# Investigation of Regio- and Stereochemistries of Microbial Biotransformation

by

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## ABSTRACT

Epoxides can be hydrolyzed by fungi to produce chiral diols. The first part of this thesis presents an investigation of the microbial hydrolysis of aziridines comparable in structure to epoxide biotransformation substrates. Biotransformation of the aziridines 1-methyl-2-phenyl aziridine, 2-phenylaziridine and N-methyl-7-aza bicyclo[4.1.0] heptane was studied using *Beauveria sulfurescens*, *Aspergillus niger* and *Diplodia gossypina* but no evidence for enzymic hydrolysis was obtained.

The hydroxylation reaction performed by the fungus *Beauveria sulfurescens* ATCC 7159 has been studied for many years and several models describing the hydroxylating pattern exhibited by this fungus have been proposed. The second part of this thesis presents a test of the proposed models. The ability of *Beauveria sulfurescens* to hydroxylate thirty potential substrates was examined, and the data suggest that none of the earlier proposed models accounts for all of the bioconversion results. A possible explanation is proposed, suggesting that there is more than one enzyme responsible for the hydroxylation reactions performed by *Beauveria sulfurescens.*

ACKNOWLEDGMENT

I would like to thank Dr. H.L. Holland for everything he taught me during the course of this project.

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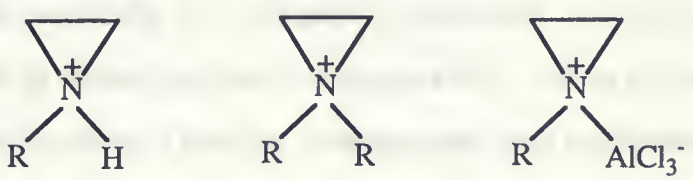
# INTRODUCTION

## PART ONE: MICROBIAL HYDROLYSIS OF AZIRIDINES

Aziridines are important chemical intermediates in the production of pharmaceuticals and chemicals, including polymers, coatings, textiles and adhesives, but they are also well known as toxic substances, especially as carcinogens.[1] Being very reactive, they exert mutagenic effects by alkylating some parts or part of the nucleic acid structures involved in cell replication. On the contrary, it is of interest that some aziridines such as mitomycins are known as useful antibacterial and carcinostatic agents.

## CHEMICAL HYDROLYSIS OF AZIRIDINES

There are two principal properties that account for all reactions of aziridines that involve the aziridine ring. These are the reactivity of the ring nitrogen atom and that of the strained three-membered ring. The reactivity properties of the ring nitrogen atom may be attributed to the unshared pair of electrons on the nitrogen and thus closely resemble such properties of other nonaromatic amines. Reactions due to the strained three-membered ring all involve ring destruction or ring opening. Nitrogen-substituted aziridines can be broadly divided in two groups of compounds. These are Group A (activated aziridines), compounds in which the substituent is capable of conjugating with the unshared pair of electrons of the aziridine nitrogen (e.g., the carbonyl group, making the aziridine ring a tertiary amide nitrogen) and Group B (basic aziridines), compounds in which there is no such substituent. Most ring-opening reactions may be regarded as substitutions involving attack of the nucleophile at an aziridine carbon atom. With basic aziridines ring-opening reactions may be ascribed to the reactivity of the protonated or quaternized aziridine or the Lewis acid-aziridine adduct:



When very reactive aziridinium ion is formed, often only catalytic amounts of relatively weak acids are necessary to promote a ring-opening reaction. In the absence of acidic reagents the basic aziridines usually do not undergo ring-opening reactions. Thus the one precaution usually necessary is to prevent formation, in even catalytic amounts, of one of the reactive species illustrated above. An activated aziridine not only is still sensitive to acidic reagents, but also undergoes ring opening with nucleophilic reagents even in the absence of acid. Maintenance of low temperatures and use of nonpolar solvents are the usual precautions taken when this type of the aziridine is prepared from a basic aziridine. As mentioned previously, the protonated aziridine is a reactive species capable of undergoing a ring-opening reaction with $X^-$, solvent, basic aziridine nitrogen, or any other nucleophilic reagent present in the medium. If, however, the reaction is carried out in dilute solution in a solvent of low nucleophilicity (such as water) with an acid the anion of which is also of low nucleophilicity (e.g., perchloric acid), the basic aziridines can form salts and ring-opening can be completely avoided.

## BIOHYDROLYSIS OF AZIRIDINES

Hydrolysis of aziridines by mammalian liver microsomes has been studied by Watabe and coworkers.[1,2] The source of enzyme activity is assumed to be the same microsomal epoxide hydrolase, since it has been shown that aziridines inhibit epoxide hydrolases, but this assumption is still an open question. The better understood hydrolysis of the epoxides is described in the following section, and then the above mentioned postulated hydrolysis of aziridines will be presented.

## HYDROLYSIS OF EPOXIDES

Epoxides are very useful compounds and are extensively employed as synthetic intermediates for the synthesis of enantiomerically pure bioreactive compounds. In living cells, aromatics and olefins can be oxidized by monooxygenases to form epoxides.[3] Due to their electrophilic character and ring tension, epoxides are very reactive. Consequently, such metabolites can bind covalently

to nucleophilic groups in many tissue constituents, including such cellular macromolecules as DNA, RNA and proteins, which makes them carcinogenic and teratogenic agents.[4] In order to eliminate them from the cell, epoxide hydrolases catalyze their conversion into biologically harmless diols, which can be further metabolized or excreted due to their higher water solubility.[3] As a consequence, most of the epoxide-hydrolase activity found in higher organisms is located in organs such as liver, which is responsible for the detoxification of xenobiotics.[4]

An important amount of work has been carried out using subcellular fractions of mammalian liver, where two distinctive forms of epoxide hydrolases, i.e. a form located in the membranes (microsomal epoxide hydrolase: MEH) and a form located in the cytosol (cytosolic epoxide hydrolase: CEH) have been purified and characterized.[3-16] Unfortunately these enzymes, which have high theoretical value for organic synthesis, cannot be used for large scale experiments because of their low availability.

Corresponding enzymes from microbial source are still not well characterized. Up to now, it has been generally assumed that bacteria are lacking epoxide hydrolases because it is possible to accumulate epoxides by bacterial epoxidation of olefins.[5] Only the purification of epoxide hydrolases from two *Pseudomonas* strains have been described.[6]

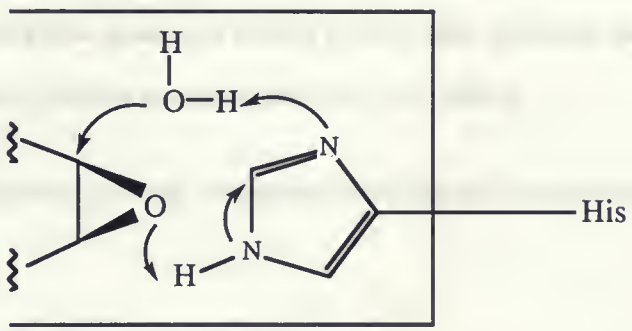
Both mammalian MEH and CEH as well as bacterial or fungal EH were shown to exhibit a remarkable capability of chiral recognition, enabling them to discriminate between enantiomers of racemic epoxides. Furthermore, these enzymes were shown to be generally highly regio- and stereoselective.

## MAMMALIAN HEPATIC EPOXIDE HYDROLASES

Two main types of epoxide hydrolases from liver tissue have been characterized: a microsomal (MEH) and a cytosolic (CEH), which are different in their substrate specificities.

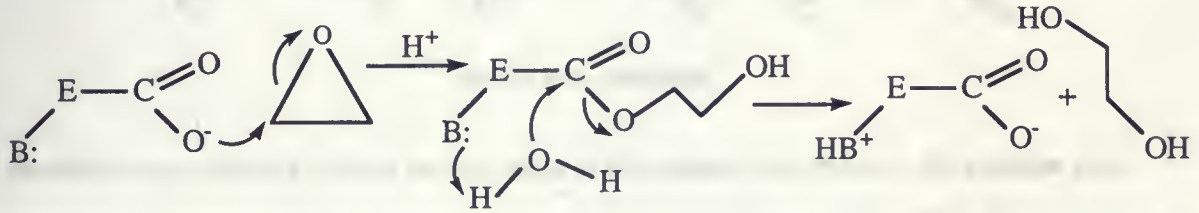
Epoxides can chemically undergo both acid- and base-catalyzed reactions. In the literature both acid catalyzed[7] and nucleophilic mechanisms[8] have been proposed. However, indirect evidence points to a general base catalyzed $S_N2$ type mechanism for epoxide hydrolase enzyme activity, as first proposed by Hanzlik and coworkers[9] on the basis of the absence of a metal ion requirement and a high regioselectivity favoring attack by water on the less substituted oxirane carbon. The imidazole unit of histidine residue in the active site (or maybe cysteine in CEH) removes a proton from the attacking water molecule to increase its nucleophilic reactivity without ever forming free hydroxide ion.[8] (Figure 1)

Figure 1: Mechanism of microsomal epoxide hydrolase




This model implies the existence of a lipophilic pocket located at the backside if the oxirane ring is oriented with the oxygen atom towards the top. Armstrong and Lacourciere have shown recently, from single turnover experiments in $H_2O^{18}$, that the oxygen atom incorporated in the product, though ultimately derived from water, is proximally derived from the enzyme by way of an ester intermediate (probably aspartate).[10] (Figure 2)

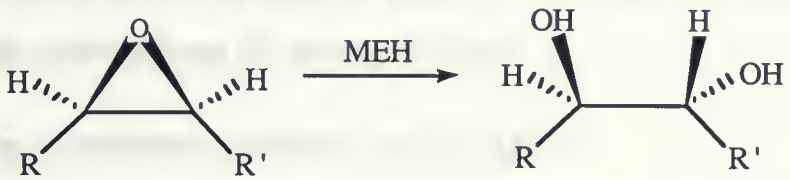
Figure 2: Alternative mechanism of MEH



From the relatively large amount of data available for microsomal EH, the following general rules can be deduced:

-MEH catalyses the hydration of both alkene and arene oxides to *trans*-dihydrodiols.[4] (Figure 3)

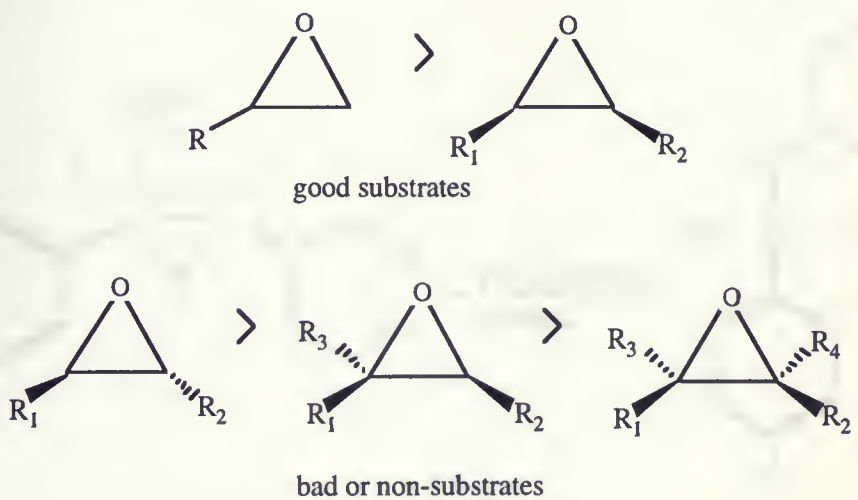
Figure 3: Reaction catalyzed by MEH




-MEH has been shown to be highly regiospecific, since mono- and/or disubstituted *cis*-epoxides containing at least one lipophilic group are readily hydrolyzed, while the reaction with mono- and/or disubstituted *trans*-epoxides are catalyzed only very slowly.

-The sterically more demanding tri- and tetra-substituted derivatives are usually not substrates for MEH.[11] (Figure 4)

Figure 4: Substrate types for MEH

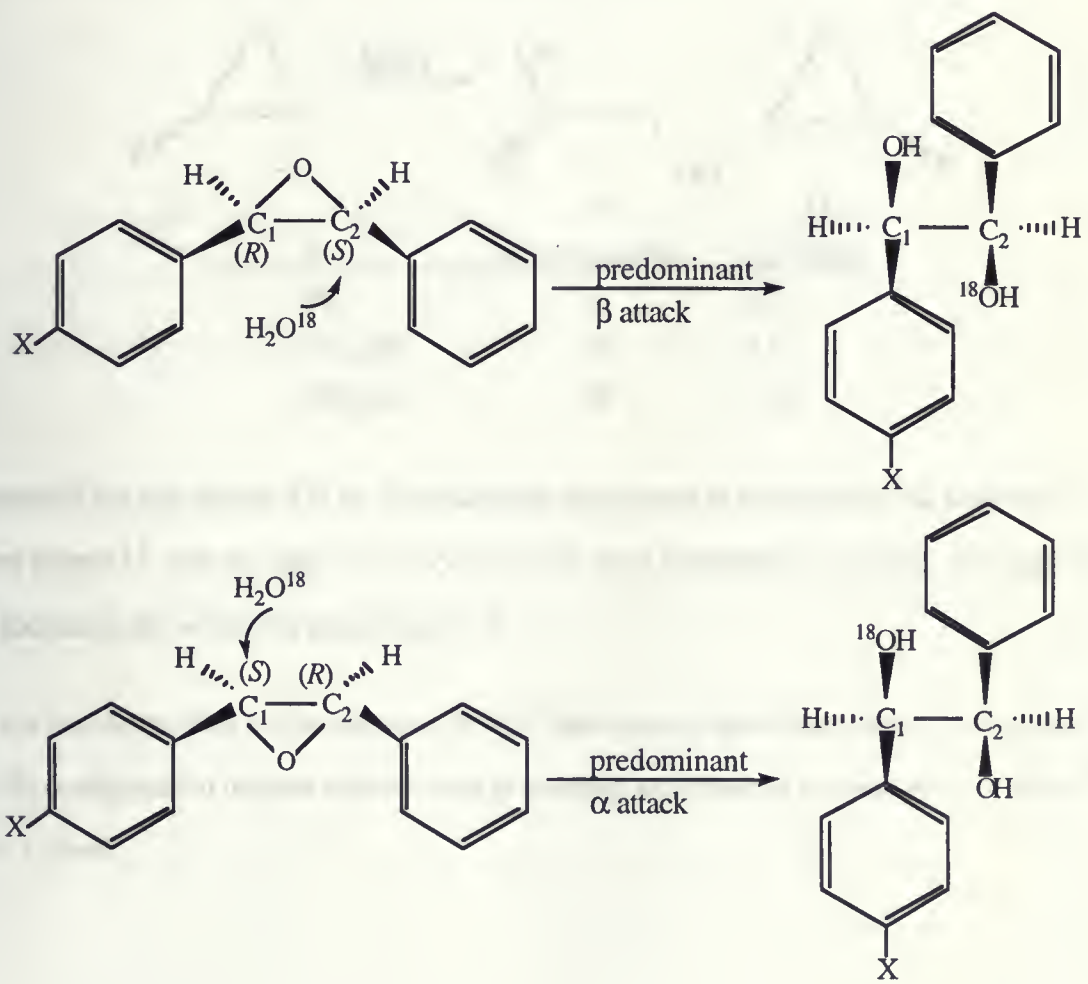



-Lipophilic aryl- and alkyl chains located close to the oxirane ring enhance the reaction rate, whereas polar groups, such as a hydroxy group, have an inhibitory effect. This latter behavior

may be explained by the fact that most xenobiotic substances are apolar compounds that need to be converted into more hydrophilic metabolites in order to facilitate their elimination.[3]

-Using $H_2O^{18}$ and/or *cis*-$^{18}O$-labelled oxides Hanzlik et al. have shown that hydration occurs preferentially at the carbon atom of the epoxide group which is less sterically hindered.[9] It has been suggested that attack by water on epoxides which are symmetrical, or nearly symmetrical occurs at the carbon atom which has *(S)* chirality.[12] (Figure 5)

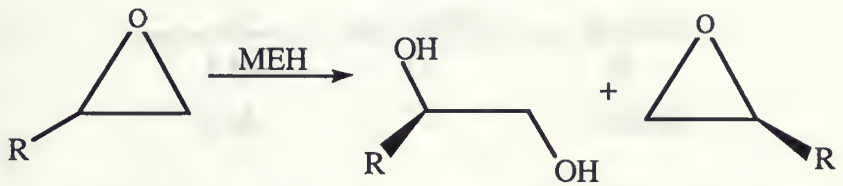
Figure 5: Hydration of substituted *cis*-stilbene oxides in $H_2O^{18}$



The substituted phenyl ring is always attached to C-1. Attack by water on C-1 is designated α attack, and on C-2 β attack. Attack by water at the *(R)* center in each enantiomer is a minor pathway.

