMB 9871[14] yielded different ratios closer to those obtained by the chemical approach: 19:1 and 10:1 respectively.

Contemporaneous studies, using whole-cells of *Pseudomonas putida* NCIMB 10007, showed a different behavior depending on the reaction time.[15] Thus, by stopping the reaction when only 60% of the substrate was transformed, only the regioisomer **2** was formed with 53 % ee recovering the unreacted ketone **1** in an optically enriched form with 70% ee. When the reaction was allowed to go to completion the only product recovered was the lactone **2** but only with 13% ee.[15]

These results show the different behaviour when several forms of the same activity are present in a strain; that it is the case of *Pseudomonas putida* NCIMB 10007 where two sets of cyclohexanone monooxigenases - MO1 and MO2 - are known to be present.[16]

In spite of the enormous efforts devoted to exploring the enantio and regioselective feature of the microbiological Baeyer-Villiger oxidation, this biotransformation presents a serious restriction. It has always been achieved in aqueous media, limiting the scope of its applicability to water-soluble substrates.

Multiphase biotransformation systems are presented as an alternative approach for several biotransformations working with poorly water-soluble substrates either using isolated enzymes[17] or whole-cells.[18] It has been proved that they enhance the reaction kinetics and yield of numerous reactions involving reactants with low solubility in water, and for which the organic phase is considered as a storage. With this procedure, high concentrations of substrates can be used. Moreover, the minimization of undesirable side reactions in organic media, substrate and product inhibition, as well as reduction of microbial contamination can be accomplished.[19] The different solubility of reactants and products in both aqueous and organic phases may also help product separation. Increases in the productivity have been assumed to derive from an increase in reactant concentration in the aqueous phase of the system, due to the cosolvency effect of the added organic phase on solubilization of the substrate.[18]

In our aim to extend the applicability of the microbiological Baeyer-Villiger oxidation to poorly soluble water substrates, in this communication we evaluate the viability of carrying out such biotransformation over (±) norbornanone (**1**) with "whole cells" of *Pseudomonas putida* NCIMB 10007 in organic medium as well as the effect on the regioselectivity of the organic solvents assayed.

Results and discussion:

The biotransformation of (±) norbornanone (**1**) using "whole cells" of *Pseudomonas putida* NCIMB 10007 has been performed in n-octane, toluene and n-decanol, as well as in mixtures of these solvents with phosphate buffer. The procedure consists of growing the microorganism in a bassalt medium,[8] containing (±) camphor as a sole carbon source, until a constant Optical Density (OD) was attained and then the cells were harvested by centrifugation and resuspended in three different media:

a. Aqueous systems: consisting of a phosphate buffer to be taken as a reference.

b. Biphasic system: consisting of a mixture of phosphate buffer:immiscible organic solvent 1:1 v/v.

c. Monophasic system: organic solvent.

The results of these biotransformations are given in Table 1. As a reference, chemical Baeyer-Villiger oxidation of racemic norbornanone (**1**) with trifluoroperacetic acid was carried out. After 1h of reaction, a quantitative yield of a mixture of lactones **2** and **3** was isolated. The yield of the lactones was quantified by GC. Because the two regioisomers are not cromatographically distinguishable in the experimental conditions used, **2** + **3** are quantified together. Moreover, the yield of lactones formed in the n-decanol experiments is not given due to the impossibility of finding isolating conditions from the cosolvent in GC.

Enzymatic Baeyer-Villiger	Yield (%)		
	2 + 3	**1**	Reaction time / h
water	100	-	1
water:octane 1:1	100	-	1.5
water:n-decanol 1:1	-	25	10
water:toluene 1:1	100	-	11
octane	96	4	11
n-decanol	-	50	>120
toluene	80	20	11
Chemical Baeyer-Villiger			
CF_3-COOOH	64	-	1

Table 1. Biotransformation of **1** to **2** and **3** by *Pseudomonas putida* NCIMB 10007 and chemical approach.

The biotransformation carried out in the aqueous medium yields, after 1h, a quantitative mixture of the two regioisomers **2** and **3**.

All the experiments carried out on a biphasic system present high yields of transformation but, whereas the presence of n-octane as a cosolvent hardly affects the rate of transformation, obtaining quantitatively the above mixture in 1.5 h, the presence of toluene delays the reaction, reaching a quantitative yield after 11 h. When n-decanol is used as cosolvent, only 75 % of substrate is transformed after 10 h of reaction, this conversion rate remaining unchanged although the reaction is maintained for 120 h.

