Stereospecific Biohydroxylations of Protected Carboxylic Acids with *Cunninghamella blakesleeana*

M. KREINER,¹ G. BRAUNEGG,^{1*} A. DE RAADT,² H. GRIENGL,² I. KOPPER,¹ M. PETSCH,²
P. PLACHOTA,² N. SCHOO,² H. WEBER,² AND A. ZEISER¹

Institute of Biotechnology¹ and Institute of Organic Chemistry,² Spezialforschungsbereich F01 Biocatalysis, Technical University Graz, 8010 Graz, Austria

Received 1 November 1995/Accepted 11 April 1996

Cunninghamella blakesleeana DSM 1906 was found to be an efficient biocatalyst for the biotransformation of cycloalkylcarboxylic acids into hydroxy and oxo derivatives. When cultivated in submerged culture, the fungus grew in pellets. In comparison with malt extract-glucose-peptone-yeast extract medium (medium E), Czapek-Dox medium was found to reduce pellet size. Cycloalkylcarboxylic acids were protected against microbial degradation by chemical transformation into 2-cycloalkyl-1,3-benzoxazoles. The transformations of protected cyclopentyl-, cyclohexyl-, cycloheptyl-, and cyclooctylcarboxylic acids by *C. blakesleeana* were investigated. The biotransformations were performed in medium E by using an aerated, stirred-tank bioreactor. The transformation of 2-cyclopentyl-1,3-benzoxazole yielded (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol as the main product. The main by-product was (1*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-one, and 2-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol was also obtained in small amounts. During the experiment, the enantiomeric excess of the main product increased up to 64%. 2-Cyclohexyl-1,3-benzoxazole was hydroxylated to 4-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol. 2-Cycloheptyl-1,3-benzoxazole and 2-cyclooctyl-1,3-benzoxazole were transformed into several alcohols and ketones, all in low yields (2 to 19%).

The hydroxylation of nonactivated carbon atoms is a difficult task. Only a few chemical methods have been reported recently (1, 2). The regio- and stereospecificity of such reactions have not been thoroughly investigated to date. Therefore, biohydroxylation is a promising alternative. Among the classes of compounds which have been extensively investigated are the commercially important steroids and terpene substrates (12, 14). In general, biohydroxylations play a cardinal role in the detoxification pathways of cells. These aspects are discussed within the concept of “microbial models of mammalian metabolism” proposed by Rosazza and Smith (20).

A drawback of microbial hydroxylation is its limited predictability. Attempts to elucidate the hydroxylating systems in specific microorganisms are being made. Active-site models for hydroxylation of monoterpenes by *Bacillus cereus* (15), steroids by *Calonectria decora* (3), and amides by *Beauveria bassiana* (9) have been developed. On the other hand, substances like carboxylic acids, ketones, and alcohols are often completely degraded by the microorganisms (11, 21). A new strategy using reversibly introduced anchor and/or protecting groups for different functionalities (6, 7, 8) is discussed in this paper. Such groups protect the substances from consumption and may provide a special target for many microorganisms. Cycloalkylcarboxylic acids were used as substrates for *Cunninghamella blakesleeana* in the form of 2-cycloalkyl-1,3-benzoxazoles.

Biotransformations can be performed either with isolated enzymes or with whole cells. Working with whole cells has the advantage of producing sufficient amounts of the biocatalyst in a comparatively easy and fast manner. In addition, required cosubstrates are recycled in the cells. This is important, since for *C. blakesleeana* the enzyme system responsible for the presented hydroxylation reactions is not known. On the other

hand, secondary reactions of the hydroxylation product generally result in lower yields and complicate downstream processing.

In order to find microorganisms that accept the 2-cycloalkyl-1,3-benzoxazoles as substrates for hydroxylation, strains previously reported to hydroxylate steroids, terpenes, and xenobiotics (12, 14, 20) were screened. *C. blakesleeana* was found to be a promising microorganism for the hydroxylation of the protected carboxylic acids, as presented in this work.

MATERIALS AND METHODS

Strain and cultivation. *C. blakesleeana* DSM 1906 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. Stock cultures of the organism were maintained on potato-dextrose (PD) agar slants, stored at 4°C, and subcultured every 3 weeks at 30°C.