Racemic monosubstituted aryl- or alkyl epoxides may be resolved by MEH in accordance with the above mentioned mechanism, wherein the more accessible C-2 carbon atom of the *(R)*-epoxide is preferentially attacked to give the *(R)*-diol leaving the *(S)*-epoxide behind. (Figure 6)

Figure 6: Resolution of monosubstituted epoxides by MEH

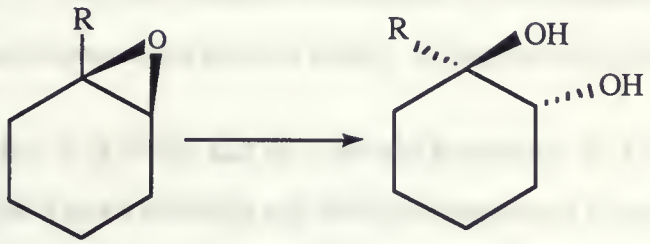



| | (*R*) | (*S*) |
|---|---|---|
| R | conversion[%] | ee diol[%] |
| Ph | 18 | <5 |
| n-C4H9- | 16 | 14 |
| (CH3)3C | 38 | 92 |

The nature of the substituent R is of the significant importance in determining the selectivity. Whereas phenyl[13]- and straight-chain alkyl epoxides were inefficiently resolved, branched alkyl chains increased the selectivity dramatically.[14]

Cyclic *cis-meso*-epoxides can be asymmetrically hydrolyzed to give *trans*-diols. In this case, once again, *(S)* configuration oxirane carbon atom is attacked and inverted to yield an (*R*,*R*)-diol.[15] (Figure 7, R=H)

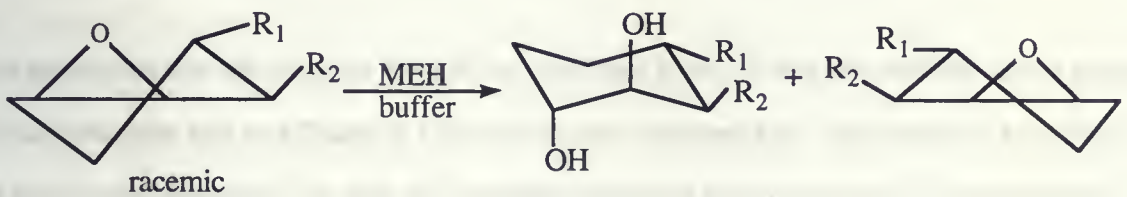
Figure 7: The hydrolysis of cyclohexene oxide **1** (R=H) and (±)-1-methylcyclohexene oxide **2** (R=$CH_3$) by MEH and CEH




| | MEH | CEH |
|---|---|---|
| R | ee diol[%] | ee diol[%] |
| **1** H | 94 | 22 |
| **2** Me | 94 | racemic |

During a study on the topology of the active site of MEH using substituted cyclohexene oxides[16,17] it has been shown that the preferentially hydrolyzed enantiomer is the one in which the cyclohexene ring has 3,4-M helicity. The assumption is that diaxial opening of the epoxide takes place. (Figure 8)

Figure 8: Hydrolysis of the substituted cyclohexene oxides




racemic

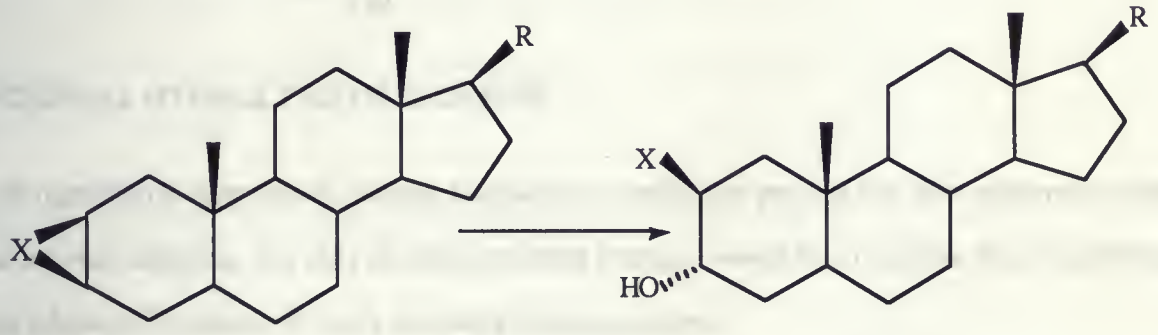
| R1 | R2 | conversion[%] | ee diol[%] |
|---|---|---|---|
| t-Bu | H | 47 | 88 |
| H | t-Bu | 53 | 74 |

HYDROLYSIS OF AZIRIDINES

As was mentioned earlier, it has been suggested that hydrolysis of aziridines by mammalian liver microsomes has the same source of the enzyme activity as microsomal epoxide hydrolase.[1, 2]

The unsubstituted aziridine **3** (X=NH), and its N-methyl homologue **4** (X=NMe) were hydrolyzed to the aminols **5** and **6** (X=$NH_2$ and NHMe respectively) by rabbit liver microsomes in the same stereochemical sense as for the corresponding epoxide. (Figure 9) The corresponding episulfide (X=S) was not hydrolyzed at all.

Figure 9: Hydrolysis of aziridines by mammalian liver microsomes

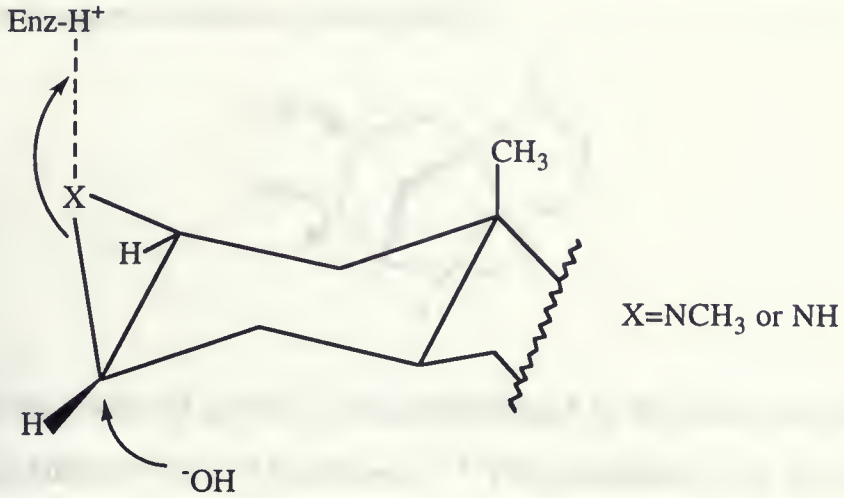



**3**, X=NH
**4**, X=NMe

**5**, X=$NH_2$
**6**, X=NHMe

It was postulated that the nitrogen atom in the aziridines interacts with the protonic active center of aziridine hydrolase and ring fission is followed by the concerted back-side attack of a hydroxyl anion from water selectively on only one aziridine carbon to form *trans*-diaxial aminoalcohol. (Figure 10)

Figure 10: A possible mechanism of hydrolysis of aziridines by hepatic microsomes. Enz-$H^+$ represents a protonic active center of the enzyme



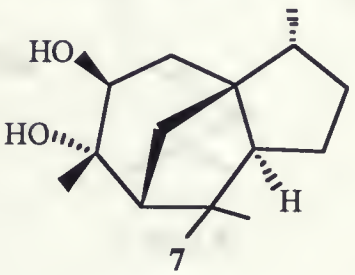

## MICROBIAL HYDROLYSIS OF EPOXIDES

Using mammalian enzymes to produce large-scale quantities of product has little preparation value. Another very tempting, but only recently explored strategy, would be to achieve these hydrolyses using whole-cell cultures of easily available microorganisms.

As previously mentioned, in the case of mammalian MEH and CEH, as well as in bacterial enzyme catalyzed hydrolyses, the reaction involves *trans*-addition of water with inversion of configuration at the oxirane carbon being attacked.[4,8,9] However, in the case of two fungi, a mechanism of *cis*-hydration has been described. Kolattukudy and Brown have reported that *Fusarium pisi* contained an epoxide hydrolase that catalyzes the *cis*-hydrolysis of epoxy acids.[18] Similarly, Suzuki and Maruno described a selective *cis*-hydration of one enantiomer of 10,11-epoxyfarnesol by *Helminthosporium sativum*.[19]

The most probable explanation for the observed stereochemistry of the product is that the transient carbonium ion generated by protonation of the oxygen is held on the back side by a nucleophilic functional group of the enzyme until solvolysis of this adduct occurs by the attack of a water molecule on the same side where the oxirane ring was attached.[18]

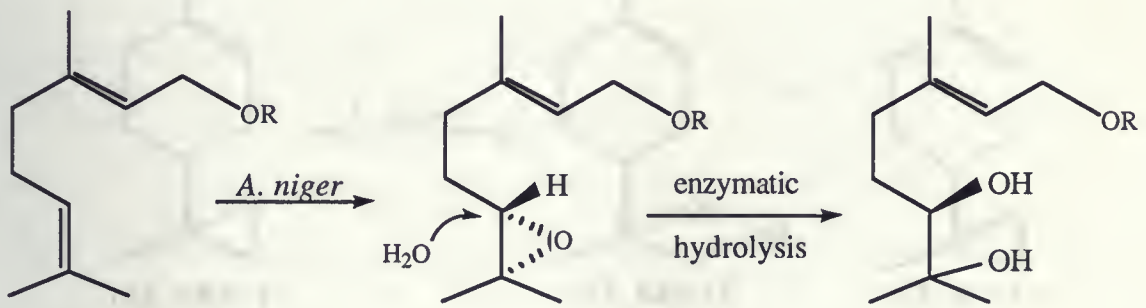
The formation of *trans* vicinal diols from olefins by microbial biotransformation is not a common process, but several examples are known. The biotransformation of cedrene to the diol **7** is performed by *Corynespora cassiicola* DSM 62474.[20]




**7**

The microbial hydroxylation of geraniol N-phenylcarbamate **8** by the fungus *Aspergillus niger* leads to dihydroxylation of C-6=C-7 double bond. [21] The mechanistic study with $O^{18}$ showed that *6(S)* epoxide **9** was formed in the first step and then enzymatically hydrolyzed to *6(R)* diol **10** at pH 6-7. (Figure 11)

Figure 11: Microbial oxygenation of geraniol N-phenylcarbamate **8**




**8**
R=CONHPh

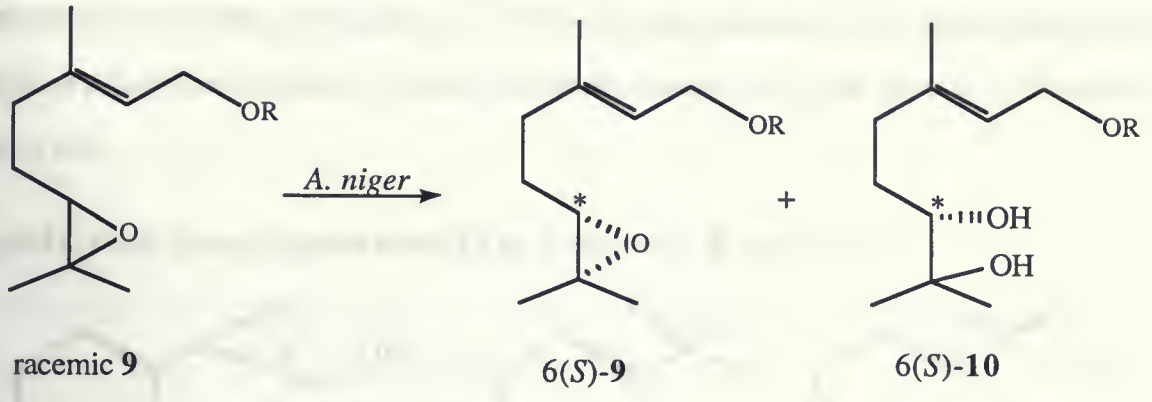
6(*S*)-**9**

6(*R*)-**10**

It was suggested that the enzymatic hydrolysis occured *via* a *trans* opening process, water attacking at the less substituted oxirane carbon C-6.

The hydrolysis of the racemic epoxide **9** was highly enantioselective, giving *6(S)*-**10** diol and the remaining *6(S)*-**9** epoxide.[22] The mechanism indicated preferential hydrolysis of the *6(R)* epoxide **9** leading to *6(S)* diol **10** *via* a *trans* water addition at C-6 and inversion of configuration at this carbon atom. (Figure 12)

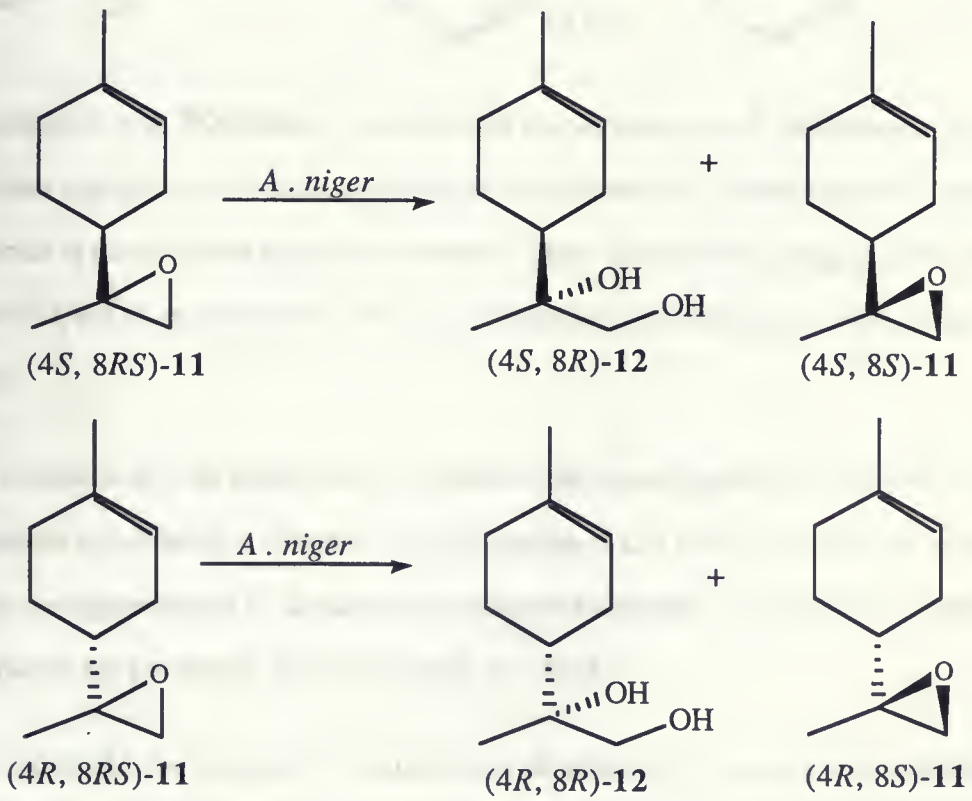
Figure 12: Hydrolysis of racemic 6,7-epoxy geraniol N-phenylcarbamate **9**




racemic **9** 6(*S*)-**9** 6(*S*)-**10**

The hydrolysis catalyzed by the intracellular EH of *Aspergillus niger* is highly diastereoselective, which was used for separation of the two diastereoisomeric epoxide mixtures of 8,9-epoxylimonene **11** (de 98%).[22] (Figure 13)

Figure 13: Hydrolysis of 8,9-epoxylimonene **11** with *A. niger*

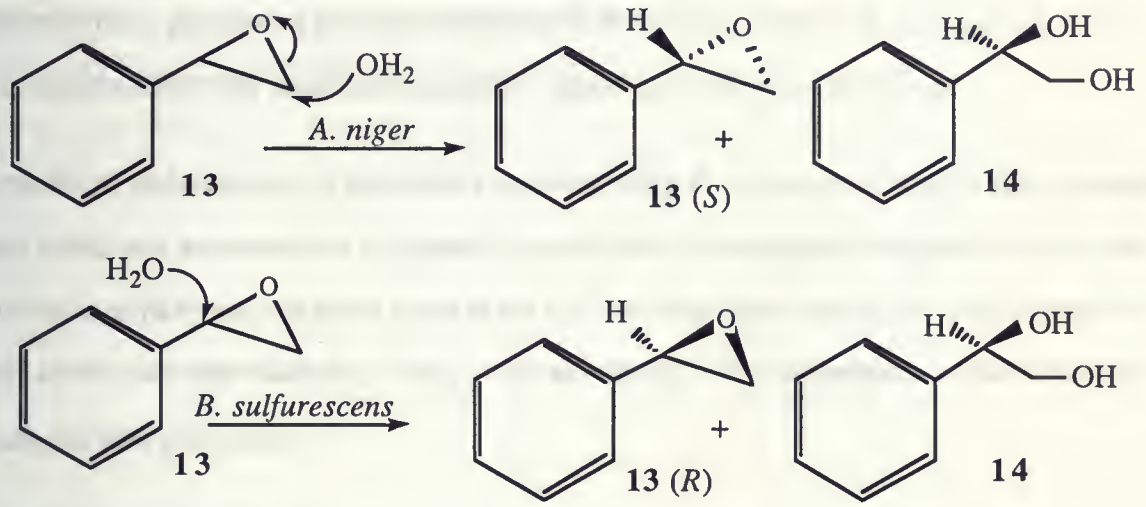



(4*S*, 8*RS*)-**11** (4*S*, 8*R*)-**12** (4*S*, 8*S*)-**11**

(4*R*, 8*RS*)-**11** (4*R*, 8*R*)-**12** (4*R*, 8*S*)-**11**

The microorganism *Beauveria sulfurescens* shows epoxide hydrolase activity of the opposite enantioselectivity for the same substrates.[23] These biotransformations are enantiocomplementary since they achieve the hydrolysis of the styrene oxide enantiomers of the opposite configuration. (Figure 14).

Figure 14: Hydrolysis of styrene oxide **13** by *A. niger* and *B. sulfurescens*




Studies conducted with $^{18}O$-labelled water showed that *A. niger* and *B. sulfurescens* operate each with a different regioselectivity during opening of the oxirane ring. In the case of *A. niger* the water molecule is incorporated at the less hindered carbon of the oxirane ring, as in the case of the mammalian EH, but *B. sulfurescens* leads to incorporation of labelled water at the more substituted carbon atom.

Enzymatic hydration of both enantiomers of styrene oxide was suggested to occur *via* a *trans* opening process which led to a retention of configuration at C-1 in the case of *A. niger* and to an inversion of configuration for *B. sulfurescens* mediated hydrolysis. It seems that *A. niger* EH active site model fits previously described model for MEH.[8]

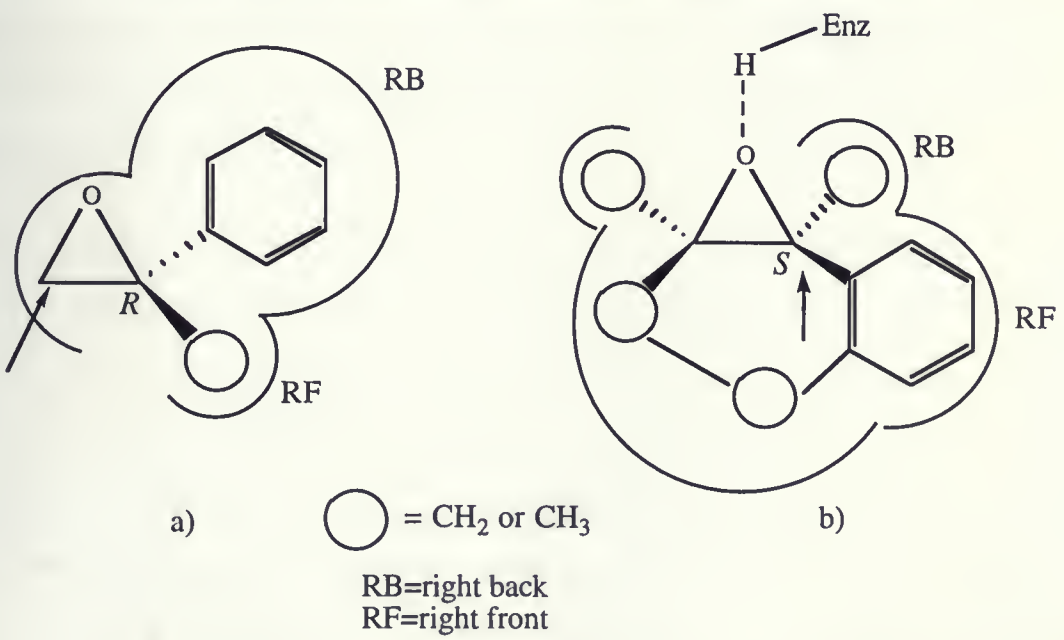
The further studies by the same authors using series of substituted oxiranes confirmed that the formed diol **14** is always of *(R)* configuration.[24] It was suggested that biohydrolysis mediated by *B. sulfurescens* may imply an acid-catalyzed process. Such a mechanism has been recently

suggested for CEH, where it seems that epoxide protonation of phenyl substituted epoxides, favoring opening at the benzylic carbon is more important for CEH than for MEH.[25] Independently of the hypothesis that each fungus could have more than one EH, Furstoss et al. proposed active site models for *A. niger* and *B. sulfurescens* epoxide hydrolase.[24] (Figure 15)

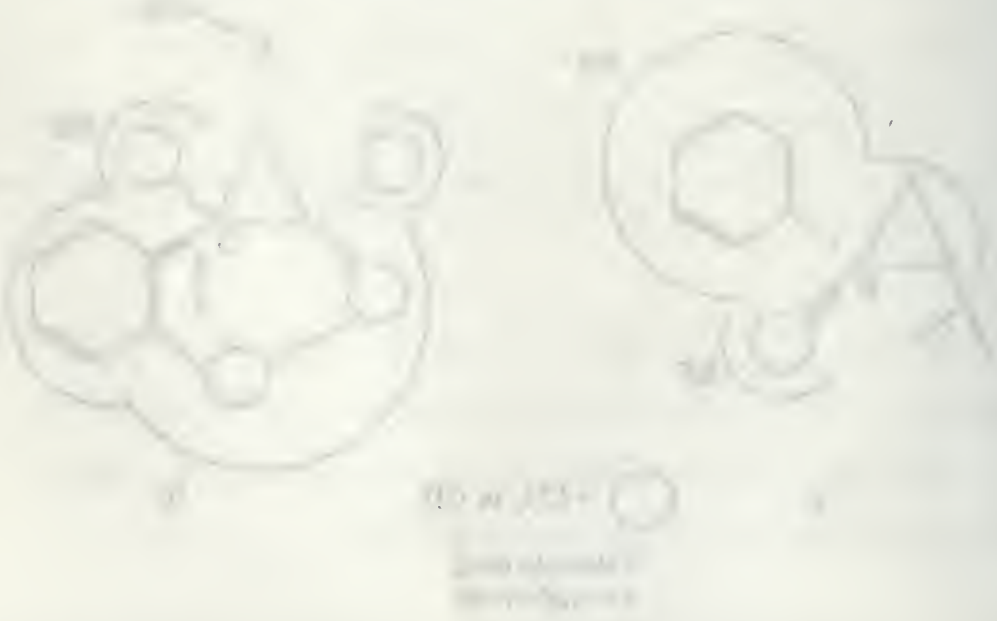
According to the results using substituted oxiranes, it seems that *A. niger* has very limited substrate stereoselectivity, processing only monosubstituted derivatives, whereas *B. sulfurescens* can accommodate differently substituted epoxides, regardless of the steric hindrance.