Results from monophasic systems present important differences on yields and reaction times with respect to biphasic systems. In general, all the biotransformations carried out in a single solvent cause an important delay in the reaction time and, even in some cases, the reaction is stopped. Thus, when n-octane or toluene are used, the reaction is stopped after 11 h, but arriving nearly at completion in the case of n-octane, with 20% of substrate remaining untransformed in the case of toluene. When n-decanol is used, no end of the reaction is observed and the amount of substrate gradually decreases, 50% of substrate remaining unreacted after 120 h of reaction. Therefore, the retarding and even termination of the reaction when cells are resuspended in a single organic solvent seems to show the important role that the amount of water plays in biocatalysis .

With respect to the influence on the regioselectivity of the presence of organic solvents in the biotransformation system, interesting results have been obtained with the solvents assayed (Table 2). The regioisomeric ratio of the lactones obtained in each biotransformation is determined by ^{1}H-NMR integration of relevant signals such as 1-H signal for **2** and 4' endo-H signal for **3** (see Experimental section).

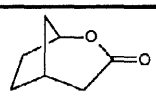
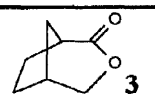
Enzymatic Baeyer-Villiger	 **2**	 **3**
Water	7	1
Water:octane 1:1	7	1
Water-n-decanol 1:1	2	1
Water:toluene 1:1	2	1
Octane	**1**	**0**
Toluene	10	1
Chemical Baeyer-Villiger		
CF_3-COOOH	14	**1**

Table 2. Regioisomeric ratio of lactones **2** and **3.**

Thus, the chemical Baeyer-Villiger oxidation yields the regioisomers **2** and **3** with a ratio of 14:1, favourable towards the isomer resulting from the rearrangement of the α-carbon to the carbonyl group with higher migration feasibility.

The biotransformation approach shows a different behaviour. When the reaction is carried out in a phosphate aqueous system an increase in the chemically unfavoured regioisomer **3** is obtained and the regioisomeric ratio between **2** and **3** is shifted to 7:1. Experiments carried out in biphasic systems work in a singular way. Thus, whereas the presence of n-octane does not affect the proportion achieved in phosphate aqueous system, the use of n-decanol and toluene as cosolvent leads to a higher increase in the proportion of the more chemically unfavoured lactone **3** with a **2/3** ratio of 2:1. Nevertheless, in monophasic systems, the results are contrary to above. Whereas in toluene systems the main lactone obtained is the chemically favoured **2** in a **2/3** ratio of 10:1, when n-octane is used the methine-migrated lactone **2** is exclusively formed.

Summarizing, we have demonstrated that the microbiological Baeyer-Villiger oxidation of racemic Norbornanone **1** using *Pseudomonas putida* NCIMB 10007 can be achieved in the presence of water-immiscible organic solvents with high yields. Nevertheless, the type of solvent and the amount of water used have been shown to play an important role in the biotransformation, even stopping it in some cases. Therefore, they should be carefully selected to get a good biotransformation yield. These results represent a valuable tool in expanding the scope of applicability of such important biotransformation to poorly water-soluble substrates.

In addition, we have demonstrated that the regioisomeric ratio of the lactones obtained changes depending on the type of solvent and the amount of water used. An explanation of it could be found if one takes into account that different organic solvents may affect each enzyme present in the microorganism in different ways.

As a conclusion, the results obtained in a microbiological Baeyer-Villiger oxidation not only depend on the characteristics of the substrate and on the enzyme or whole-cell used, as it was commonly assumed, but also on the type of organic solvent used as cosolvent. Therefore, Solvent Engineering can be applied in microbiological regioselective Baeyer-Villiger oxidation of ketones, extending its applicability.

Experimental section:

FID gas chromatography analyses were done with a Perkin-Elmer Chromatograph equipped with a UV-101 column. The oven temperature program was 85°C for 5 min, 85°C to 160°C ramp at 10°C min^{-1}, 165°C for 2.5 min. The carrier gas was nitrogen (flow rate of 25 cm^3 min^{-1}). The temperature of the injection oven was set at 285°C and that of the detector (FID) at 285°C. IR spectra were recorded on a Perkin-Elmer 683 spectrophotometer. ^{1}H-NMR spectra were recorded on a Varian-Gemini (300 MHz) spectrometer in $CDCl_3$ solutions. Chemical shifts (δ) are given in ppm with Me_4Si as reference and *J* values are given in Hz.