Media. Medium E consisted (per liter) of 15 g of malt extract (Merck), 10 g of glucose, 5 g of peptone (Merck), and 2 g of yeast extract (Oxoid). Modified medium E1 was prepared (per liter) with 20 g of malt extract, 10 g of glucose, 5 g of peptone, and 1 g of yeast extract. Modified medium E2 was made (per liter) of 5 g of malt extract, 10 g of glucose, 2 g of peptone, and 2 g of yeast extract. Czapek-Dox medium, used for precultures, contained (per liter) 2 g of NaNO₃, 1 g of KH₂PO₄, 0.5 g of MgSO₄ · 7H₂O, 0.5 g of KCl, 0.1 g of Fe(III)-NH₄-citrate, 20 g of glucose, and 1 g of yeast extract (Oxoid). PD broth (PDB) was obtained from Difco. All other chemicals were obtained from Merck (per analysis quality). Media were sterilized at 121°C for 30 min. In order to avoid precipitates, C and N sources were sterilized separately.

Experimental determination of the optimum time for the addition of substrate. Biotransformations were performed in 12 shake flasks (1 liter) containing modified medium E1 (250 ml) at 30°C with agitation at 140 rpm. Each flask was inoculated with *C. blakesleeana* (10 ml) previously grown in modified medium E1 for 2 days. After 10.5, 20, 31, and 44 h of growth, 650 µl of an ethanolic solution of the substrate 2-cyclopentyl-1,3-benzoxazole was added to the flasks in three different concentrations (200, 400, and 1,000 mg liter⁻¹).

Fermentor and fermentation procedure. The biotransformations were performed in a Bioengineering AG model 1523 fermentor (Bioengineering, Wald, Switzerland) with a 16-liter stainless-steel vessel with four baffles and a working volume of 11 liters. Agitation was provided by two Rushton impellers. The pH was measured with a combined glass electrode (Ingold, Urdorf, Switzerland), and the temperature was determined with a Pt-100 sensor. Dissolved oxygen was measured with a dissolved-oxygen electrode (Ingold). The assembled vessel, containing glucose, malt extract, and 1 ml of polypropylene glycol 2000, was sterilized in situ with saturated steam at 121°C for 30 min. Yeast extract and

* Corresponding author. Mailing address: Institute of Biotechnology, Technical University Graz, Petersgasse 12, 8010 Graz, Austria. Phone: 43-316-873-8412. Fax: 43-316-8434.

peptone were sterilized separately and added aseptically. During the fermentation, the temperature was kept at 30°C, agitation was at 320 rpm, and dissolved oxygen was automatically controlled at 50% of air saturation. The inoculum was prepared by adding a spore suspension (8 ml) from an agar slant containing a culture grown for 4 days. This spore suspension was aseptically added to a 1-liter shake flask containing Czapek-Dox medium (250 ml). After 24 h of growth at 30°C on a rotating (140 rpm) shaker (Infors AG, Bottmingen, Switzerland), 1 liter was used as the inoculum for the fermentor. After 2 days of growth in the fermentor, an ethanolic solution of 2-cycloalkyl-1,3-benzoxazole (1 ml of ethanol liter of medium⁻¹) was added aseptically, giving a final concentration of 340 mg liter⁻¹ (2-cyclopentyl-1,3-benzoxazole) or 320 mg liter⁻¹ (2-cyclohexyl-, cycloheptyl-, and 2-cyclooctyl-1,3-benzoxazole).