The results of biohydrolysis of substituted epoxides using *B. sulfurescens* suggest that a lipophilic pocket which will accommodate the phenyl group of the (1*S*) enantiomer must be located at the right front side (RF) and that some space at the left front side must also be available around the C-2 carbon atom to accommodate the β-alkyl group substituents. The nucleophile would be located beneath the benzylic carbon.

Figure 15: Proposed models for the active sites of *A. niger* a) and *B. sulfurescens* b)




Unlike the biotransformation of terpenes, steroid biotransformation rarely results in conversion of an unsaturated steroid substrate directly to a diol, although the formation of 5α,5β-diols from 5(6)-



epoxy steroids[26] and the microbial hydrolysis of exocyclic C-3-oxiranes[27] have been observed. The rearrangement of steroid epoxides when used as substrates for bioconversions has also been reported[28], but there is no evidence to suggest that these reactions can proceed directly from olefinic substrates.

## MICROBIAL HYDROLYSIS OF AZIRIDINES

Microbial hydrolysis of aziridines is still not reported. As described in the introduction, it has been suggested that there is a parallel in the enzyme activity of microsomal epoxide hydrolase and aziridine hydrolysis by mammalian liver microsomes. The purpose of this project is therefore to investigate whether a similar observation can be made for microbial hydrolysis.

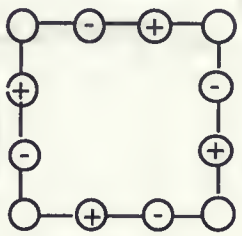
## PART TWO: A RATIONAL APPROACH TO THE HYDROXYLATING ENZYMES OF *Beauveria sulfurescens* ATCC 7159

Microbial monooxygenase enzymes are able to perform direct conversion of a saturated or unsaturated carbon-hydrogen bond to a carbon-hydroxyl. Although the biohydroxylation of unactivated carbons has great potential, since it is impossible to perform the same reaction by means of classical organic chemistry, it is still not widely used in synthetic chemistry. The problem is that isolated hydroxylase enzyme preparations are not available commercially, and a whole cell biotransformation technique has to be employed. Whole cell biotransformation, although considered to be inconvenient, offers several advantages such as: it can be used on a preparative scale (as opposed to isolated enzymes), and the expensive enzyme cofactors are not required. A disadvantage of this technique is inability to predict the regio- and stereochemistry of oxidation, since the characteristics of the hydroxylase enzyme's active site are generally not known. It is even very hard to predict the outcome of the biocatalysis of the new substrate using a microorganism whose oxidative bioconversions on different compounds are known. In order to be able to use this very useful microbial ability effectively, it is desirable to develop a "model" of the enzyme's active site by "feeding" the microorganism with carefully designed substrates with selectively modified functionalities.

Alcohols were among the first compounds whose biotransformation using the fungus *Beauveria sulfurescens* ATCC 7159 was studied.[29] Cyclododecanol was readily hydroxylated to produce a mixture of 1,6- and 1,7-dihydroxycyclododecanol as a major products. Although cyclododecanol was readily transformed by the microorganism, cyclododecane was not. This result indicated that a polar functional group was needed for transfer of the substrate to a cell, or it possibly serves for binding to the enzyme's active site. Using a previously proposed stereochemical model of cyclododecane in the crystal state (represented two dimensionally in Figure 16)[30], and aligning the hydroxyl group of the substrate hydroxycyclododecane on either of the corner atoms (in one plane designated by O), or on those carbons that lie above the plane of the ring (+), or below it (-), the

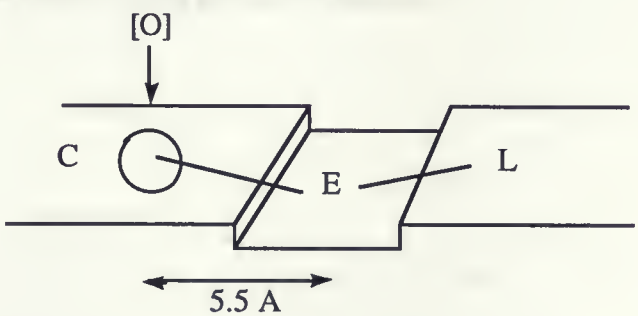
distance between the original hydroxyl group of cyclododecanol and the positions that were hydroxylated represented approximately 5.5 Å (Dreiding models). Of course, the measurement was done with the assumption that cyclododecanol will retain preference for this conformation in solution and, more importantly, in the enzyme-substrate interaction.

Figure 16: Crystal state conformation of cyclododecane



On the basis of this result it was proposed that an electron-rich functional group of a substrate, such as hydroxyl group, amine or amide, binds to the enzyme and directs hydroxylation at a point 5.5 Å away from the site of binding. This was rationalized in the "first generation" model of the enzyme's active site. (Figure 17)

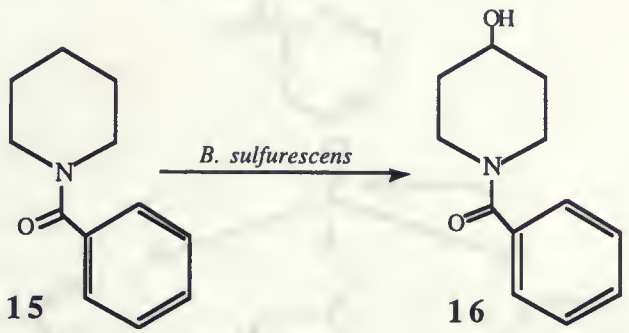
Figure 17: Original model for hydroxylation by *B. sulfurescens*. C=cyclic system; E=electron rich group; L=lipophilic group; [O]=site of hydroxylation




Microbial oxygenation of a series of heterocyclic compounds was done in order to check the above proposed model.[31] The substrates included piperidine and hexa-, hepta-, and octamethyleneimine as their benzoyl derivatives as well as the p-toluenesulfonyl derivative of hexamethyleneimine. In these substrates, the electron-rich center was considered to be the oxygen of the carbonyl or sulfonyl groups. The yields of the hydroxylated products were in the range of 25-60%. The

representative compound from the piperidine series was 1-benzoylpiperidine **15** which was hydroxylated to 1-benzoyl-4-hydroxypiperidine **16.** (Figure 18)

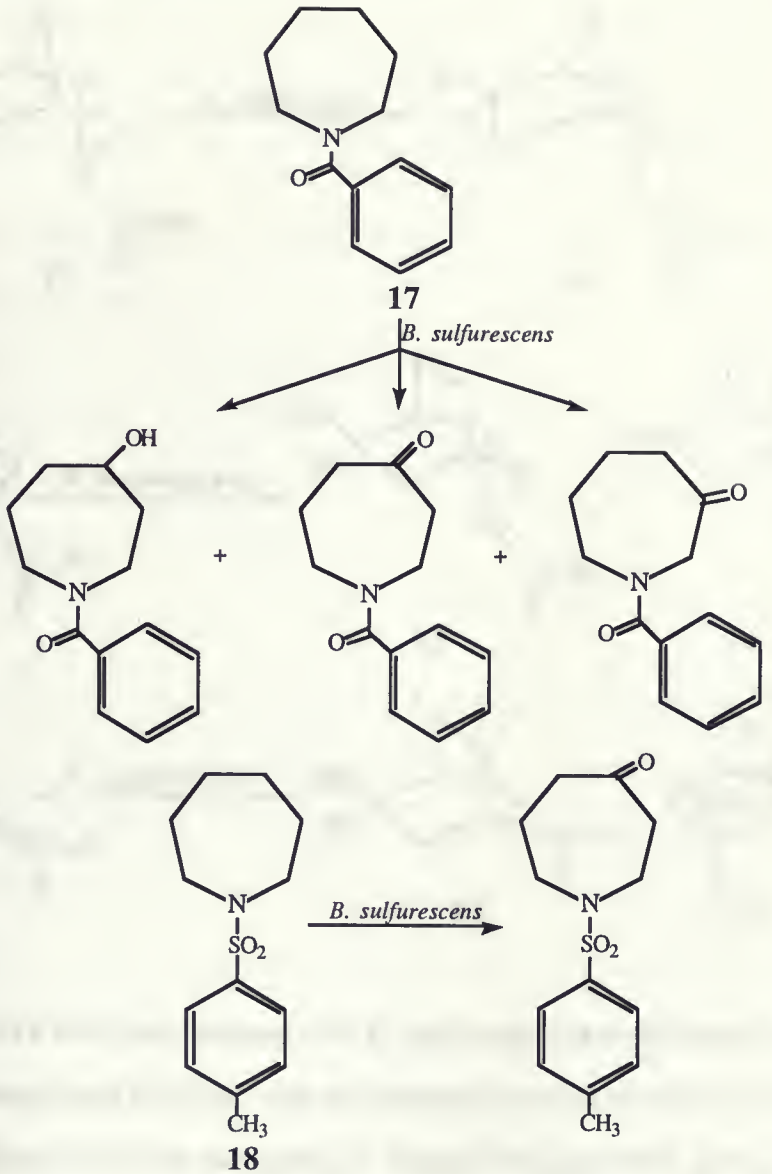
Figure 18: Hydroxylation of piperidine series




The hexamethyleneimine series (represented in Figure 19 with benzoyl (**17**) and p-toluenesulfonyl (**18**) derivatives of hexamethyleneimine) indicated that the nature of the acyl function had little effect on the outcome of the bioconversion since similar products were isolated.

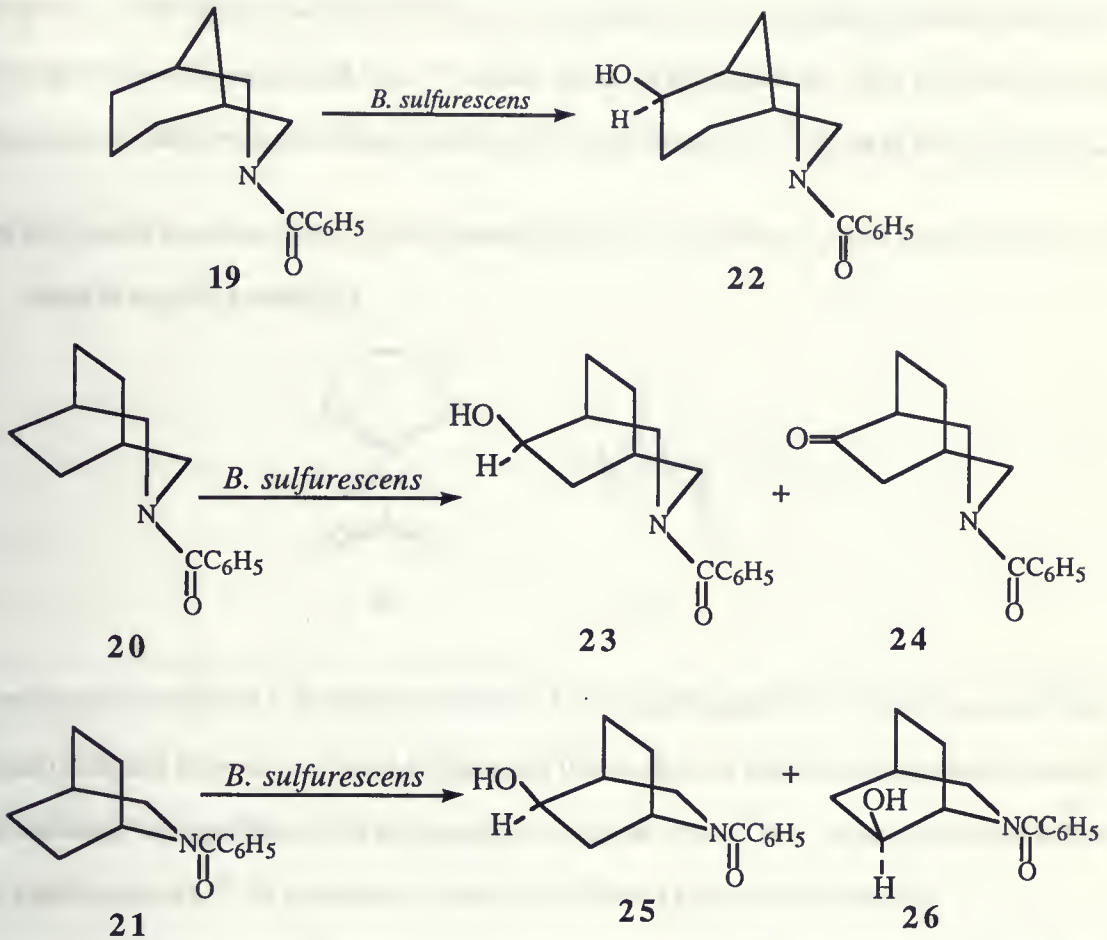
The microbial oxygenation of both substrates (**17** and **18**) occurred primarily at 4-position. Similar results were obtained when compounds of the hepta- and octamethyleneimine series underwent bioconversion using *B. sulfurescens*. Again, oxygenation occurred mainly at the 4-position giving rise to the hydroxylated product or ketone.[31]

Figure 19: Hexamethyleneimine series




The same research group studied the bioconversion of the amides of azabicycloalkanes.[32] Data from the oxygenation of 3-benzoyl-3-azabicyclo[3.3.1]nonane **19**, 3-benzoyl-3-azabicyclo[3.2.2]nonane **20** and 2-benzoyl-2-azabicyclo[2.2.2]octane **21** are presented in Figure 20.

Figure 20: Oxygenation of amides of azabicycloalkanes

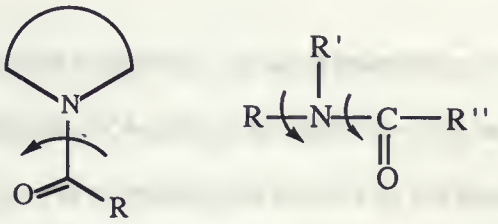



The bicyclic amide **19** when bioconverted with *B. sulfurescens* gave the single monooxygenated product **22** in excellent yield (60-70%) with the hydroxyl group in the axial position. The substrate **20** was transformed into two products: alcohol **23** (yield 50%; hydroxyl group was found to be *endo* with respect to the six-membered ring) and ketone **24** (22% yield). The major and minor hydroxylated products obtained from bioconversion of **21** were *endo* alcohols **25** (45% yield) and **26** (6% yield).

The hydroxylation of a series of adamantanamines[33] was studied along with the bioconversion of *trans*-decahydroquinoline[34]. The requirements of an approximate 5.5 Å spacing between the electron-rich center and the site of hydroxylation appeared to be largely met by these substrates, assuming the amide carbonyl oxygen as the binding point to the enzyme. However, the amide

group can have different conformations, making the proposed model more difficult to use. The amide group, if nitrogen is part of the ring (i.e. piperidine), has two preferred conformations, depending on the rotation around the C-N amide bond. If the amide nitrogen is primary, or not in a cyclic system, additional rotational freedom will exist around the C-N alkyl bond. (Figure 21)

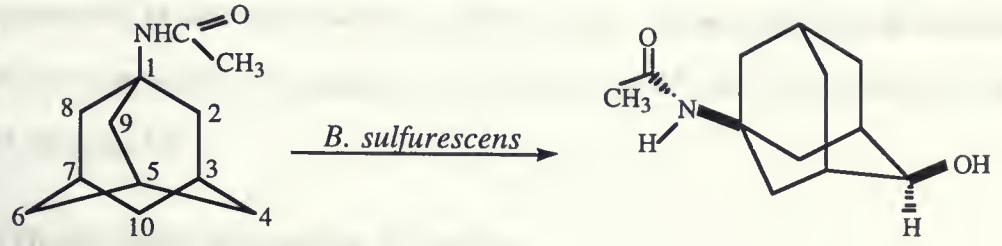
Figure 21: Amide bond preferred conformations in the cyclic system (a) and free rotation of C-N bond in acyclic system (b)




a) b)

The previously mentioned 1-benzoylpiperidine **15** was hydroxylated at the 4 position and the maximum distance between carbonyl oxygen and the methylene carbon at 4 position is about 5.3 Å (when carbonyl is perpendicular to the piperidine ring) or about 5.0 Å (when the amide is in a planar conformation).[35] In the case of azabicycloalkane **19** which also contains a 1-benzoylpiperidine ring system, hydroxylation occurs outside of the piperidine ring, at the distance 5.3 Å or greater from the amide carbonyl, depending on the amide conformation. According to these results, it was suggested that 5.5 Å spacing between electron-rich center and the site of hydroxylation, although not necessary, is still preferred in the substrates containing the amide functional group.[35] The hydroxyl group introduced into the substrate molecule was found to be oriented *trans* with respect to the amide functional group in all examples studied up to that point.[35] One of the examples is presented in Figure 22 where the major product of bioconversion of N-acetyl-1-adamantanamine **27** was the 4-hydroxy compound **28**.[33] The *trans* relationship of the introduced hydroxyl group to the amide group is illustrated with the heavy line.

