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Microbial Hydroxylation of 2-Cycloalkylbenzoxazoles. Part I. Product Spectrum Obtained from *Cunninghamella blakesleeana* DSM 1906 and *Bacillus megaterium* DSM 32

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Abstract: 2-Cycloalkyl-1,3-benzoxazoles and -thiazoles (ring sizes C-3 to C-8) were biotransformed using the title microorganisms. Products of preparative importance were (1*S*,3*S*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-ol **6**, (1*R*)-3-(benz-1,3-oxazol-2-yl)cyclopentan-1-one **8**, (1*R*,2*R*)-2-(benz-1,3-oxazol-2-yl)cyclohexan-1-ol **14** and the corresponding cycloheptanol and cycloheptanone derivatives.

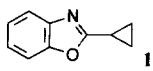
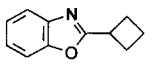
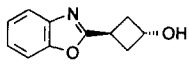
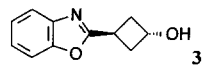
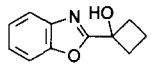
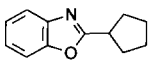
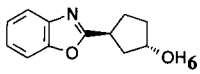
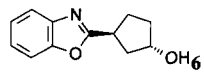
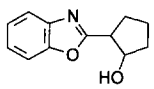
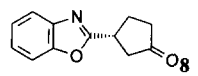
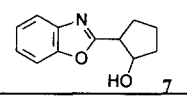
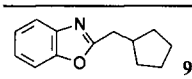
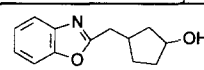
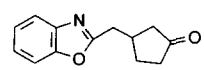
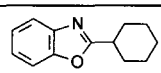
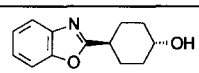
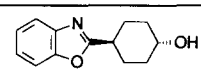
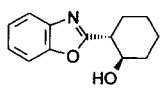
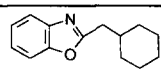
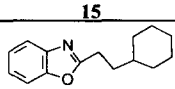
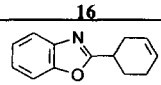
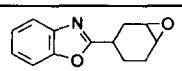
INTRODUCTION

The regio- and stereoselective introduction of oxygen into an unactivated carbon-hydrogen bond still poses an unresolved problem[1,2] in the field of chemical synthesis. In the last decade several chemical hydroxylation systems have been developed, which still need some further refinement.[3-10] Apart from these systems microbial hydroxylation is an often used tool to introduce a hydroxyl group into a hydrocarbon.[11-14] This approach is most prominent in the field of steroids and terpenes.[15,16]

We were looking for a general method to carry out this reaction with different classes of low-molecular weight compounds to generate products which might be useful in asymmetric synthesis. Therefore a special concept of anchor and protecting groups was developed. It is known from previous studies that with certain microorganisms hydroxylation in substrates carrying functional groups like benzamides and urethanes always occurs in a specified distance from these "anchoring" substituents.[12] By introducing groups like these it should be possible to lower the number of potential hydroxylation sites in a given substrate to some extent and thereby enhance regio- and maybe also stereoselectivity. The second role that this group has to fulfill is the role of a protective group which is well known in organic chemistry. The compounds we were examining were either susceptible to hydrolytic or oxidoreduction reactions, which could be prevented by protection, or as in the case described here, were very polar and might not enter the microbial cells readily. Herein we want to describe the hydroxylations obtained for differently substituted 2-benzoxazoles, which serve as nonpolar substitutes for carboxylic acids.

RESULTS AND DISCUSSION

The preparation of the starting materials is described in the following publication.[17] An exhaustive screening program including 50 strains[18] suggested two microorganisms which would give hydroxylated products from benzoxazoles. These two were *Cunninghamella blakesleeana* DSM 1906 and *Bacillus megaterium* DSM 32. Products obtained by the fermentations are shown in Table 1.