Analytical methods. For determination of the cell dry mass, 10 ml of culture broth was centrifuged (10 min at 3,220 × g, 4°C) in preweighed polypropylene test tubes. The supernatant was saved for other analysis. After washing of the pellets with physiological NaCl solution (0.9%), they were lyophilized and weighed. The glucose concentration in the supernatant was measured by the hexokinase method (Dipro, Wiener Neudorf, Austria). Complex nitrogen sources in the supernatant were determined by the Folin method (5). For the determination of the total amount of reducing sugars, the DNS method was used (17). Concentrations of 2-cycloalkyl-1,3-benzoxazoles and metabolites were determined by gas chromatography (GC). Supernatant (5 ml) was extracted with ethyl acetate (2 ml), and the organic phase was transferred to GC vials for the autosampler (HP 7673; Hewlett-Packard). GC was performed on an HP 5890 Series II Plus gas chromatograph, equipped with a deactivated empty capillary (5 m by 0.32 mm) as a retention gap, an HP-5 analytical column (25 m by 0.32 mm by 0.52 µm), and a flame ionization detector. Helium, flowing at 1.5 ml min⁻¹, acted as the carrier with a split of 1:20. The oven temperature was kept at 170°C for 1 min and then raised to 220°C at a rate of 5°C min⁻¹. After 1 min, the temperature was increased to 250°C (10°C min⁻¹). The final phase was 3 min at 250°C. The detector and injector temperatures were 250 and 210°C, respectively. Prior to the chromatography, 2-cycloalkyl-1,3-benzoxazole metabolites were identified by nuclear magnetic resonance. Enantiomeric excess (e.e.), defined as percent excess of the major enantiomer in relation to the minor enantiomer, was determined by chiral high-pressure liquid chromatography (HPLC) as described by de Raadt et al. (7). The determination of the absolute configurations of the products is described by de Raadt et al. (6).

Synthesis of substrates and cleavage of the anchor and/or protecting group. Synthesis of 2-cycloalkyl-1,3-benzoxazoles and cleavage of the anchor and/or protecting groups are described by de Raadt et al. (7, 8).

Adsorption of substrate and products. *C. blakesleeana* was grown in 1-liter shake flasks with baffles containing 250 ml of modified medium E2 at 30°C and 135 rpm. After 45 h of growth, the cultures were combined and aseptically distributed into 16 300-ml shake flasks with baffles to give 70 ml of culture in each flask. A 150-µl volume of an ethanolic substrate (2-cyclopentyl-, 2-cyclohexyl-, 2-cycloheptyl-, or 2-cyclooctyl-1,3-benzoxazole) solution was added to each flask to give a starting substrate concentration of 280 mg liter⁻¹. At 1 min, 4 h, 23 h, and 49 h after substrate addition, the contents of two flasks were extracted. The total content of one flask (pellets and liquid phase) was extracted twice with 30 ml of ethyl acetate. The pellets of a second flask were separated by a sieve (stainless steel; mesh width, 1.5 mm). After filtration, the aqueous phase was extracted twice with ethyl acetate (30 ml). The separated pellets were freeze-dried, ground, and extracted three times with ethyl acetate (once with 10 ml and twice with 5 ml). The organic phases were combined. Substrate and product concentrations were measured by GC.

RESULTS

Choice of the fungal strain. A screening of 44 microorganisms, mostly fungi, indicated that several microorganisms have the ability to transform 2-cycloalkyl-1,3-benzoxazoles. Further work indicated that the following organisms hydroxylated 2-cyclopentyl-1,3-benzoxazole with a reasonable yield: *Bacillus megaterium* CCM 2037 and DSM 32, *C. blakesleeana* DSM 1906, *Mortierella alpina* ATCC 8979, *Rhizopus stolonifer* DSM 907, *Curvularia lunata* CBS 11534, and *Chaetomium cochlioides* DSM 831. From these microorganisms, *C. blakesleeana* DSM 1906 was chosen for further investigations.

Time of 2-cycloalkyl-1,3-benzoxazole addition and optimum concentration of 2-cycloalkyl-1,3-benzoxazole. *C. blakesleeana* formed pellets (up to 8 mm in diameter) when grown in modified medium E1. When the substrate was added after 10.5 h of growth, neither the main nor the secondary hydroxylation products were detected. However, the substrate concentration decreased within the sampling time, indicating that the substrate was adsorbed to the surface of the pellets. When the substrate was added 20 h after inoculation, small amounts of

FIG. 1. Biohydroxylation of 2-cyclopentyl-1,3-benzoxazole with *C. blakesleeana*. Shown is the time course of formation of the main product, (1S,3S)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol, as a function of time of substrate addition and substrate concentration. (A) Substrate addition after 20 h of growth. (B) Substrate addition after 31 h of growth. (C) Substrate addition after 44 h of growth.