Substrates: (±)-Norbornanone was purchased from Aldrich Chemical. Its lactones, 2-oxabicyclo [3.2.1] octan-3-one (**2**) and 3-oxabicyclo [3.2.1] octan-2-one (**3**) were prepared by a Baeyer-Villiger oxidation with trifluoroperacetic acid: to a stirred solution of ketone **1** (1.1g, 0.01 mol) in dry $CHCl_3$ (20 cm^3) at 0° C in innert atmosphere was added cautiously in a dropwise fashion a solution of trifluoroperacetic acid (42 cm^3, 0.16 mol) generated *in situ* from trifluoroacetic anhydride (26 cm^3, 0.19 mol) and H_2O_2 30% (16 cm^3, 0.16 mol). The mixture was stirred for 1 h and, after complete reaction, was stirred vigorously with 10% aqueous sodium tiosulphate for up to half hour. Then, the solution was washed with aqueous $NaHCO_3$ until neutral, separated and the aqueous layer extracted with $CHCl_3$ (4 x 200 cm^3). The combined extracts were washed with brine and dried ($MgSO_4$) and evaporated under reduced pressure to afford the lactone mixture **2** + **3** (0.80g, 64%). ν_{max}(film)/cm^{-1} 2920, 1730, 1370, 1220, 1190, 1120 and 1060; δ_H (300 Mhz; $CDCl_3$) 4.87 (1H, br s, 1-H), 4.32 (1H, ddd, *J* 10.5, 3.0 and 1.2, 4'endo-H), 4.12 (1H, ddd, *J* 10.5, 1.8 and 1.2, 4'exo-H), 2.92 (1H, br s, 1'-H), 2.73 (1H, ddd, *J* 18.3, 4.8 and 2.1, 4endo-H), 2.49 (1H, ddd, *J* 18.3, 2.1 and 2.1, 4exo-H). The

isomeric lactones ratio was based on integration of 1-H signal (methine-migrated lactone **2**) to 4'endo-H (methylene-migrated lactone **3**).

Maintenance and growth of the microorganisms: *Pseudomonas putida* NCIMB 10007 was provided by National Culture of Industrial and Marine Bacteria, Aberdeen, UK. Stock cultures were grown on nutrient agar slopes at 28°C and stored at 4°C. Before transformation the organism was routinely grown in a basal salts medium previously described;[8] (±)-camphor (2.5 g dm^{-3}) was added prior to autoclaving the medium at 15 psi for 20 min. A 100 cm^3 culture of bacteria was inoculated from a slope and after 24h of growth at 28°C in an orbital shaker (150 rpm) was transferred to 1 dm^3 of medium. Growth of the microorganism was monitored by taking 2 cm^3 aliquots of growing cell suspension and reading the absorbance at 490 nm on a Jenway 6050 Colorimeter.

General procedure for the biotransformation using whole-cells of *Pseudomonas putida* NCIMB 10007:*Pseudomonas putida* NCIMB 10007 inoculuum was grown as described earlier until an OD of 0.9 at 490 nm was obtained. Then the cells were harvested by centrifugation (30 min, 700 rpm, 15°C) and resuspended in the transformation medium.

a. Reference: 50 cm^3 of phosphate buffer (0.1 M).

b. Biphasic systems: 50 cm^3 of organic solvent:phosphate buffer (0.1M) 1:1 v/v.

c. Monophasic systems: 50 cm^3 of the solvent chosen .

In all cases 50 mg of (±)-norbornanone **(1)** was dissolved (1mg cm^{-3}).

Transformation conditions were 325 rpm and 30°C. The biotransformation was monitored by periodic sampling of aliquots (5 cm^3) which were extracted with $CHCl_3$, solvent removed and diluted with 5 cm^3 of $CHCl_3$ to inject into the GC analyses.

Acknowledgements:

This work was supported by the Comissió Interdepartamental de Recerca i Innovació Tecnológica (CIRIT. QFN95-4716).

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