Substrate	Product with *Bacillus megaterium* DSM 32		Product with *Cunninghamella blakesleeana* DSM 1906	
 1	no product detected		no product detected	
 2	 3	yield: 14 % stereochemistry: *trans*[a]	 3	trace
	 4	yield: 6%		
 5	 6	yield: 30 % stereochemistry: 1*S*,3*S*[a] e.e.: 35 %	 6	yield: 20 - 40 %[b] stereochemistry: 1*S*,3*S*[a] e.e.: 25 - 95 %[c,d]
	 7	yield: 6 % e.e.: 35 %	 8	yield: 0 - 23 %[e] stereochemistry: 1*R* e.e.: 50 - 95 %[e]
			 7	yield: 3 % e.e.: 8 - 68 %[f]
 9	no product detected		 10	yield: 33 % mixture of diastereomers 1:1
	no product detected		 11	yield: 14 %
 12	 13	yield: 28 % stereochemistry: *trans*[a]	 13	yield: 25 %
	 14	yield: 17 % stereochemistry: 1*R*,2*R*[a] e.e.: 65 %[d]		
 15	7 products in very low yields		not investigated	
 16	9 products in very low yields		not investigated	
 17	 18	yield: 18 % stereochemistry: *trans*[g] racemic	no product detected	

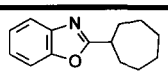
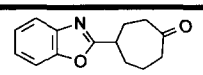
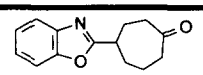
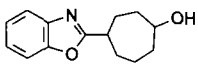
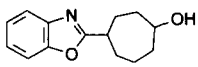
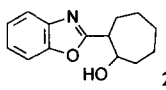
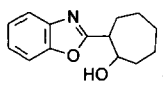
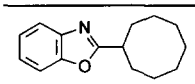
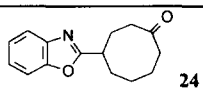
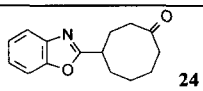
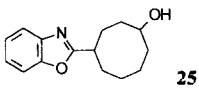
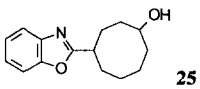
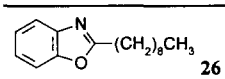
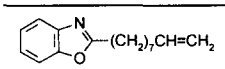
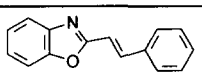
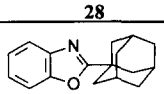
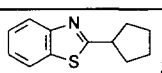
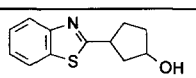
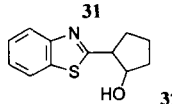
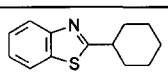
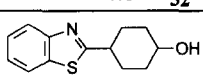
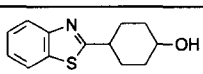
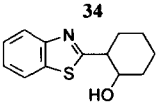
Substrate	Product with *Bacillus megaterium* DSM 32		Product with *Cunninghamella blakesleeana* DSM 1906	
 **19**	 **20**	yield: 17 % e.e.: 64 % sp. rot.: + 39.4	 **20**	yield: 6 % e.e.: 19 % sp. rot.: -12.2
	 **21**	yield: 22 % e.e.: 44 %[d] sp. rot.: + 7.9	 **21**	yield: 7 % mixture of diastereomers
	 **22**	yield: 5 % racemic	 **22**	yield: 1 % racemic
 **23**	 **24**	yield: 7 % sp. rot.: +10.5 e.e.: 7 %	 **24**	yield: 3 %
	 **25**	yield: 9 % mixture of diastereomers	 **25**	yield: 2 % mixture of diastereomers
 **26**	fully metabolized		fully metabolized	
 **27**	fully metabolized		fully metabolized	
 **28**	no product detected		no product detected	
 **29**	no product detected		no product detected	
 **30**	 **31**	yield: 12 %	not investigated	
	 **32**	yield: 5 %		
 **33**	 **34**	TLC[h]	 **34**	TLC[h]
	 **35**	TLC[h]		

Table 1. Results obtained by the Microbial Hydroxylation of Benzoxazoles [a] The determination of the stereochemistry is described in the following paper. [b] The yield obtained changes with fermentation time; the highest yield is found after approximately 24 h (40 %) and later goes down due to oxidation of alcohol **6** to ketone **8**. [c] The enantiomeric excess of this product depends both on fermentation time and conditions; the highest e.e. can be obtained with immobilized *Cunninghamella blakesleeana* and long fermentation times. [d] The e.e. of this compound can be enhanced up to > 98 % by lipase catalyzed hydrolysis of the respective acetate; the procedure is described in the following paper.[17] [e] The yield obtained for this product varies with the fermentation time. [f] The e.e. of this compound also changed with the fermentation conditions, however no plausible explanation could be found to date. [g] Assumed by analogy to compound **13**. [h] Assigned by analogous behavior as products from **12**; TLC-conditions: Silica gel 60 F_{254} aluminum plates (Merck), eluent: ethyl acetate / cyclohexane 1:1; R_f **34** 0.15; R_f **35** 0.45.