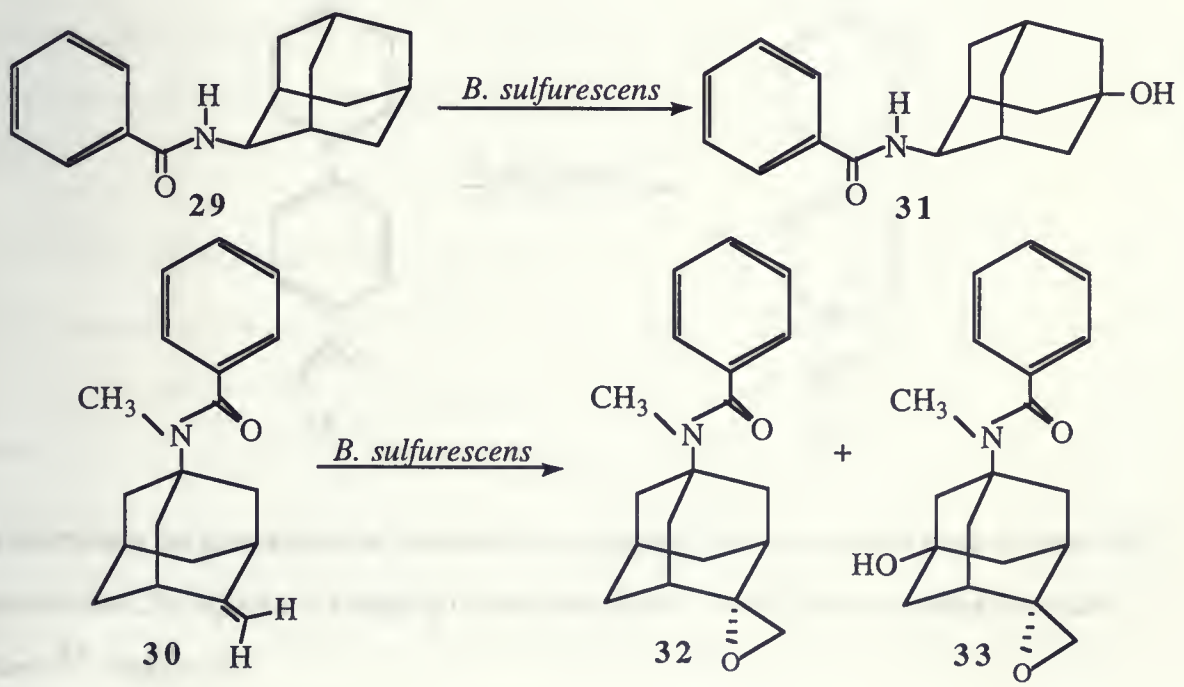
Figure 22: Stereochemistry of the biohydroxylated product




**27** **28**

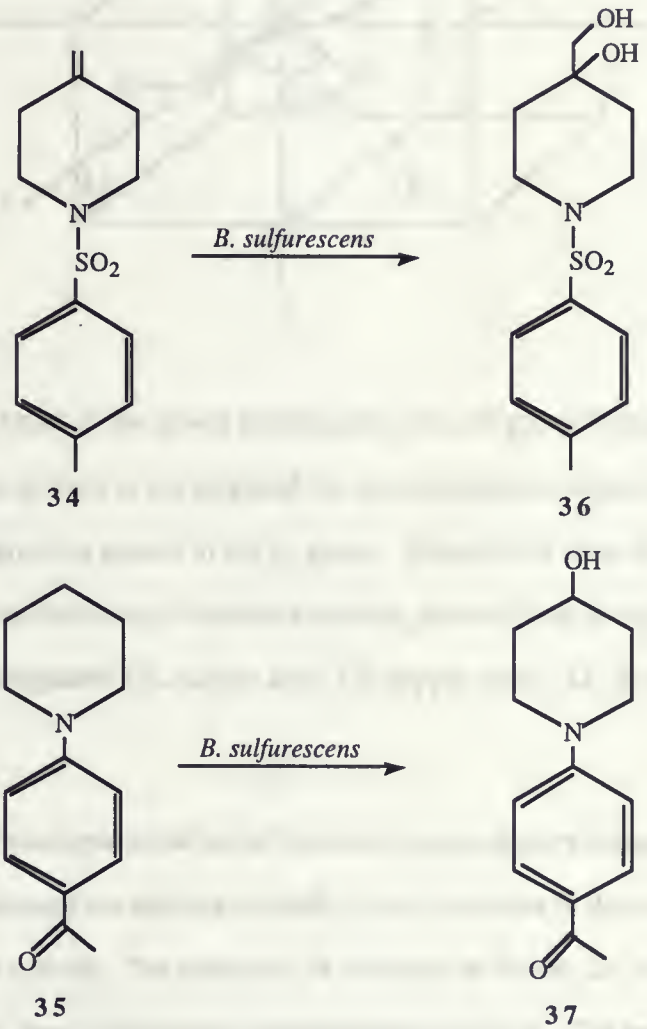
In order to further test this aspect of selectivity, the same research group used N-benzoyl-2-aminoadamantane **29** and N-methyl-N-(4-methylene adamant-1-yl) benzamide **30** as substrates.[36] As expected, the hydroxyl group was oriented *trans* relative to the benzamide group in product **31**. (Figure 23) The epoxides **32** and **33** were products of bioconversion of substrate **30**, with oxygen delivered to the olefin from the opposite direction (*trans*) from the benzamido substituent. (Figure 23)

Figure 23: Bioconversion of adamantanes



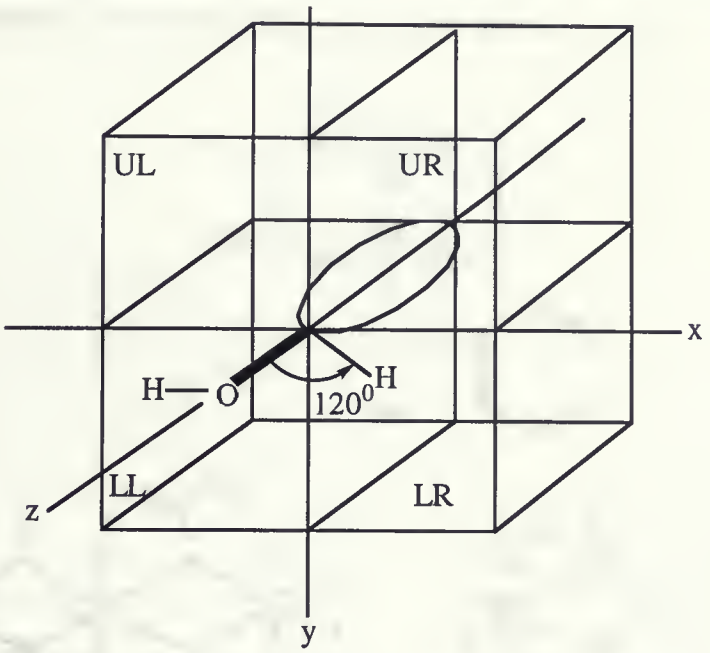
The same paper reported bioconversion of two piperidine derivatives: 4-methylene-1-(p-toluene sulfonyl) piperidine **34** and 4-piperidino acetophenone **35**. The substrate **34** was converted into the diol **36** (*via* an epoxide intermediate), and the compound **35** was hydroxylated at C-4 giving product **37**. (Figure 24)

Figure 24: Hydroxylation of piperidine derivatives




By examining the stereochemical features of various rigid molecules which were successfully hydroxylated, the attempt of mapping the enzyme contours was carried out using the octant system[35]. (Figure 25)

Figure 25. Octant system for defining orientation of substrate-product molecules in space




In this system, the C-O bond of the newly introduced hydroxyl group is extended along the z axis, the hydroxylated carbon is fixed at the origin of the xyz coordinate system, while the C-H bond of the hydroxymethylene group is placed in the yz plane. When this is done the hydroxylated products appear similar to Newman projection formulas, and the bulk of each molecule falls into one of four quadrants designated UL (upper left), UR (upper right), LL (lower left), or LR (lower right).

The products obtained from hydroxylation of 1-benzoyl-*trans*-decahydroquinoline **38**[34] and its analogs indicated a preference for placing the bulk of the molecules in the upper right (UR) rear octant of the coordinate system. The substrate **38** is shown in Figure 26, with arrows indicating sites of hydroxylation in three different biotransformation products. The projection formulas of two major products (4a*S*, 5*S*, 8a*R*)-1-benzoyl-*trans*-decahydroquinolin-5-ol **39** and (4a*S*, 6*S*, 8a*S*)-1-benzoyl-*trans*-decahydroquinoline-6-ol **40** are also presented in the Figure 26. The results obtained with this compound and the others suggest that more space is available to the substrate molecule in the UR octant with respect to the incoming C-O bond.

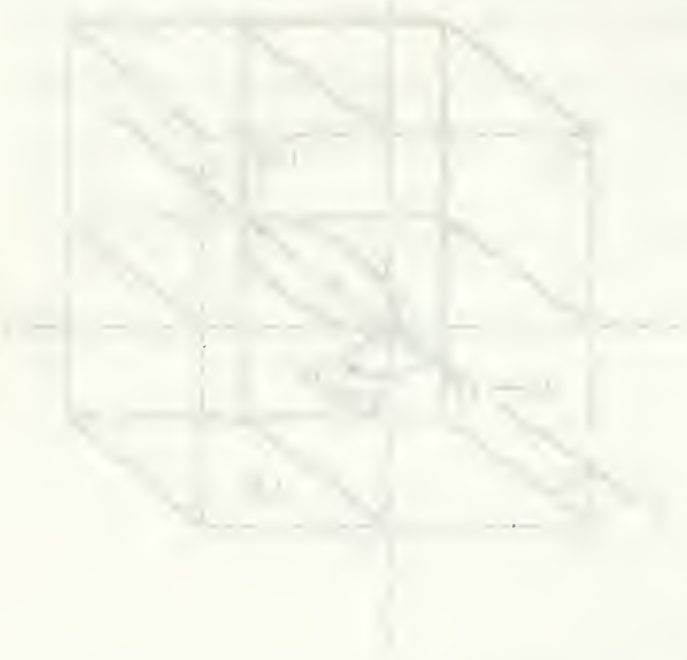
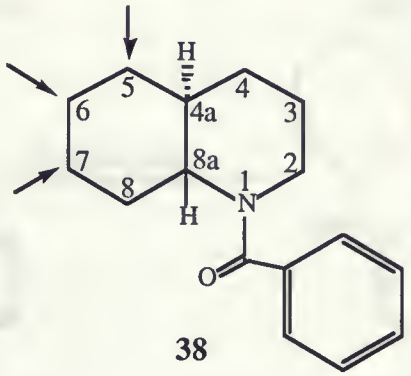
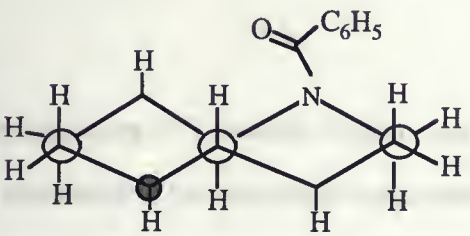


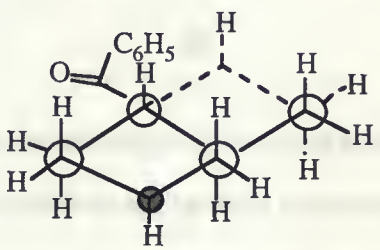
Figure 26: Projection formulas of bioconversion products of 1-benzoyl-*trans*-decahydroquinoline **38**. Heavy dot indicates C-O bond projecting toward the viewer




**38**




**39**

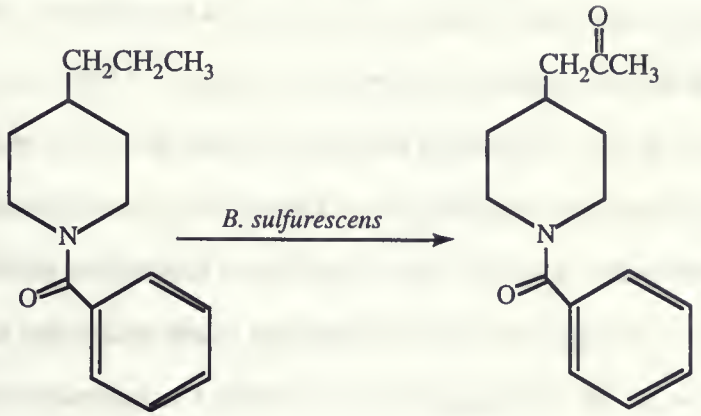



**40**

It was noted[35] that oxygenation of some compounds gives only hydroxylated products, while some also give ketones. The ketone groups were found at the same position, suggesting that the hydroxy compound is an intermediate in the formation of the ketone. The rigid molecules give predominantly hydroxylated products, whereas molecules with higher degree of conformational mobility tend to give ketones as a biotransformation products. The suggestion here was that the greater conformational mobility allows hydroxylated compounds to be converted to ketones by alcohol dehydrogenase enzymes of the microorganism .[35]

One of the examples which confirm the theory of further oxidation of conformationally mobile substrates was the bioconversion of 1-benzoyl-4-n-propylpiperidine **41** to 1-benzoyl-4-(2-oxo)-propylpiperidine **42** as the only product. This was also first example of oxygenation occurring in the noncyclic portion of a substrate.[37] (Figure 27)

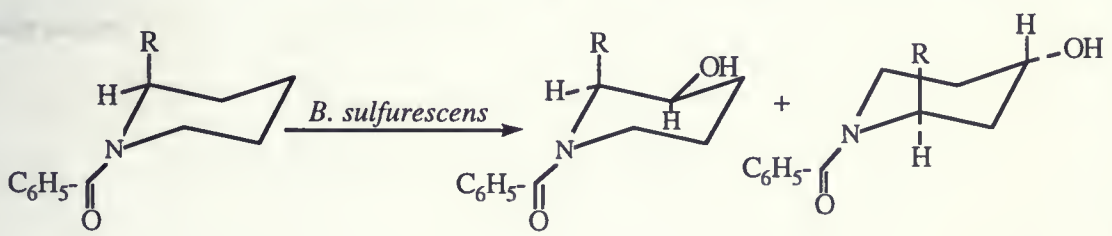
Figure 27: Oxygenation of 1-benzoyl-4-n-propylpiperidine **41**




**41** **42**

The homologous series of 2-methyl, 2-ethyl- and 2-n-propyl-substituted piperidine substrates (**43, 44, 45**) was submitted to the microbial oxygenation and similar products were obtained from each.[37] The preferred conformation of the substrate molecules is the one in which the 2-alkyl group is in an axial configuration. Two major products isolated from each bioconversion are a 3-hydroxy and a 4-hydroxy compound in which the configuration of the hydroxyl group is equatorial in each case. This observation is consistent with the previously mentioned *trans* relationship between the introduced hydroxyl group and the electron-rich amide group. The substrates and the major products of their bioconversion are shown in Figure 28.

Figure 28: Microbial oxygenation of 1-benzoyl-2-alkylpiperidines




**43**, R=$CH_3$ **46**, R=$CH_3$ **47**, R=$CH_3$

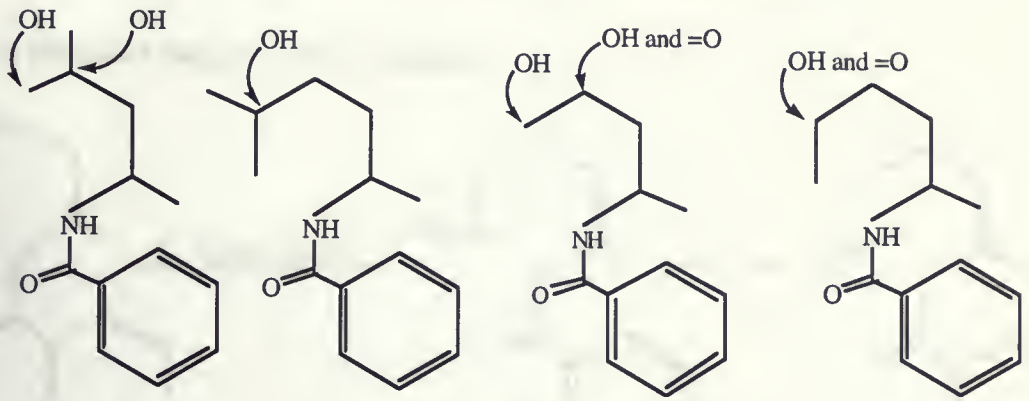
**44**, R=$C_2H_5$ **48**, R=$C_2H_5$ **49**, R=$C_2H_5$

**45**, R=n-$C_3H_7$ **50**, R=n-$C_3H_7$ **51**, R=n-$C_3H_7$

Another similarity between the products is their optical activities; all 3-hydroxylated products had positive optical rotation, and all three 4-hydroxylated products had negative optical rotation, measured in the same solvent.[37] Because of this the authors suggested that the formation of optically active products from these racemic substrates followed the analogous reaction pathway for each substrate: either because of preferential hydroxylation of one enantiomer of the substrate at a single position or further preferential metabolism (and loss) of one enantiomer of the product molecules. The spatial orientation model outlined previously was applied in the attempt to predict the outcome of the bioconversion of 1-benzoyl-2-methylpiperidine **43**. As was expected, the bulk of the processed enantiomer was placed in the upper right rear octant in **46**, and in **47**, besides occupied UR, methyl group was placed slightly in the lower right octant in the converted enantiomer, suggesting that this area of space is also available to the substrate molecule when in contact with the hydroxylating enzyme.

A series of acyclic N-alkylbenzamides of four to seven carbon chain length (branched and straight chain) was also submitted to the biotransformation with *B. sulfurescens*[38] in order to investigate whether a fragmented cyclic system like **15** (Figure 18) could still be hydroxylated. It was found that the longer chain substrates were oxygenated mainly at the subterminal carbon furthest from the benzamido group, whereas shorter chain length substrates were bioconverted at either the terminal or the subterminal carbon atom. Some examples are shown in Figure 29, with arrows indicating site of the oxygenation. Yields were in the range of 22-59%, and almost all products were optically active.

Figure 29: The microbiological oxygenation of acyclic N-alkylbenzamides




**52** **53** **54** **55**

It was found that the 1-*(S)*-enantiomer reacted faster in each case than the 1-*(R)*-enantiomer of the racemic substrate. Additionally, oxygenation of the five-carbon chain substrates, **52** and **54**, occurred at a different position in each enantiomer, suggesting that stereochemistry at C-1 influences the oxygenation reactions. Again, the oxygenation occurred at carbon not closer that 5.5 Å from the amide group. Preference for oxygenation of the more substituted carbon (next to methyl group) was evident in most of the substrates studied .