product were formed (Fig. 1A). Hydroxylation did not start until an additional 12 h had passed. At this point, the culture was 32 h old. With substrate addition after 31 h (Fig. 1B), biohydroxylation started immediately after addition of 200 mg of substrate liter⁻¹. Main-product formation peaked at approximately 169 mg liter⁻¹. With 400 mg and 1 g of substrate liter⁻¹, only small amounts of the measured product were formed after a lag phase of about 40 h. Figure 1C summarizes the biotransformation with the substrate addition 44 h after inoculation; 400 mg of substrate liter⁻¹ led to a maximum

amount of product, but only after a 40-h period of very slow product formation. The 200 mg of substrate liter⁻¹ was transformed much faster to the hydroxylated product, which was, as usual, partially oxidized to the corresponding ketone. One gram of substrate liter⁻¹ resulted again in hardly any hydroxylation.

From this, it can be concluded that *C. blakesleeana* becomes less sensitive to the substrate with increasing age of the culture. Metabolic changes as the culture ages may account for this, as may a lower substrate-to-biomass ratio when more biomass is present at the time of substrate introduction.

Preparation of the inoculum. In shake flask experiments, *C. blakesleeana* was found to be able to generate pellets with diameters up to 8 mm. Medium E tended to maximize the pellet size. Inoculating Czapek-Dox medium with the same amount of spores as used for medium E led to an increased number of smaller pellets. In PDB the size of the pellets was intermediate between those of pellets from medium E and Czapek-Dox medium. Pellets in Czapek-Dox medium looked fluffy, whereas they were compact in PDB and medium E. Consequently, Czapek-Dox medium was used for growing *C. blakesleeana* as an inoculum for fermentation cultures. In the fermentor, however, medium E was preferred for the cultivation of *C. blakesleeana*, because biotransformation of the substrate seemed to be maximized under these conditions. Higher shear forces in the fermentor probably helped to keep the pellet size down.

Biotransformation of 2-cycloalkyl-1,3-benzoxazoles in the bioreactor. The transformations of 2-cyclopentyl-1,3-benzoxazole, 2-cyclohexyl-1,3-benzoxazole, 2-cycloheptyl-1,3-benzoxazole, and 2-cyclooctyl-1,3-benzoxazole with *C. blakesleeana* DSM 1906 were investigated. Since 2-cyclopentyl-1,3-benzoxazole was the most intensively investigated substrate, most of the data shown result from experiments with this compound.

Figures 2A and B show the consumption of the sugars and complex nitrogen sources and the increase in biomass. In addition, the change in pH during the fermentation with 2-cyclopentyl-1,3-benzoxazole is presented. After a short lag phase, sugars and complex nitrogen sources started to be consumed. 2-Cycloalkyl-1,3-benzoxazole was added after 44 h in the late exponential phase, when a cell dry mass of 12 g liter⁻¹ had been reached. The addition of substrate was followed by a short phase of stagnation in glucose consumption. The complex nitrogen sources were consumed at a slower rate after the addition of substrate. After the apparent complete consumption of sugars, there was a rapid increase in pH. The culture behaviors of *C. blakesleeana* seemed to be similar after the addition of every 2-cycloalkyl-1,3-benzoxazole tested.

The time course of the transformation of 2-cyclopentyl-1,3-benzoxazole is presented in Fig. 2C. The substrate concentration decreased very fast. (1*S*,3*S*)-3-(Benz-1,3-oxazol-2-yl)cyclopentan-1-ol was formed at up to 320 mg liter⁻¹ within the first 10 h after substrate addition. The e.e. of the alcohol increased up to 65%, which would be explained by the further stereoselective oxidation of this alcohol to the corresponding ketone. The e.e. of the corresponding ketone was almost constant during the fermentation. The hydroxylation was not totally regioselective. Therefore, small amounts of 2-(benz-1,3-oxazol-2-yl)-cyclopentan-1-ol were also found as by-products.