Substrate Specificity

By inspection of Table 1 several features of the substrates which are preferred by the enzyme system become clear. Firstly, the best substrates seem to be the ones with relatively low conformational flexibility bearing medium sized carbocycles. Substrates **2**, **5**, **12**, **17** and **19** are converted in fair yields to a small number of products with reasonable stereoselectivity. Smaller-ring substrates such as 2-cyclopropyl-1,3-benzoxazole **1** or larger-ring compounds such as 2-cyclooctyl-1,3-benzoxazole **23** or 2-adamantyl-1,3-benzoxazole **29** are either not substrates at all or converted unselectively. Compounds with higher conformational flexibility having a spacer between the benzoxazole moiety and the carbocycle are also converted less successfully. This is the case for **9**, **15** and **16**, which give a whole range of products in low stereoselectivity. Benzoxazoles substituted with long alkyl chains (**26** and **27**) are not suitable substrates. They tend to undergo reactions of the β-cleavage pathway leading to complete degradation of the chain. Only traces of UV-active material could be found after the fermentation of these compounds. Aromatic substrates such as the cinnamyl derivative **28** are not converted by either microorganism.

For purposes of comparison, two benzothiazoles **30** and **33** were also investigated. Easier cleavage of the benzothiazole moiety[19] after hydroxylation would make them more attractive for synthetic purposes. Unfortunately, the yields obtained with the benzothiazoles were considerably lower than those for the corresponding benzoxazoles. Hydroxylation occurs on the same carbon atoms as with the benzoxazoles. For *B. megaterium* the products seem to inhibit further hydroxylation leading to low yields.

Regio- and Stereoselectivity

As far as the anchoring role of the benzoxazole group is concerned, it can be found that in many cases the hydroxylation occurs at a carbon remote from this moiety. This could imply the same role for the benzoxazole as it has already been suggested for benzamides and carbamates for hydroxylations of organic substrates with *Beauveria bassiana* ATCC 7159. Furthermore, the steric relationship between the benzoxazole group and the hydroxyl moiety is *trans* in all products which is also in accordance with the previous work.[20]

Diastereoselectivites are generally high for the medium sized benzoxazoles, giving almost exclusively diastereomerically pure products. Enantioselectivities for the hydroxylation reactions, on the other hand, are low and generally range between 35 - 65 %, a fact also known for many other microbial hydroxylations.[21] Careful choice of fermentation conditions, however, can lead to higher optical purities.

The best studied example is the conversion of 2-cyclopentyl-1,3-benzoxazole **5**. It was found that as first product alcohol **6** was formed as almost a racemate sometimes with the prevailing (1*R*,3*R*)-enantiomer. This alcohol then underwent a second stereoselective oxidation with presumably an abundant alcohol dehydrogenase to give ketone **8** with an e.e. of typically 70 % and predominance of the (*R*)-enantiomer. With the course of the fermentation the yield of the ketone increased thereby increasing the e.e. of alcohol (1*S*,3*S*)-**6**.

Consequently, after 24 h the e.e. of **6** was 35 % and after 72 h it was 70 %. Details of these fermentations with identification of all product e.e. by HPLC are given in the subsequent publications.[17]

This enhancement procedure could not be applied to the bioconversion of **5** with *Bacillus megaterium*, as no subsequent reaction of the initially formed alcohols took place. With 2-cyclohexyl-1,3-benzoxazole **12**, however, the highest e.e. for a hydroxylation in this series could be obtained (65 % e.e. for alcohol **14**). In addition both strains give the achiral *trans*-4-hydroxyalcohol **13** in good yields.

Interestingly compound **17**, which differs from **12** just by a double bond, was only oxidized by *B. megaterium* to the epoxide **18**. **18** was found to be diastereomerically pure but racemic.