N-(1-Methylhexyl) benzamide **56** was bioconverted into a secondary hydroxylic product **57**, a ketonic product **58** and two carbon shorter product **59**[38] (Figure 30). It was proposed that reaction analogous to the chemical Baeyer-Villiger reaction took place, and that the acetate intermediate was hydrolyzed to give primary alcohol as a product.

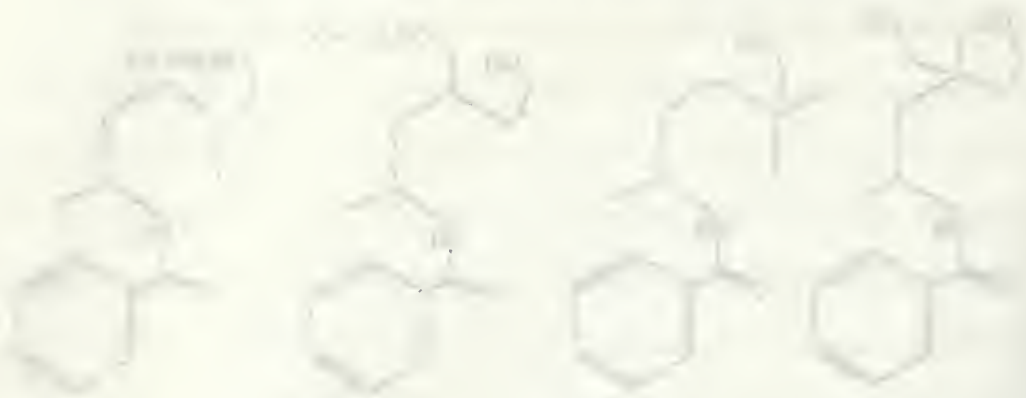
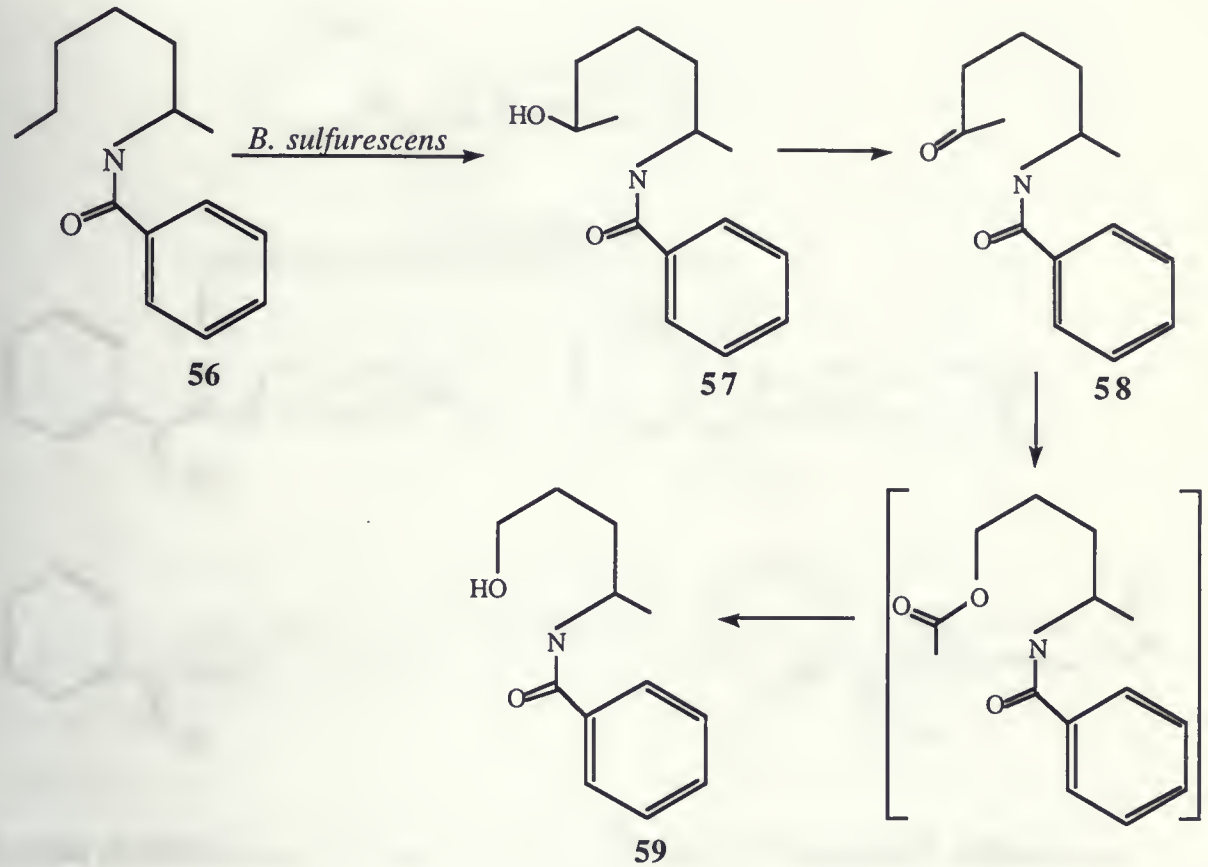


Figure 30: Microbial Baeyer-Villiger oxidation




Johnson et al. studied oxygenation of several N-cycloheptyl benzamides by fermentation with *B. sulfurescens*.[39] Biotransformation of N-cycloheptylbenzamide **60** gave N-[(1*S*)-4-oxo cycloheptyl] benzamide **62** and N-[(1*S*, 4*S*)-4-hydroxycycloheptyl] benzamide **63**. (Figure 31)

N-methylated analog **61** gave, under the same conditions, ketone **64** with a reversed enantioselectivity (methylated **62** and product **64** are enantiomers). (Figure 31)

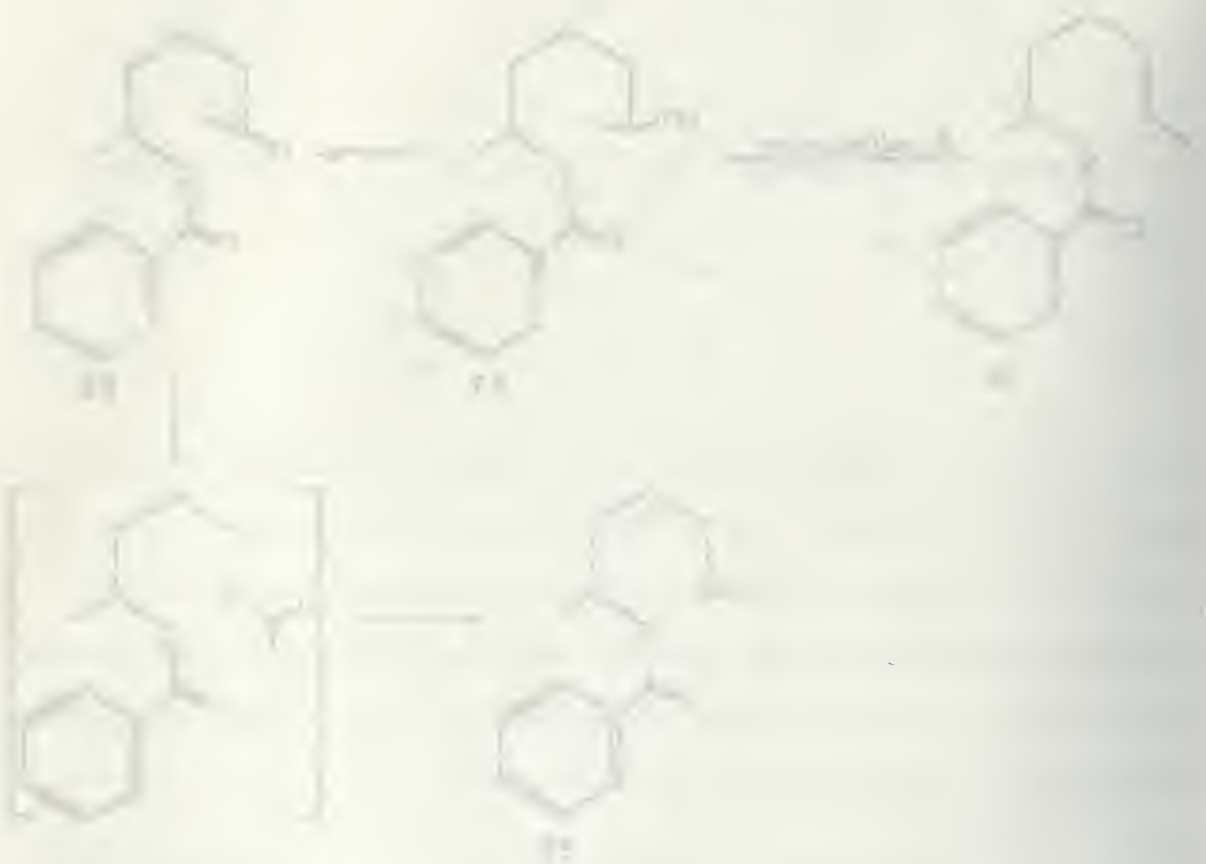
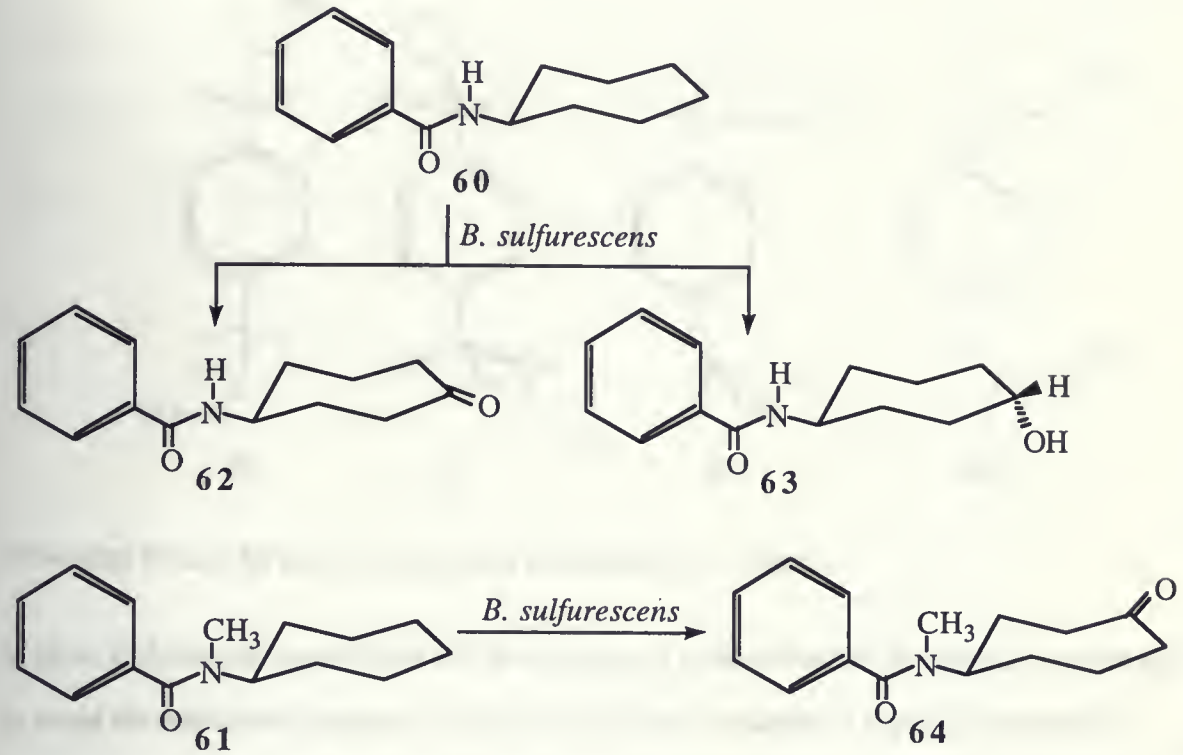


Figure 31: Oxygenation of N-cycloheptyl benzamides




A series of dialkylbenzene substrates was subjected to bioconversion by *B. sulfurescens*. Although these compounds do not have the electron-rich amide group thought to be necessary for the enzyme binding, side chain hydroxylation was observed in most of the products.[40] (Figure 32) Comparing the oxygenation of substrates containing isopropyl (i.e. **66**, **67**) and those with isobutyl groups (i.e. **68**) suggested that the former are oxygenated exclusively or predominantly on the primary carbon of the methyl group, whereas oxygenation of the latter occurs predominantly on the tertiary carbon of the isobutyl group. This was explained as a preference for hydroxylation at more highly substituted carbon, but that this preference is counteracted by the benzene ring when it is too close to the tertiary center.

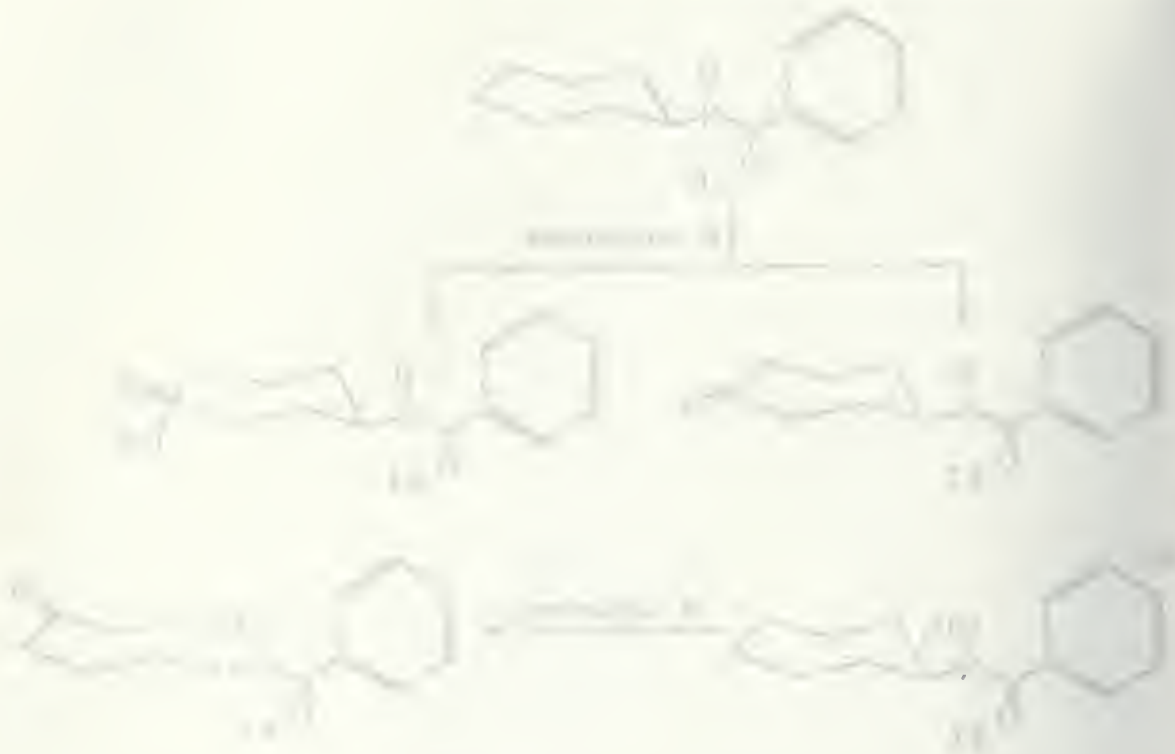
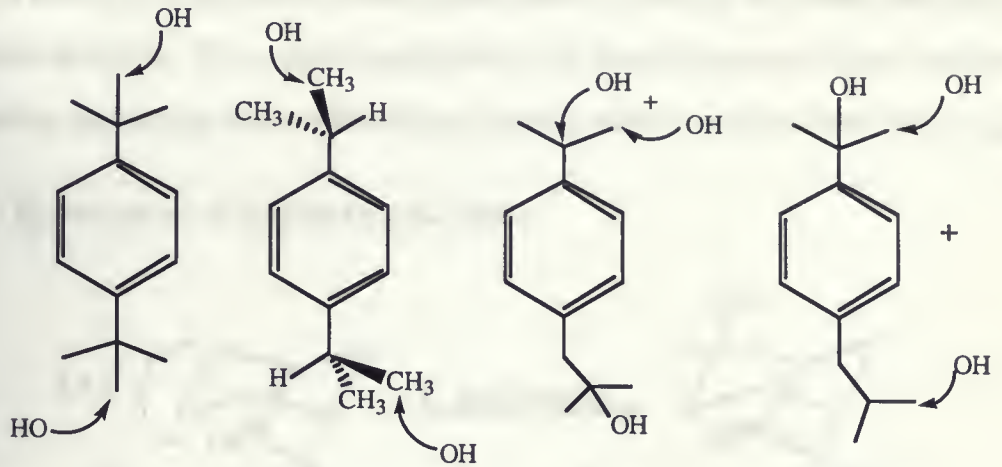


Figure 32: Microbial oxygenation of dialkybenzenes




**65** **66** **67** **68**

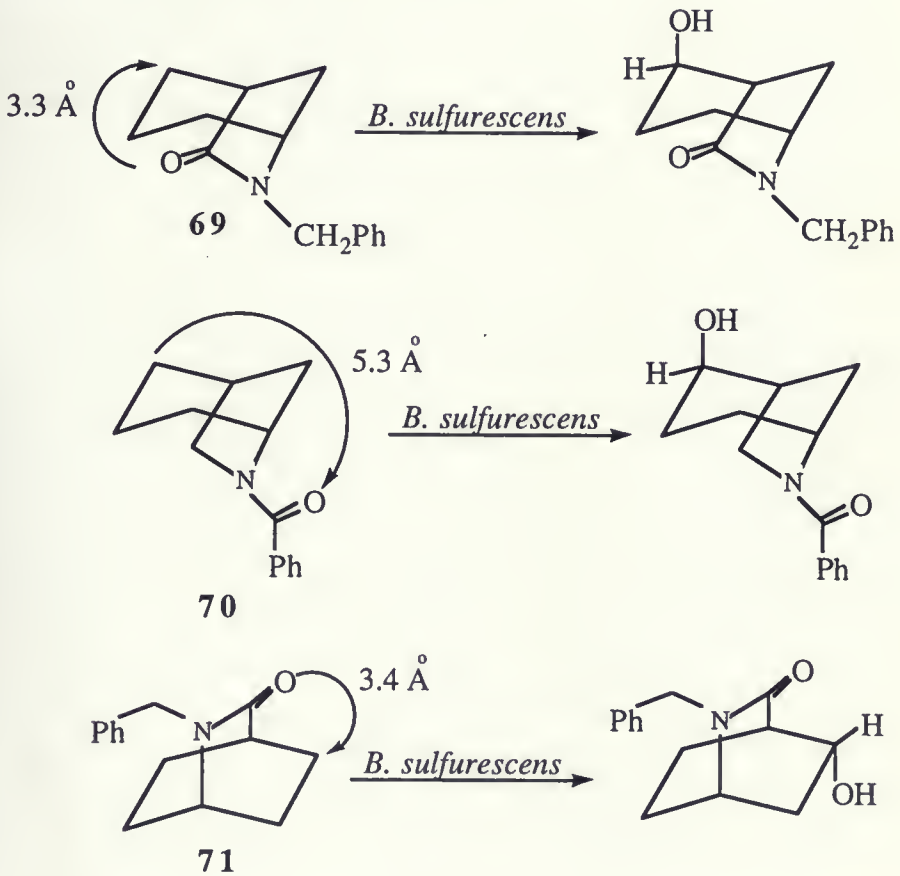
Substrates **67** and **68** gave two products (indicated with + sign).