Chiral HPLC allowed a convenient separation of the enantiomers of the products formed from 2-cycloalkyl-1,3-benzoxazoles. Figure 3 shows chromatograms of the products in samples taken at different times during the fermentation.

Figure 4 shows the time course of the biotransformation of 2-cyclohexyl-1,3-benzoxazole. Again, the substrate concentration decreased rapidly because of adsorption on the cells. The

FIG. 2. (A) Consumption of complex nitrogen sources (CNS), total amount of reducing sugars, and glucose. (B) Cell dry mass (CDM) and behavior of pH. (C) Time course of substrate consumption, product formation, and the e.e.'s of the two main products during fermentation of *C. blakesleeana* transforming 2-cyclopentyl-1,3-benzoxazole. Symbols (C): ●, 2-cyclopentyl-1,3-benzoxazole; ■, 2-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol; ▲, (1*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol; ▼, (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol; △, e.e. of (1*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol; ▽, e.e. of (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol.

main product was 4-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol. Compared with the production of the main hydroxy product of the 5-carbon ring (Fig. 2C), the concentration of 4-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol increased slowly, but even 50 h after the addition of substrate, there was a slight increase in product concentration. The yield (38%) of this hydroxylated product is low in comparison with that achieved with the metabolites of the 5-carbon ring (Table 1). In addition to 4-(benz-1,3-oxazol-2-yl)-cyclohexan-1-ol, traces of a ketone were produced.

With 2-cycloheptyl-1,3-benzoxazole, poor product yields were obtained (Table 1). Figure 5 presents the time course of the formation of the main products 4-(benz-1,3-oxazol-2-yl)cycloheptan-1-ol and the corresponding ketone. The substrate concentration decreased rapidly, because of adsorption, and the products were formed within the first 5 h after substrate addition. Small amounts of a second alcohol, 2-(benz-1,3-oxazol-2-yl)cycloheptan-1-ol, were detected.

Concerning overall yields, the transformation of the 2-cyclooctyl-1,3-benzoxazole was similar to that of the 7-carbon

FIG. 3. Chiral HPLC elution profile of metabolites formed from 2-cyclopentyl-1,3-benzoxazole by *C. blakesleeana*. Samples were taken 1, 14, 28.5, and 58 h after substrate addition.

ring, and the substrate concentration decreased as swiftly as with the other substrates. However, product concentrations were even lower with this substrate. Yields of isolated products were between 2 and 3% (Table 1). The main product was 4-(benz-1,3-oxazol-2-yl)cyclooctan-1-one, which was produced within the first 7 h after substrate addition. Afterwards, its concentration declined slightly. Five hours after substrate addition, 4-(benz-1,3-oxazol-2-yl)cyclooctan-1-ol was found in the culture broth. After another 2 h, traces of a second ketone were detected.

Adsorption of substrate and products. At the beginning of the experiment, 230 mg of 2-cyclopentyl-1,3-benzoxazole liter⁻¹ was found when both pellets and nutritional medium were extracted. When the extraction was done separately for

FIG. 4. Transformation of 2-cyclohexyl-1,3-benzoxazole with *C. blakesleeana*. Shown are the time courses of substrate consumption and formation of the main product. ●, 2-cyclohexyl-1,3-benzoxazole; ■, 4-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol.

pellets and the liquid phase, the substrate concentration in the pellets (23 mg liter⁻¹) was only about 8% of the concentration found in the liquid phase. After 4 h, the situation had changed completely: now the substrate concentration in the pellets (117 mg liter⁻¹) was three times higher than the substrate concentration in the filtrate (40 mg liter⁻¹), and the same partitioning was found after 23 h. At 49 h after substrate addition, all of the residual substrate was measured in the pellets. This behavior strongly indicates that the substrate was adsorbed on the cells. The main product (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol was found almost exclusively in the liquid phase; only 3 to 6% was found in the pellets. Comparable data were found for the secondary product (1*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-one (5 to 8% in the pellets). There seems to be hardly any adsorption of the products on the biomass, which can be explained by the higher water solubility of the products relative to the substrate. Similar results were found for 2-cyclohexyl-, 2-cycloheptyl-, and 2-cyclooctyl-1,3-benzoxazole.