Ketones as products were also found in the fermentations of 2-cycloheptyl-1,3-benzoxazole **19** with both microorganisms. In this case oxidation of alcohol **21** to ketone **20** also occurred with *B. megaterium* and with opposite stereochemistry compared to the product of *C. blakesleeana*. *B. megaterium* gave **20** in higher e.e.. However, no enhancement of the e.e. of alcohol **21** could be obtained, because the predominant enantiomer of **21** is oxidized leading to racemization of this alcohol with longer fermentation times. The same is true for the biotransformation of **19** with *C. blakesleeana*, although here **21** was produced as mixture of diastereomers. This situation is also found with 2-cyclooctyl-1,3-benzoxazole **23** where both microorganisms form diastereomeric mixtures. Therefore, these are not preparatively useful.

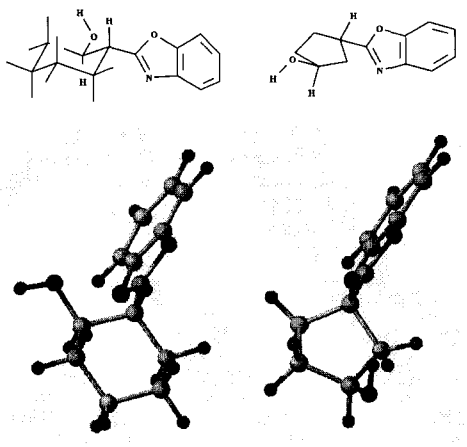


Figure 1. Ball-and-stick drawing of the major enantiomers of the hydroxylated benzoxazoles **14** and **6** (Compounds were drawn with SYBYL and minimized using the Tripos forcefield)

Inspection of Figure 1 readily explains the low e.e. encountered with these hydroxylations assuming an anchoring role of the benzoxazole moiety. According to previous reports, the hydroxylation system is relatively flexible compared to the anchoring part of the enzyme system leading to different possible approaches on the target molecule.[22] This is especially true for benzoxazole **5** where the two carbon atoms (C-3 and C-4) leading to the enantiomeric products **6** are very close together. Therefore it is obvious that the enzyme might hydroxylate the substrate at both carbon atoms with only little discrimination thereby leading to

a product of low e.e.. For benzoxazole **12**, however, the two carbon atoms (C-2 and C-6) leading to enantiomeric products **14** are much further apart and the hydroxylation site would be more energetically different. Here a higher e.e. can be found for alcohol **14** after hydroxylation.

EXPERIMENTAL

Fermentation Conditions: Transformations with *Cunninghamella blakesleeana* DSM 1906 were performed in 1-L shaking flasks containing 250 mL of Czapek-Dox medium or medium E. After 2 days of growth an ethanolic solution of substrate (0.3 g/L) was added to the culture. Biohydroxylations of 2-cyclopentyl-, 2-cyclohexyl-, 2-cycloheptyl- and 2-cyclooctyl-1,3-benzoxazole were done in a bioreactor as described elsewhere.[23]

Biohydroxylations with *Bacillus megaterium* DSM 32 were performed in 1-L shake flasks containing 250 mL of buffered medium E. 2-Cyclohexyl-1,3-benzoxazole was transformed in a bioreactor (Bioengineering L1523) containing 11 L of medium K. Substrate (0.3 g/L) was added after 16 hours of growth (5.0 g/L of biomass).

After 2 - 4 days the culture broth was extracted twice with ethyl acetate. The organic phase was evaporated after drying with Na_2SO_4. Products were separated by column chromatography on silica gel.

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). Czapek-Dox medium contained (per liter) 2 g of $NaNO_3$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4.7H_2O$, 0.5 g KCl, 0.1 g of Fe(III)NH_4-citrate, 20 g of glucose, and 1 g of yeast extract (Oxoid). Medium K was made of 4.5 g/L $Na_2SO_4.2H_2O$, 1.5 g/L KH_2PO_4, 3.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4.7H_2O$, 0.05 g/L Fe(III)-NH_4-citrate, 0.02 g/L $CaCl_2.2H_2O$, 1 g/L Na-acetate, 1 g/L yeast extract, 20 g/L glucose, 1 mL/L trace solution.

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