In order to determine geometrical and stereochemical requirements of the enzyme's active site, and to avoid the considerable number of degrees of freedom available to linear or monocyclic compounds, Furstoss et al. studied bridged bicyclic and polycyclic amides.[41] Preliminary results showed that only aromatic compounds led to hydroxylated products, which was explained by either specific interaction of the phenyl group with the hydrophobic site of the enzyme or by lipophilic character of aromatics allowing penetration of the substrate through the cytoplasmic membrane of the fungi, (or both). Some of the results are shown in Figure 33. The regio- and stereoselectivity of the hydroxylation was compared in bioconversions of substrates **69**, **70**, **71**, and the previously reported bioconversion of **21** (Figure 20). Two conclusions were made:

- the position of the carbonyl group is not the determining factor for regioselectivity of hydroxylation (the directed hydroxylation cannot be obtained by moving the carbonyl function from one carbon to another)

- the distance between the carbonyl oxygen and the site of hydroxylation is not necessary 5.5 Å. This can be seen in the examples in Figure 33: Hydroxylation of **69** and **71** takes place at the carbons 3.3 and 3.4 Å respectively, although other carbons closer to the earlier proposed desired distance were available. The authors suggested the here observed regioselectivity could be determined by the nitrogen atom or phenyl ring position, rather than that of the carbonyl group.

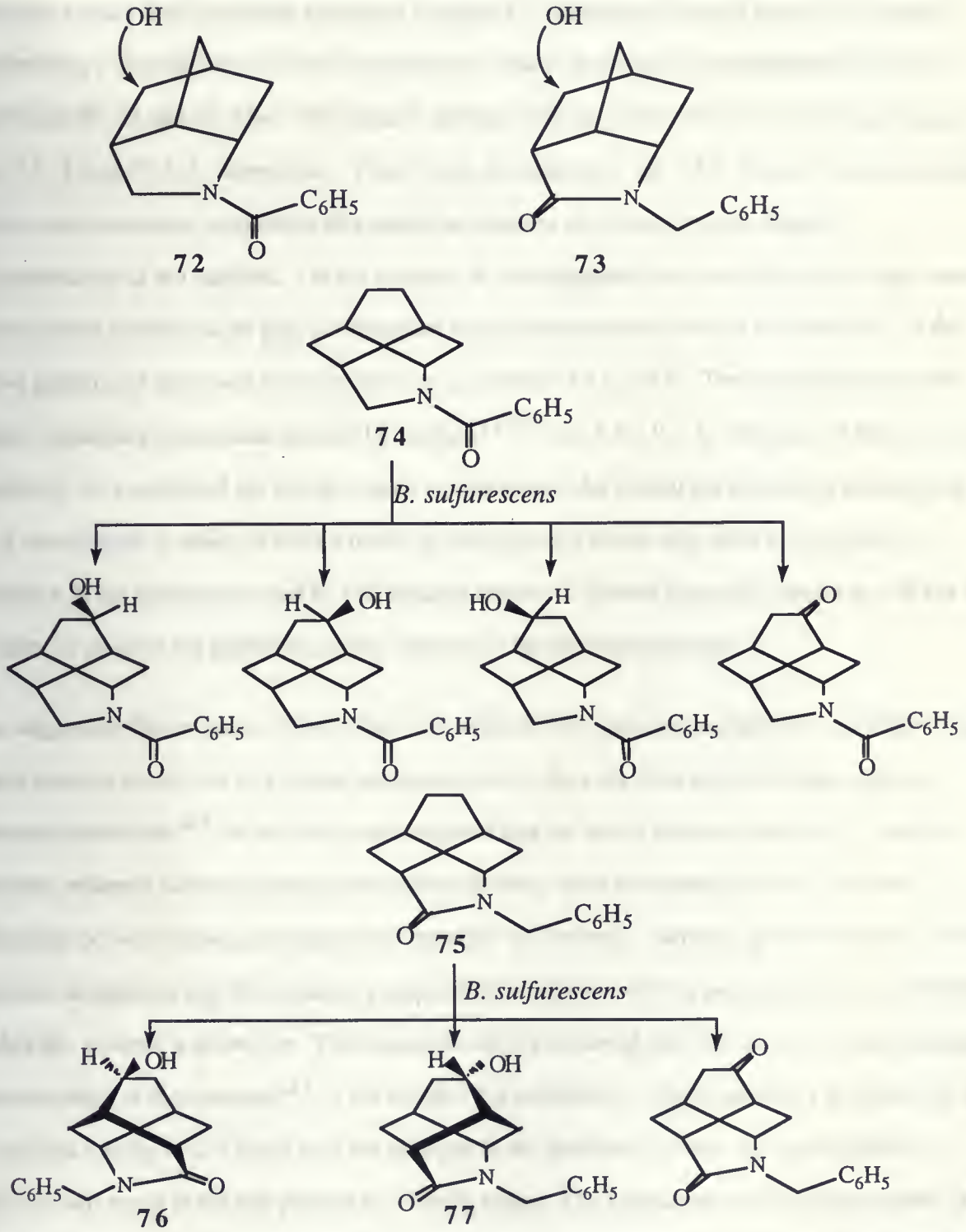
Figure 33: Hydroxylation of bridged bicyclic amides

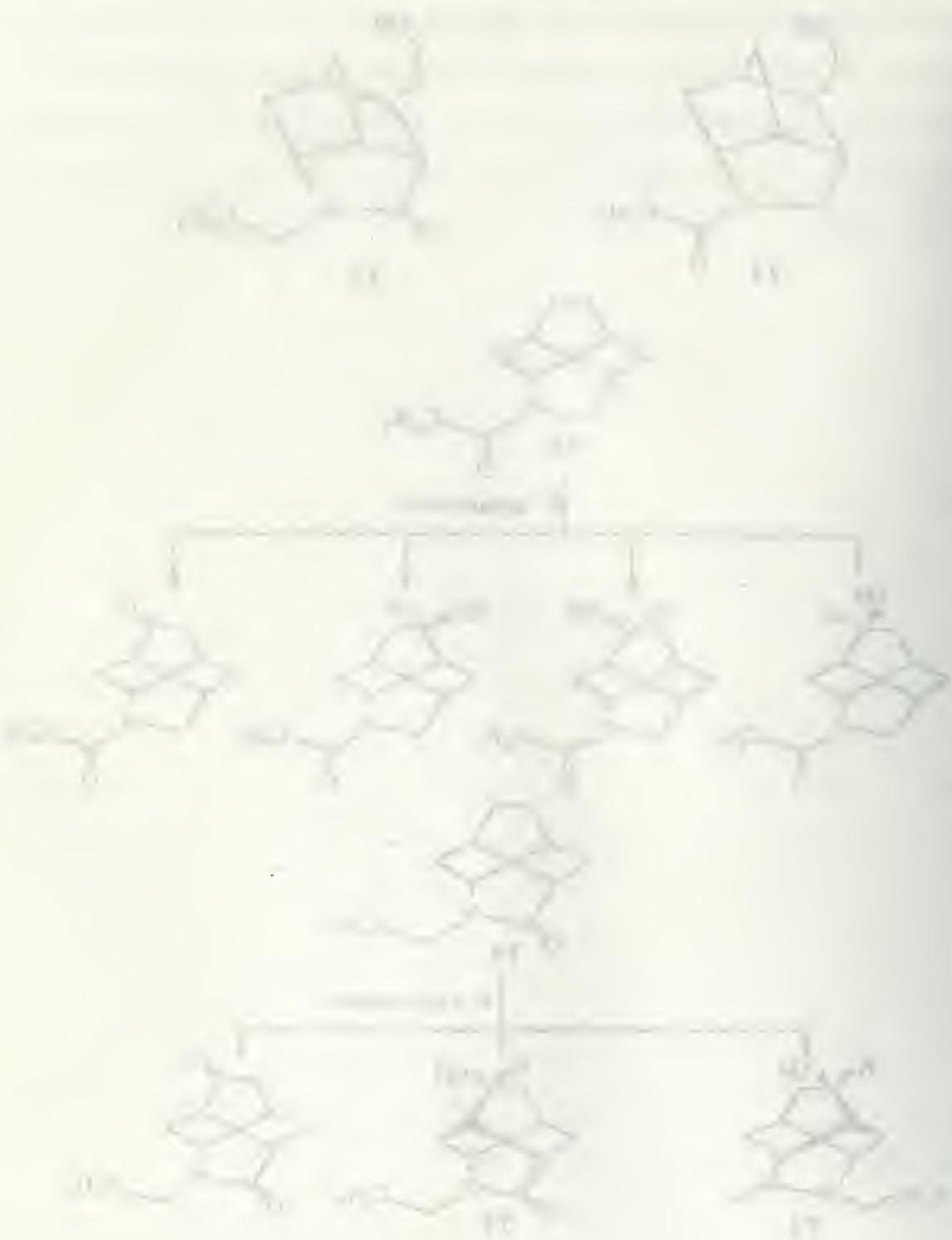



In order to further examine biohydroxylation using *B. sulfurescens* Furstoss et al. studied bioconversion of some globular type amides and lactams.[42] They were interested to see if higher lipophilicity of the globular compounds (globular here referring to the geometry of the substrate) would have any influence on regio- and stereoselectivity of the hydroxylation, especially when compared with bioconversion of the corresponding lactams. Some results of this study are shown

in Figure 34. It was observed that, despite their sterically crowded skeleton, globular type molecules are good substrates for hydroxylation with *B. sulfurescens*. The yields and regioselectivities of the hydroxylations were not altered by the lipophilic character of these compounds. Finally, carbonyl oxygen location did not influence the regioselectivity of the hydroxylation. The stereoselectivity was higher for lactams (less byproducts formed) than for "external" amide substrates, which was attributed to the rigidity of the amide moiety (no rotation around N-CO bond).

Figure 34: Regioselective biohydroxylation of azabrendane (**72, 73**) and azatwistane (**74, 75**) derivatives and corresponding lactams



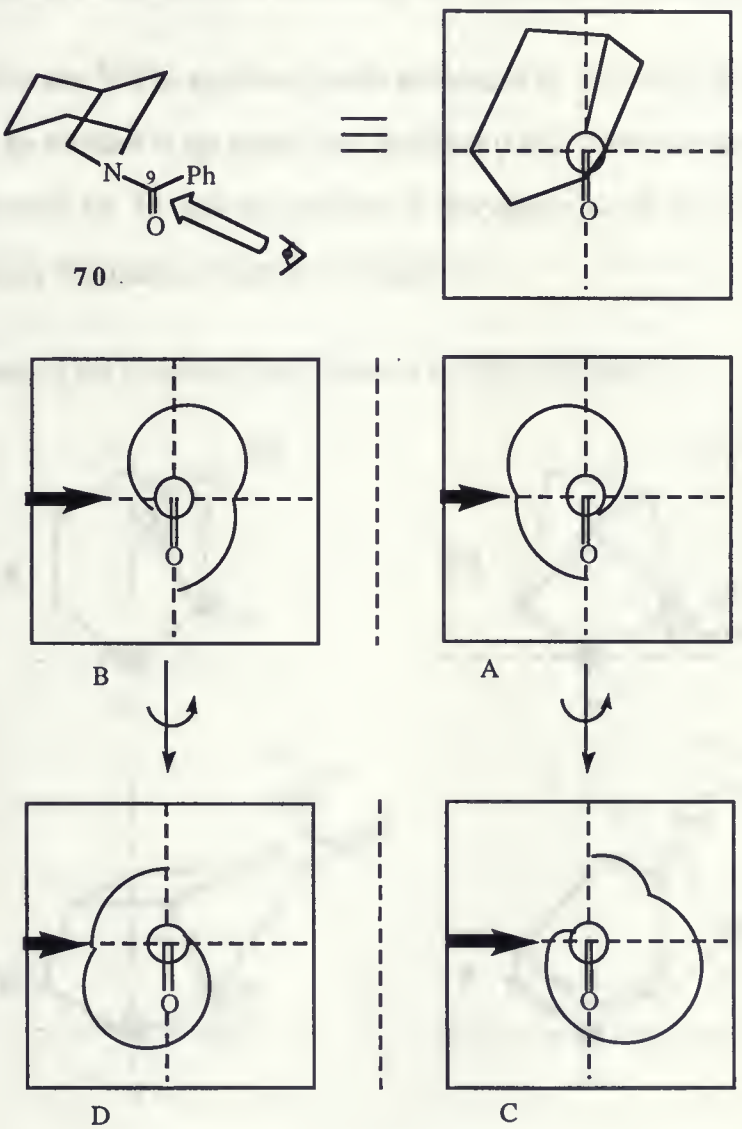


A more complete analysis of these results was done by Archelas and Furstoss[43] by comparing the distances between the site of hydroxylation and amide or carbonyl group, or nitrogen atom. It was concluded again that previously suggested required 5.5 Å distance does not seem to be a factor determining regioselectivity of the hydroxylation. This is illustrated in the biohydroxylation of substrates **69**, **21** and **72** where the distance between hydroxylated carbon and carbonyl oxygen was 3.3, 3.4 and 3.3 Å respectively. Even more, the carbons at the 5.5 Å distance were available in the same molecules, suggesting this particular distance as a base for predicting the regioselectivity is not justified. On the contrary, it was suggested that the distance (nitrogen atom-hydroxylated carbon) might play an important role in the regioselectivity of oxygenation. In the above mentioned substrates this distance was an average $3.4 \pm 0.6$ Å. The same distance in the amide containing compounds studied by Fonken[31-37] was $3.5 \pm 0.7$ Å. Because of this striking similarity, the position of the nitrogen atom was thought to be crucial for binding to the enzyme. If so, it would make it easier to build a model of the enzyme's active site, since in that case the existence of the rotamers caused by free rotation around C-N bond in acyclic amides would not be a matter of concern for prediction of the outcome of the biotransformation.

The suggestion that position of the carbonyl function is not a determining factor in regioselectivity of the reaction could lead to a wrong assumption that it does not have any role in the enzyme-substrate interaction.[43] In fact, the results showed that the amide bioconversion led to racemic alcohols, whereas lactams gave optically active alcohols upon biotransformation. The only difference between these substrates is the "internal" or "external" carbonyl group within the carbon skeleton, suggesting that the carbonyl group indeed interferes with the positioning of the substrate within the enzyme's active site. The enantioselectivity observed with the lactams comes from the non-existence of the rotamers.[43] If the amide **70** is positioned in the quadrant as in Figure 35, and if one looks along N-C-9 bond with the nitrogen in the quadrant's center, the hydroxylation reaction will occur at the site pointed by a heavy arrow. The enantiomers of **70** are presented as A and B in Figure 35, and their rotamers, obtained by rotation around N-CO bond by $180^0$ are C and

D respectively. Overall, A, B, C and D represent all possible spatial configurations of **70**. Each enantiomer will have a rotamer which has bulky part of the molecule positioned in such a way that facilitates the hydroxylation, resulting in a racemic mixture of alcohols as products. On the other hand, lactams do not have rotamers, and each enantiomer has just one possible position, yielding optically active alcohol.

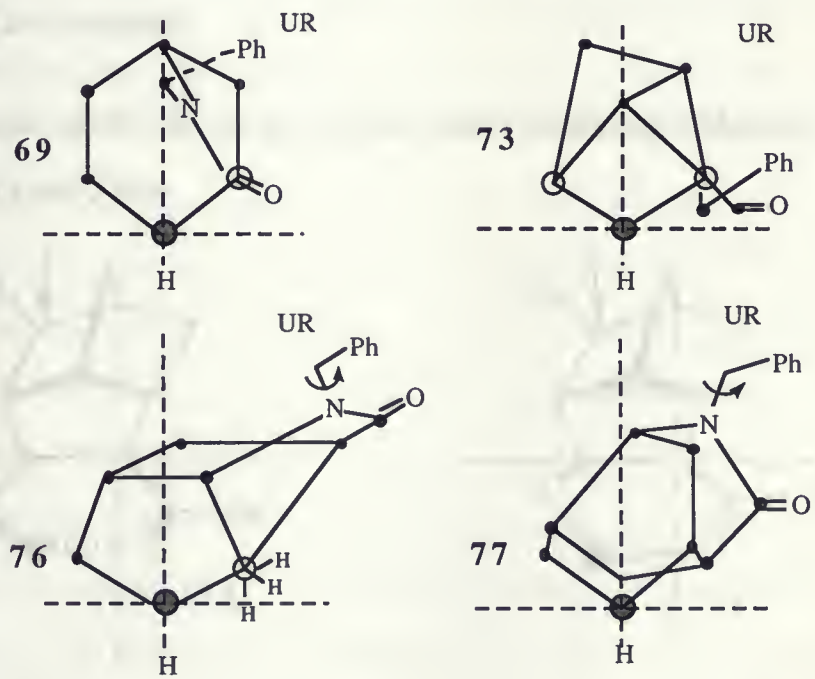
Figure 35: Possible spatial orientation of the substrate **70** and hydroxylation



Stereospecific production of one alcohol suggests that the site of hydroxylation is determined by spatial factors.[43] This does not seem to be supported in the case of bioconversion of **75** to two epimers **76** and **77** (Figure 34). It appears that there is no stereospecificity at all. The absolute configuration at the hydroxylated carbon is *(S)* in both cases. These two products are diastereomers, and their formation is explained in this way: each enantiomer positions itself identically in the enzyme's active site, then both of them are stereoselectively hydroxylated. This means that the reaction is stereoselective, but there is no substrate enantioselectivity.[43]

Topology of the active site:[43] The quadrant model introduced by Fonken[35] proposed that the bulk of the substrate will be situated in the upper right quadrant (UR). The absolute configurations of the hydroxylated lactams **69**, **73**, and the products of bioconversion of **75** (two epimers **76** and **77**) are presented using the quadrant system in Figure 36.