DISCUSSION

C. blakesleeana is an appropriate system for the biohydroxylation of protected carboxylic acids because it is a versatile and rapidly growing fungus that transforms the substrates with good yields into hydroxylated compounds.

In submerged cultures, *C. blakesleeana* formed pellets, a major advantage of which is the significant decrease in viscosity in comparison with that of a filamentous suspension. Small pellets are desired (13, 16) to minimize limitations of diffusion of oxygen or other nutrients into the interior parts of the pellets and maximize active surface. Pellet formation and size depend, among other factors, on the fungal strain, the medium employed, the agitation speed, and the inoculum (22). For *C. blakesleeana*, the change from a rich, complex medium to a glucose and mineral salt medium, containing 1 g of yeast extract liter⁻¹ only, decreased the diameter of the pellets by more than 50%.

TABLE 1. Products of transformations of 2-cyclopentyl-, 2-cyclohexyl-, 2-cycloheptyl-, and 2-cyclooctyl-1,3-benzoxazole with *C. blakesleeana*, yields, and e.e.'s

Substrates:					
Product ^a	Time [h] ^b	Yield [%] ^c (e.e. [%])	Product ^a	Time [h] ^b	Yield [%] ^c (e.e. [%])
 (1 <i>S</i> ,3 <i>S</i>)-3-R1-cyclopentan-1-ol	25 58	83 (40) 47(64)	 4-R1-cycloheptan-1-one	25	13 (19)
 (1 <i>R</i>)-3-R1-cyclopentan-1-one	25 58	17 21(68)	 4-R1-cycloheptan-1-ol	25	19
 2-R1-cyclopentan-1-ol	25 58	2 0	 2-R1-cycloheptan-1-ol	25	1
 4-R1-cyclohexan-1-ol	54	38	 4-R1-cyclooctan-1-ol	25	2
			 4-R1-cyclooctan-1-one	25	3
^aR1 = ^bTransformation time ^cOverall yields calculated from GC data					

The transformation of 2-cyclopentyl-1,3-benzoxazole yielded (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol as the main product. The enzyme involved seems to act almost regioselectively, as the second hydroxylated product, 2-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol, was found in traces only. The ratio of 2-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol to (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol was 1:40. (1*S*,3*S*)-3-(Benz-1,3-

oxazol-2-yl)cyclopentan-1-ol was probably oxidized to the corresponding ketone. In addition to the oxidation to the ketone, another reaction with (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol must have occurred, because its concentration decreased faster than the ketone concentration increased, especially when the sugars in the medium were exhausted (Fig. 2).

The e.e. of the hydroxylated compound increased, probably

FIG. 5. Transformation of 2-cycloheptyl-1,3-benzoxazole with *C. blakesleeana*. Shown are the time courses of substrate consumption and formation of the two main products. ●, 2-cycloheptyl-1,3-benzoxazole; ●, 4-(benz-1,3-oxazol-2-yl)cycloheptan-1-one; ■, 4-(benz-1,3-oxazol-2-yl)cycloheptan-1-ol.

because of the stereoselective oxidation of (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol to the corresponding ketone. As a consequence, the overall yield and e.e. of (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol evolved in inverse proportion to one another. This may enable one to choose between rapid production of large amounts of low-e.e. product and slower production of smaller quantities of high-e.e. protected hydroxycarboxylic acids.

The yield of 4-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol, the main product of the transformation of the 6-carbon ring substrate, was lower than the yield of the main product of the 5-carbon ring substrate (38 versus 83% [Table 1]). In addition, its initial production rate was lower (Fig. 4). One explanation could be further degradation reactions, resulting in simultaneous synthesis and degradation of the hydroxylated compound.

2-Cycloheptyl-1,3-benzoxazole and 2-cyclooctyl-1,3-benzoxazole were transformed into several alcohols and ketones, all with low yields. These results are possibly the consequence of the greater flexibility of the cycloheptyl and cyclooctyl systems. More carbon atoms present more potential targets for hydroxylation. Additionally, these ring systems can show a large number of conformations. Therefore, the hydroxylation reactions do not proceed as regio- or stereoselectively.