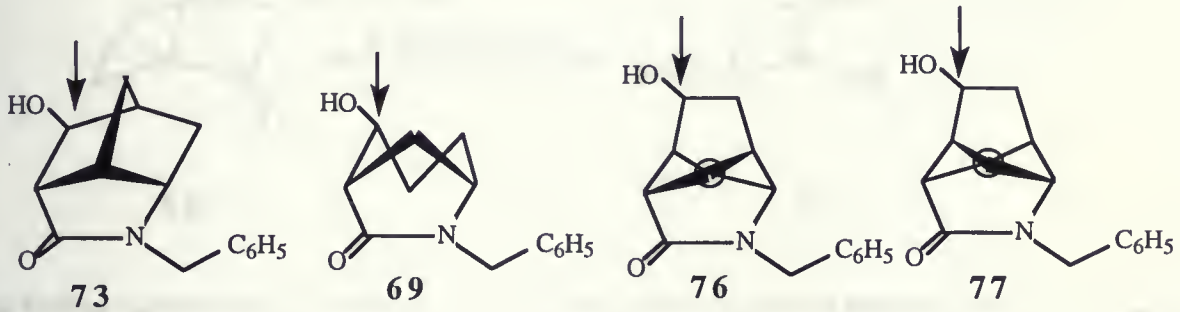
Figure 36: Positioning of the lactams in the Fonken's quadrant system.




Since it is not easy to predict regioselectivity of the hydroxylation using this model, Archelas et al. suggested that by positioning the amide group in plane, with the carbonyl function on the left side,

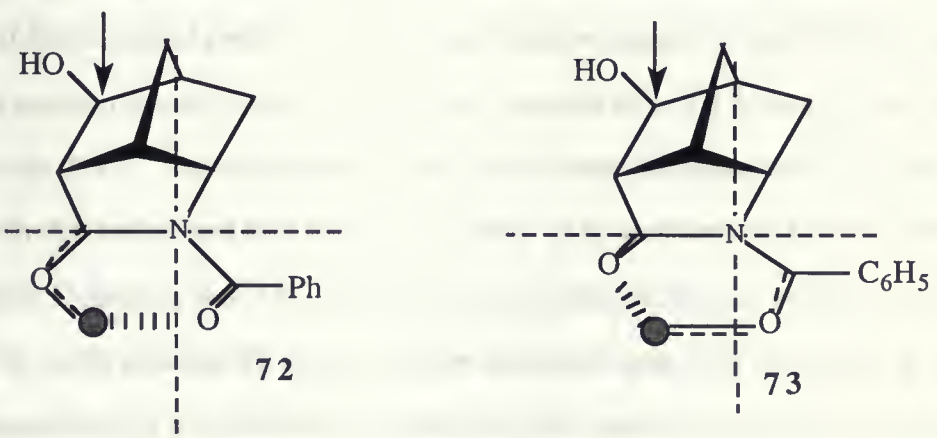
the hydroxyl group clearly comes from the upper left in each substrate, making it easier to predict the outcome of the biotransformation.[43] This is illustrated with the same lactams in Figure 37.

Figure 37: Determination of the topology of the active site; the arrow shows direction of the hydroxyl group introduction




This representation can be applied to other types of amides if the nitrogen atom position is fixed, whereas the carbonyl group position can be changed without affecting the regioselectivity of the reaction (providing that nothing else has changed). This is illustrated in Figure 38, where lactam **73** and amide **72** are compared.

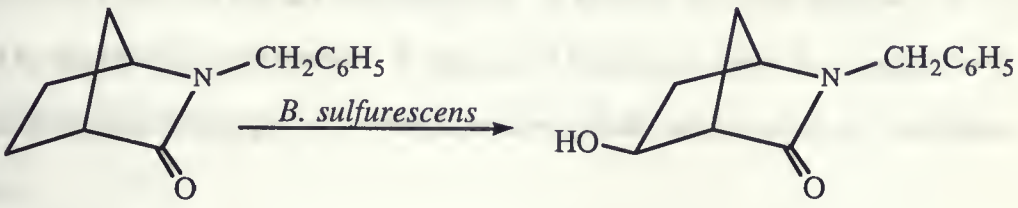
Figure 38: Interaction of the carbonyl group with a coordinating group of the active site (represented with a heavy dot)




As can be seen in this figure, the carbonyl group can be positioned on the either side of the nitrogen atom and still equally interact with the enzyme's binding site (represented with a dotted line).

Biohydroxylation of 2-azabicyclo [2.2.1] heptane **78** gave the optically active 5-exo hydroxylated product **79**, a precursor of carbocyclic 2'-deoxy nucleotides.[44] (Figure 39)

Figure 39: Biohydroxylation of 2-aza bicyclo[2.2.1] heptane **78**




**78** **79**

The hydroxylation using *B. sulfurescens* was further investigated by studying five, six and seven membered lactams, amides and imides.[45] The results obtained from biotransformation of these compounds suggested two conclusions: a) hydroxylation occurred at a non-activated carbon, b) hydroxylation at a position α to the nitrogen atom occurred when process a) was disfavored for geometric reasons. The products obtained were not optically active. Some of the results are shown in Figure 40, including piperidine substrate **15** previously shown in Figure 18. The hydroxylation at a position α to nitrogen atom was observed in lactam substrates and five-membered cyclic amides, whereas six- (Figure 40) and seven-membered (Figure 41) cyclic amides did not give α-hydroxylated products. These results were explained by the fact that nitrogen atom-hydroxylated carbon distance in pyrrolidine lactam substrate **80** is 2.4 Å, and in piperidine lactam substrate **83** was 2.9 Å. The piperidine lactam **83** underwent simultaneously both hydroxylation at the non-activated carbon and α to the nitrogen atom. In seven-membered cyclic substrates, the N-hydroxylated C distance was 3.5 Å and only hydroxylation of non-activated carbon was observed. The imide substrate **82** does not follow the results seen with the amide **81** and lactam **80**, since regioselectivity was different. Furthermore, the seven-membered imide substrate **86** does not give any hydroxylated product. (Figure 41) Having two types of regioselectivity does not prove the existence of two different metabolic pathways. There might be one enzyme capable of activating oxygen and then transferring the same to the substrate (lactam, amide or imide) with

different regioselectivity. Hydroxylation at the position α to nitrogen atom was observed here for the first time, probably because more complex structures have a greater number of non-activated carbons available for oxygenation. Since the isolated products generally were not optically active, the hydroxylation reaction was not stereoselective. In the case of amide substrates, this was explained by the existence of rotamers. In the case of lactams, it seems that a higher level of stereoselectivity would be required in order to obtain optically active products from these substrates.

Figure 40: Pyrrolidine and piperidine series

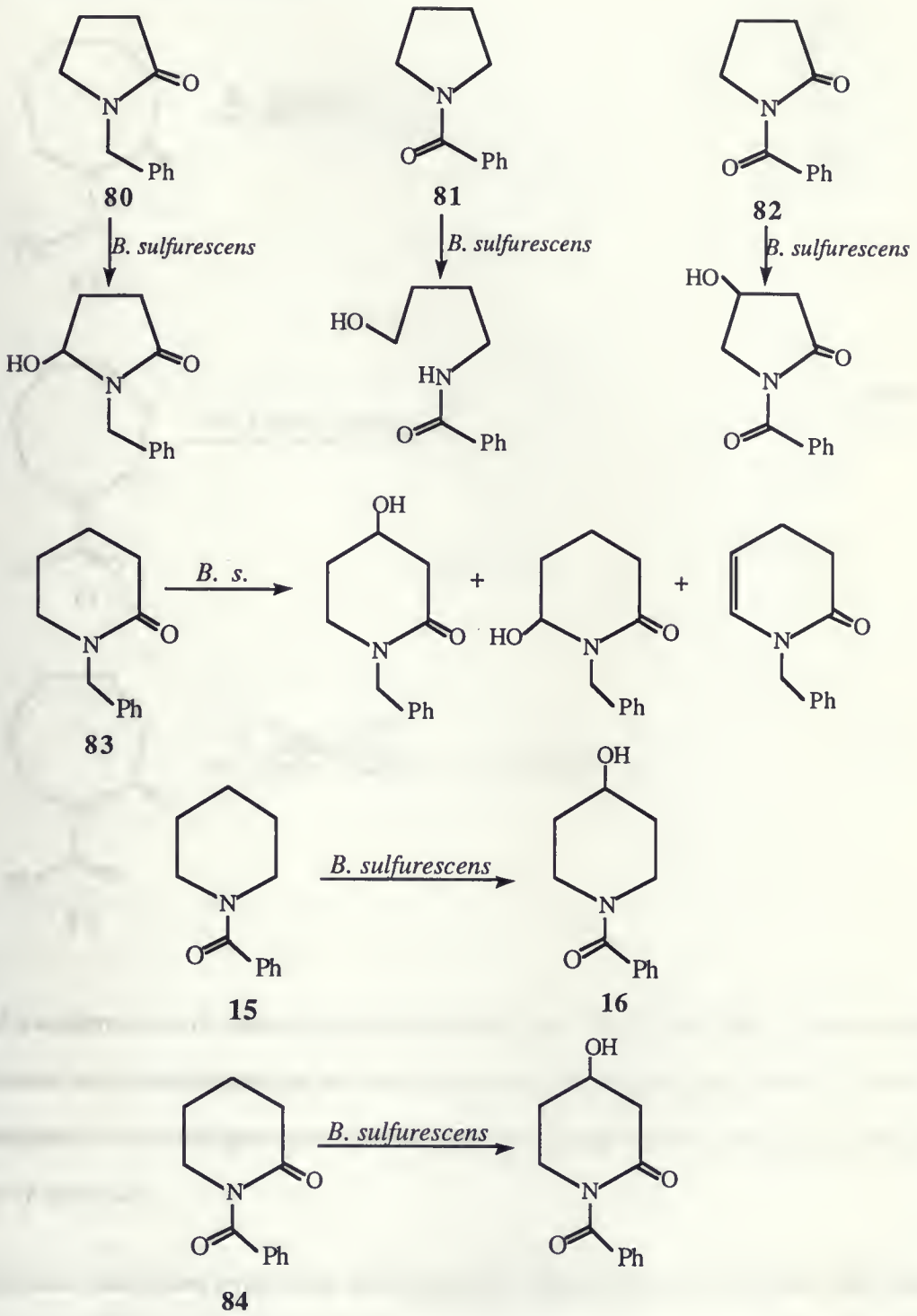
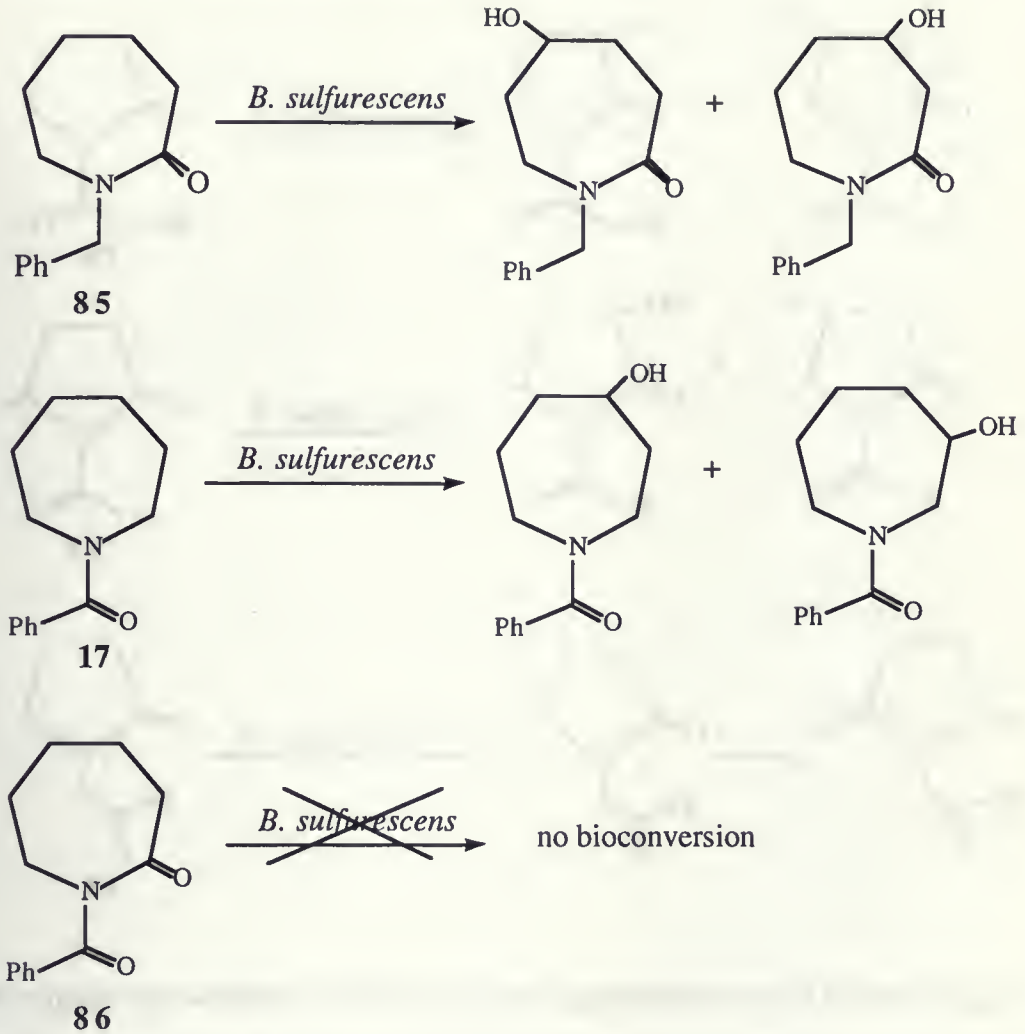


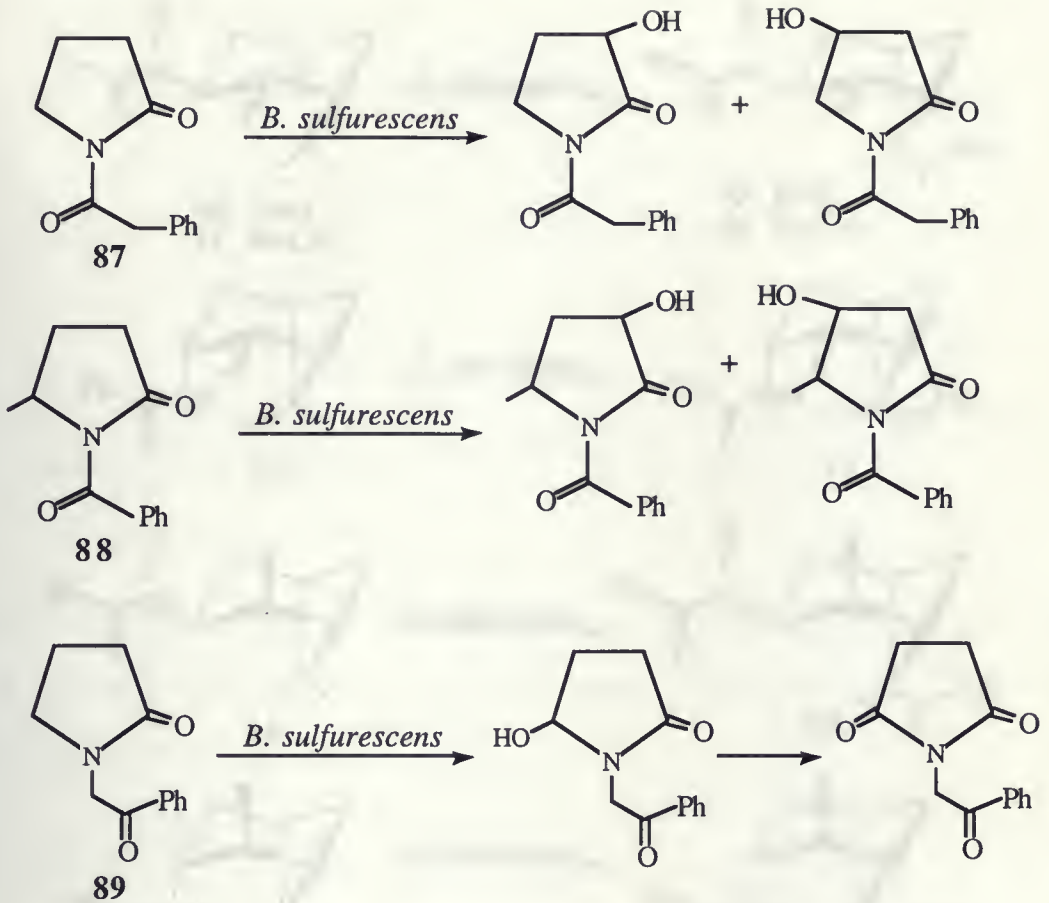
Figure 41: Hexamethyleneimine derivatives




Microbial transformation of various pyrrolidin-2-ones was carried out using *B. sulfurescens*.[46] The substrates were hydroxylated at position 3, 4, or 5 of the heterocycle, depending on the nature of the substituent on the nitrogen atom, position and number of carbonyl groups present in the molecule. (Figure 42)

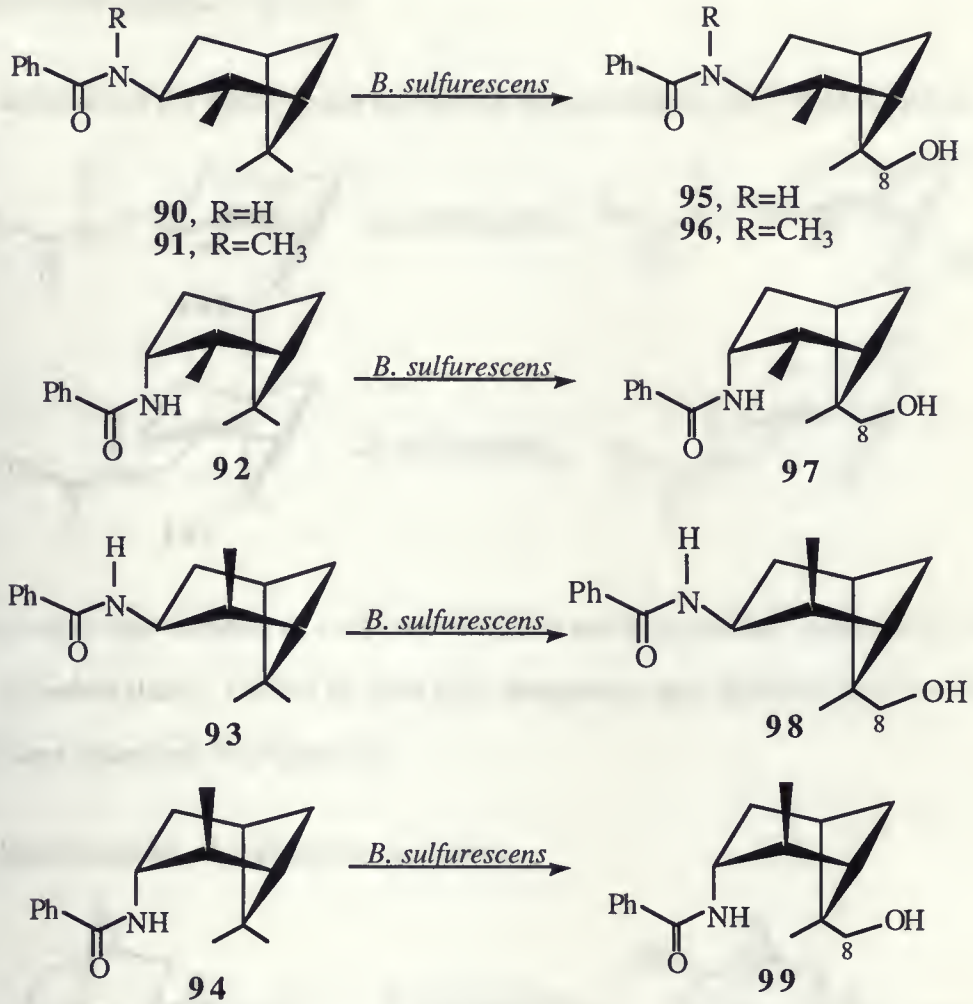
It was suggested that either pyrrolidine derivatives **87**, **88** and **82** are of minimum size limit for one hydroxylating enzyme, or there are two enzymes, one capable of hydroxylating non-activated carbon and another enzyme, which is hydroxylating carbon α to the nitrogen atom (i.e. in substrate **89**, one carbon longer substituent).