Comparison of transformation results for the four protected cycloalkylcarboxylic acids reveals that hydroxylation occurs mainly on the carbon atoms *trans* to the benzoxazole moiety and that the overall yields of products decrease with increasing ring size. A possible explanation for the decreasing yields of products could be a further degradation of the hydroxylated compounds. The decreasing recovery rates strengthen this assumption. When the nutritional medium and pellets were separately extracted 1 min after substrate addition, the total amount of substrate and product obtained accounted for 80 mol% of the total mass of 2-cyclopentyl-1,3-benzoxazole originally added. After 49 h only 62 mol% was recovered. For the 6-carbon substrate the molar recovery rate decreased from 90 to 73 mol% during the same period of time. For 2-cycloheptyl-

1,3-benzoxazole the situation was similar. A decrease in the recovery rate from 78 mol%, 4 h after substrate addition, to 53 mol% after 49 h was found. In microbial metabolism, hydroxylation is an important step in the detoxification of xenobiotics, providing a tool for improving water solubility of such compounds. At least three further reaction pathways are possible. The oxidation of the alcohol to the ketone indicates that the next step could be a lactonization of the ketone, followed by a ring fission (21). Another possible reaction for detoxification is the conjugation of the alcohol with sulfate or with a sugar compound. Cerniglia et al. (4) and Pothuluri et al. (19) identified glucopyranoside, glucuronide, and sulfate conjugates during the metabolism of polycyclic aromatic hydrocarbons by *Cunninghamella elegans*. During the transformation of the steroid lithocholic acid by *C. blakesleeana* ST-22, a glucose-conjugated metabolite was found (18). A third possible means of degradation is attack on the aromatic ring, of which *C. elegans* is capable as well (10).

Adsorption of the substrates seems to be a common phenomenon. With all compounds, the concentration of 2-cycloalkyl-1,3-benzoxazole in broth decreased rapidly to almost zero, whereas the products were formed gradually at fermentation times when the substrate concentration in the medium remained constant.

As our results show, *C. blakesleeana* is able to hydroxylate 2-cycloalkyl-1,3-benzoxazoles with a ring size of 5 to 8 carbon atoms, allowing the hydroxylation of 83% of the pentane benzoxazole derivative and 38% of the hexane derivative. The 7- and 8-carbon rings were hydroxylated in the range of 2 to 19%. Therefore, *C. blakesleeana* is a useful system for the production of cyclic hydroxycarboxylic acids.

ACKNOWLEDGMENTS

Funding within the framework of the European Community Program Human Capital and Mobility, project Biooxygenations, contract no. ERB CHRXCT 930259, by the Christian Doppler Society and Chemie Linz GmbH is gratefully acknowledged.

We also thank Gilles Lefebvre and Paul Ira Keyser for aid with the revision of the manuscript.

REFERENCES

- Barton, D. H. R., and D. Döller. 1991. The selective functionalisation of saturated hydrocarbons: Gif and all that. *Pure Appl. Chem.* **63**:1567–1576.
- Barton, D. H. R., and D. Döller. 1992. The selective functionalisation of saturated hydrocarbons: Gif chemistry. *Acc. Chem. Res.* **25**:504–512.
- Bell, A. M., P. C. Cherry, I. M. Clark, W. A. Denny, E. R. H. Jones, G. D. Meakins, and P. D. Woodgate. 1972. Microbial hydroxylation of steroids. IV. The pattern of dihydroxylation of mono-oxygenated 5 α -androstanes with cultures of the fungus *Calonectria decora*. *J. Chem. Soc. Perkin Trans.* **2**:2081–2095.
- Cerniglia, C. E., G. L. White, and R. H. Heflich. 1985. Fungal metabolism and detoxification of polycyclic aromatic hydrocarbons. *Arch. Microbiol.* **143**:105–110.
- Clark, J. M., Jr., and R. L. Switzer. 1977. *Experimental biochemistry*, 2nd ed. W. H. Freeman & Co., San Francisco.
- de Raadt, A., H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunnegg, I. Kopper, M. Kreiner, and A. Zeiser. 1996. Microbial hydroxylation of 2-cycloalkyl-benzoxazoles. II. Determination of product structures and enhancement of enantiomeric excesses. *Tetrahedron Asymmetry* **7**:473–490.
- de Raadt, A., H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunnegg, I. Kopper, M. Kreiner, and A. Zeiser. 1996. Microbial hydroxylation of 2-cycloalkyl-benzoxazoles. III. Determination of product enantiomeric excesses and cleavage of benzoxazoles. *Tetrahedron Asymmetry* **7**:491–496.
- de Raadt, A., H. Griengl, M. Petsch, P. Plachota, N. Schoo, H. Weber, G. Braunnegg, I. Kopper, M. Kreiner, A. Zeiser, and K. Kieslich. 1996. Microbial hydroxylation of 2-cycloalkylbenzoxazoles. I. Product spectrum obtained from *Cunninghamella blakesleeana* DSM 1906 and *Bacillus megaterium* DSM 32. *Tetrahedron Asymmetry* **7**:467–472.
- Furstoss, R., A. Archelas, J. D. Fourneron, and B. Vigne. 1986. Biohydroxylation of non activated carbon atoms. A model for the hydroxylation site of

- the fungus *Beauveria sulfurescens*, p. 361–370. In M. P. Schneider (ed.), *Enzymes as catalysts in organic chemistry*. Reidel, Dordrecht, The Netherlands.
10. **Gibson, D. T., and V. Subramanian.** 1984. Microbial degradation of aromatic hydrocarbons, p. 181–251. In D. T. Gibson (ed.), *Microbial degradation of organic compounds*, vol. 13. Marcel Dekker, Inc., New York.
  11. **Hasegawa, Y., H. Obata, and T. Tokuyama.** 1984. Microbial degradation of cyclomonocarboxylic acids and cyclomonoketones by *Pseudomonas*. *J. Ferment. Technol.* **62**:87–91.
  12. **Holland, H. L.** 1992. *Organic synthesis with oxidative enzymes*. VCH Publishers, Inc., New York.
  13. **Hotop, S., J. Möller, J. Niehoff, and K. Schügerl.** 1993. Influence of the preculture conditions on the pellet size distribution of *Penicillium chrysogenum* cultivations. *Process Biochem.* **28**:99–104.
  14. **Kieslich, K.** 1984. Biotransformations, p. 1–473. In H. J. Rehm and G. Reed (ed.), *Biotechnology*, vol. 6a. Verlag Chemie, Weinheim, Germany.
  15. **Liu, W.-G., A. Goswami, R. P. Steffek, R. L. Chapman, F. S. Sariaslani, J. J. Steffens, and J. P. N. Rosazza.** 1988. Stereochemistry of microbiological hydroxylations of 1,4-cineole. *J. Org. Chem.* **53**:5700–5704.
  16. **Metz, B., and N. W. F. Kossen.** 1977. The growth of molds in the form of pellets—a literature review. *Biotechnol. Bioeng.* **19**:781–799.
  17. **Miller, G. L.** 1959. Standard methods of the biochemical engineering group of the University of Waterloo. *Anal. Chem.* **31**:426.
  18. **Nihira, T., Y. Okada, and Y. Yamada.** 1992. A new bile acid glycoside produced by *Cunninghamella blakesleeana* ST-22 from lithocholic acid. *J. Ferment. Bioeng.* **74**:191–193.
  19. **Pothuluri, J. V., R. H. Heflich, P. P. Fu, and C. E. Cerniglia.** 1992. Fungal metabolism and detoxification of fluoranthene. *Appl. Environ. Microbiol.* **58**:937–941.
  20. **Rosazza, J. P., and R. V. Smith.** 1979. Microbial models for drug metabolism. *Adv. Appl. Microbiol.* **25**:169–208.
  21. **Trudgill, P. W.** 1984. Microbial degradation of alicyclic ring structural relationships and metabolic pathways, p. 131–180. In D. T. Gibson (ed.), *Microbial degradation of organic compounds*, vol. 13. Marcel Dekker, Inc., New York.
  22. **Whitaker, A., and P. A. Long.** 1973. Fungal pelleting. *Process Biochem.* **11**:27–30.