Figure 42: Microbial hydroxylation of pyrrolidin-2-ones




In order to explore the possibility of influencing the regioselectivity of the hydroxylation by varying the conformation or localization of the amide group on the substrate molecule, Archelas et al. studied some pinene derivatives.[47] The results presented in Figure 43 and 44 showed that variation of the amide conformation did not influence the stereoselectivity of the reaction, whereas changing the localization of the amide group leads to hydroxylation at a different carbon atom. In each case hydroxylation occurred at C-8, whether the amide group was in an equatorial (**90, 91, 93**) or axial (**92, 94**) position. Of course, these substrates are not very rigid molecules, and flexibility of the cyclohexane ring in the enzyme-substrate complex would allow positioning of the C-8 carbon of the methyl group in the same place in each substrate within the complex.

Figure 43: Hydroxylation of pinene derivatives

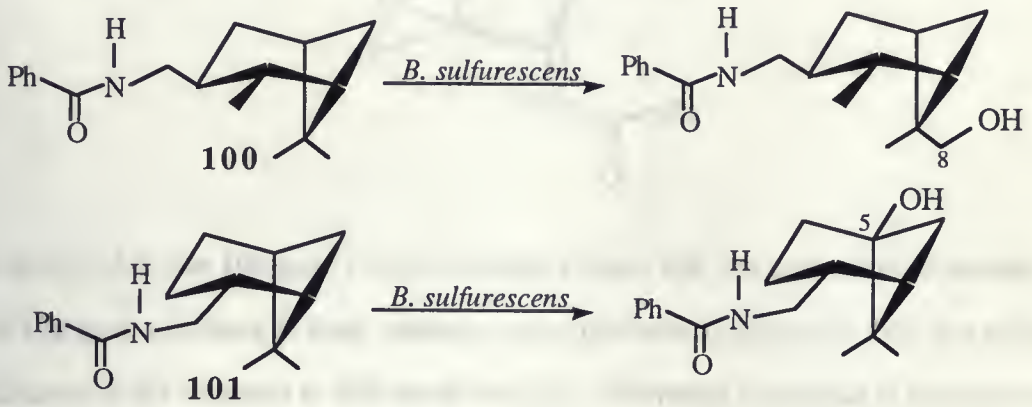



Contrary to earlier results, the hydroxylation was not enantioselective, since all of the products were optically inactive.

Altering the location of the amide group can have an influence on the regioselectivity of hydroxylation. This is illustrated in Figure 44. The substrate **100** (a one carbon longer version of **90**) gave product hydroxylated at C-8, whereas substrate **101**, with a changed position of the amide group, gave product hydroxylated at C-5. These results strengthen the hypothesis that the amide group serves as a binding point in the enzyme-substrate complex, and also show that by

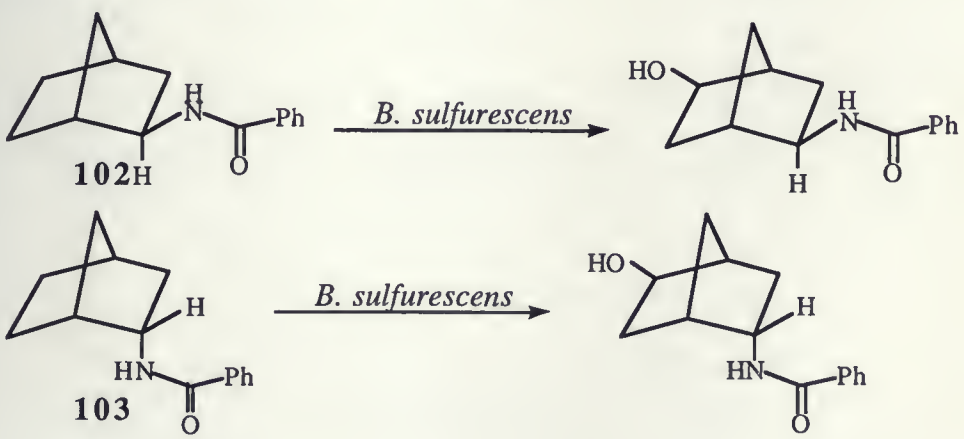
modifying the localization of the amide group within the molecule, one can "choose" the carbon atom at which hydroxylation will occur.

Figure 44: Influence of the amide group location on the regioselectivity of hydroxylation



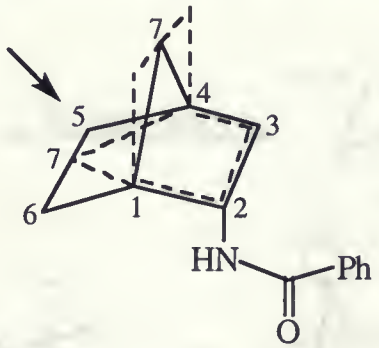

In order to confirm that variation of configuration of the amide group has no influence on the selectivity of hydroxylation, a series of more rigid compounds such as norbornane and camphane derivatives were examined.[48] (Figure 45)

Figure 45: Hydroxylation of norbornane derivatives



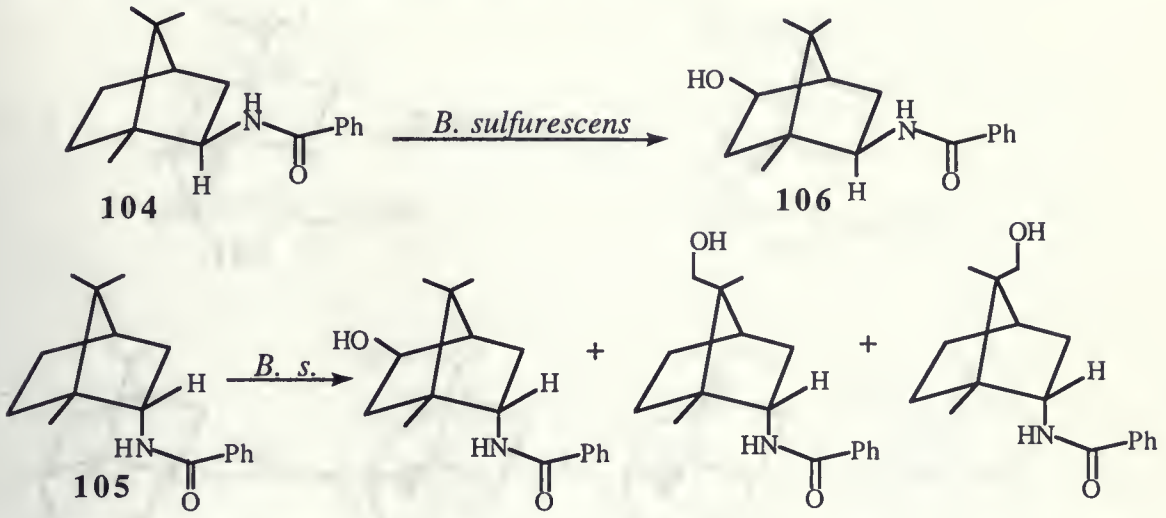

This was still found to be quite surprising, since it would be expected that **102** and **103** would be positioned differently in the enzyme's active site because of the different configurations of the anchoring amide function. The hydroxylation of **102** should have occurred at C-7, and at C-5 in the substrate **103**, when the two substrates are superimposed as shown in Figure 46.

Figure 46: Hypothetical positioning of amides **102** and **103** on the enzyme's active site



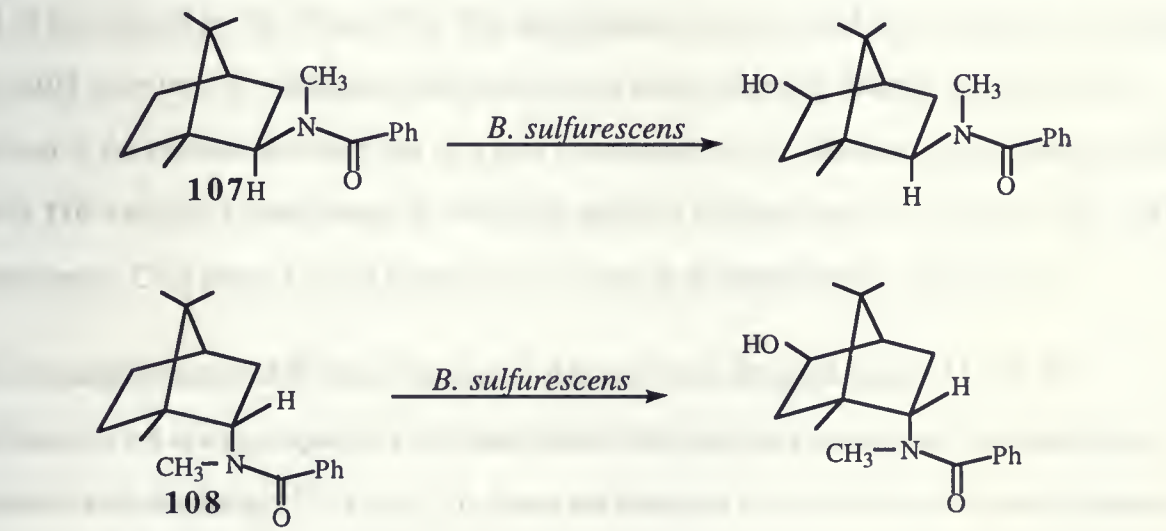

Racemic isobornyl amide **104** gave a single racemic alcohol **106**, but racemic bornylamide derivative **105** gave a mixture of three optically active products. (Figure 47) This was explained by the existence of the rotamers in **105** which would be differently positioned in the active site. It was proven that the two enantiomers of **105** were hydroxylated at the same rate (since recovered starting material was not optically active) and, also, that each of the enantiomers was preferentially hydroxylated at different positions with different rates: (1*R*) enantiomer predominantly at C-5, whereas (1*S*) at C-8. These results suggested that the hydroxylation process is only moderately enantioselective.

Figure 47: Hydroxylation of secondary amides derived from camphor



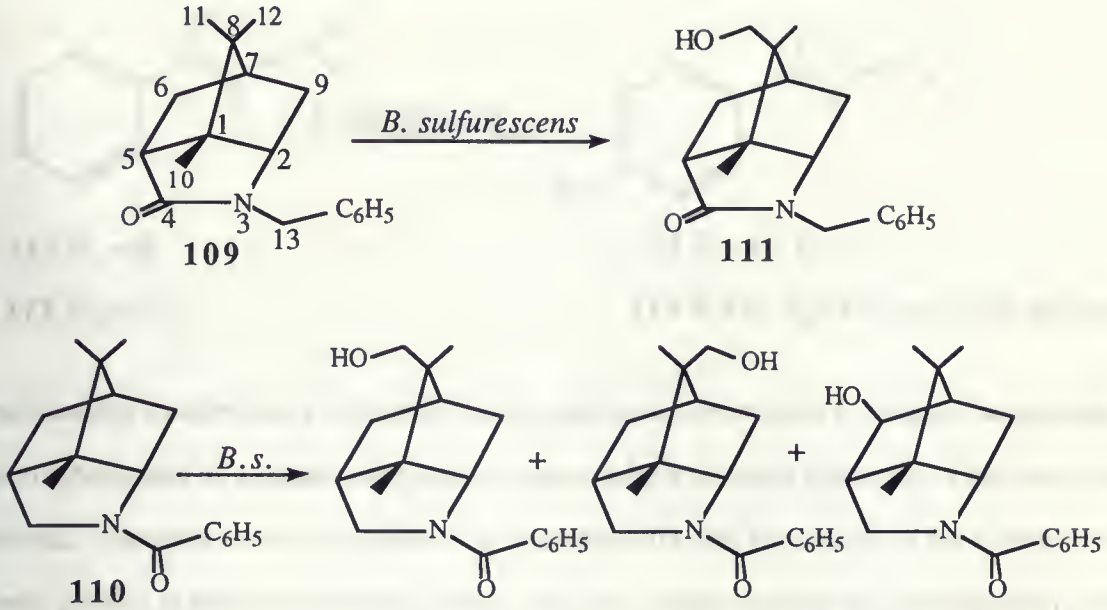

Biotransformation of N-methylated derivatives of **107** and **108** confirmed that regioselectivity could be improved if $C_2$-N rotation is disfavored, since a single product was obtained in both cases. (Figure 48) Lack of optical activity in the products suggested again that no kinetic resolution had occurred at all.

Figure 48: Hydroxylation of the N-methylated amides derived from camphor




Since it was noticed that bioconversion of bornyl amide derivatives could lead to partial enantioselective differentiation[48], rigid substrates such as azabrendane derivatives were used in order to further investigate the enantioselectivity of the hydroxylation reaction.[49] (Figure 49)

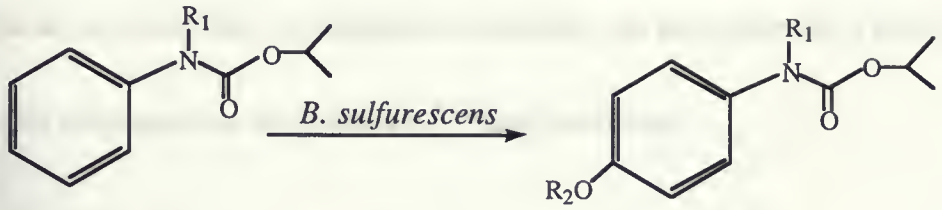
Figure 49: Hydroxylation of azabrendane derivatives




Because of the fact that the bulk of the carbon skeleton appears to be completely located on the one side of the plane of the amide moiety, the spatial difference between two enantiomers would presumably lead to different positioning in the active site of the enzyme, giving different hydroxylated products. The racemic substrate **109** was hydroxylated at C-11, affording product **111** of low optical purity. (Figure 49) The same product was obtained when either 1*R*-(+)-**109** or 1*S*(-)-**109** were used as substrates, with high optical purity, showing that the hydroxylation reaction of the racemic substrate had very poor enantioselectivity. However, bioconversion of the amide **110** was very enantioselective, affording products hydroxylated at C-11 (from 1*R*-(-)-**110** enantiomer), C-12 (from 1*S*(+)-**110**) and at C-6 (from both enantiomers). (Figure 49)

The biotransformation of N-phenylcarbamate **112** and its methylated analog **113** by *B. sulfurescens* led to a regiospecific p-hydroxylation which was the first aromatic hydroxylation observed with this fungus.[50] (Figure 50) Since the chemical hydroxylation of aromatic rings is very difficult to achieve and it is never achieved with complete p-regioselectivity, the microbial hydroxylation could be of interest for specific synthesis of phenol derivatives.

Figure 50: Bioconversion of N-phenyl carbamates




**112** $R_1$=H

**113** $R_1$=$CH_3$

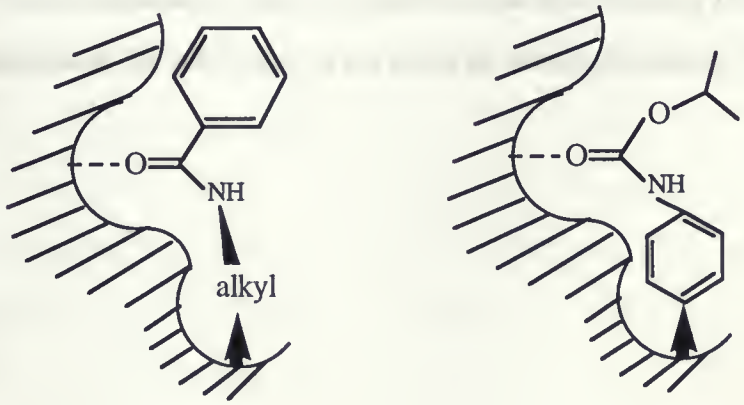
**114** $R_1$=H, $R_2$=H

**115** $R_1$=H, $R_2$=4-O-methyl-β-glucose

The mechanistic studies with p-methylated and p-deuterated derivatives confirmed the generally accepted mechanism of aromatic monohydroxylation which involves formation of an arene oxide intermediate followed by rearrangement involving the NIH shift (migration of the substituent originally present at the site of hydroxylation). It is very surprising that no hydroxylation of the alkyl group was observed, even though the distances between the oxygen atom or nitrogen atom and the isopropyl methyl group are 4.5 and 2.5 Å respectively. This could be explained either by different positioning of the urethane substrate in the active site (compared to the amide substrate, Figure 51), or by the existence of different enzymes for these two types of substrates.

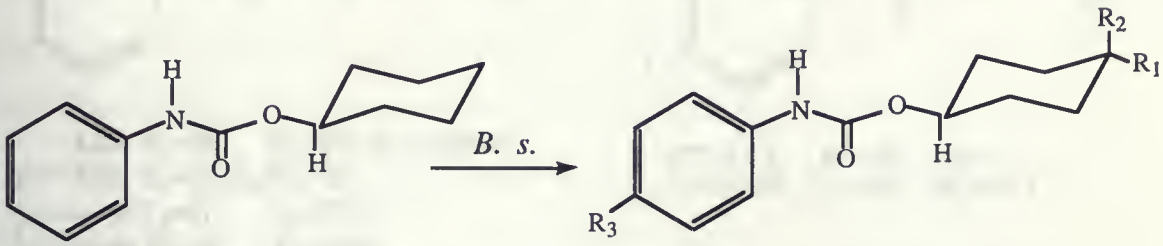
The observed p-hydroxylation mimics the mammalian hydroxylation pathway of detoxification which could be used for the synthesis of various metabolites of aromatic xenobiotics.

Figure 51: Different positioning of amide and urethane substrates in the enzyme's active site



The aromatic hydroxylation was further explored with cyclohexyl N-phenyl carbamate **116**.[51] In addition to p-hydroxylation, cyclohexyl oxygenation was also observed. (Figure 52)

Figure 52: Bioconversion of cyclohexyl N-phenyl carbamate



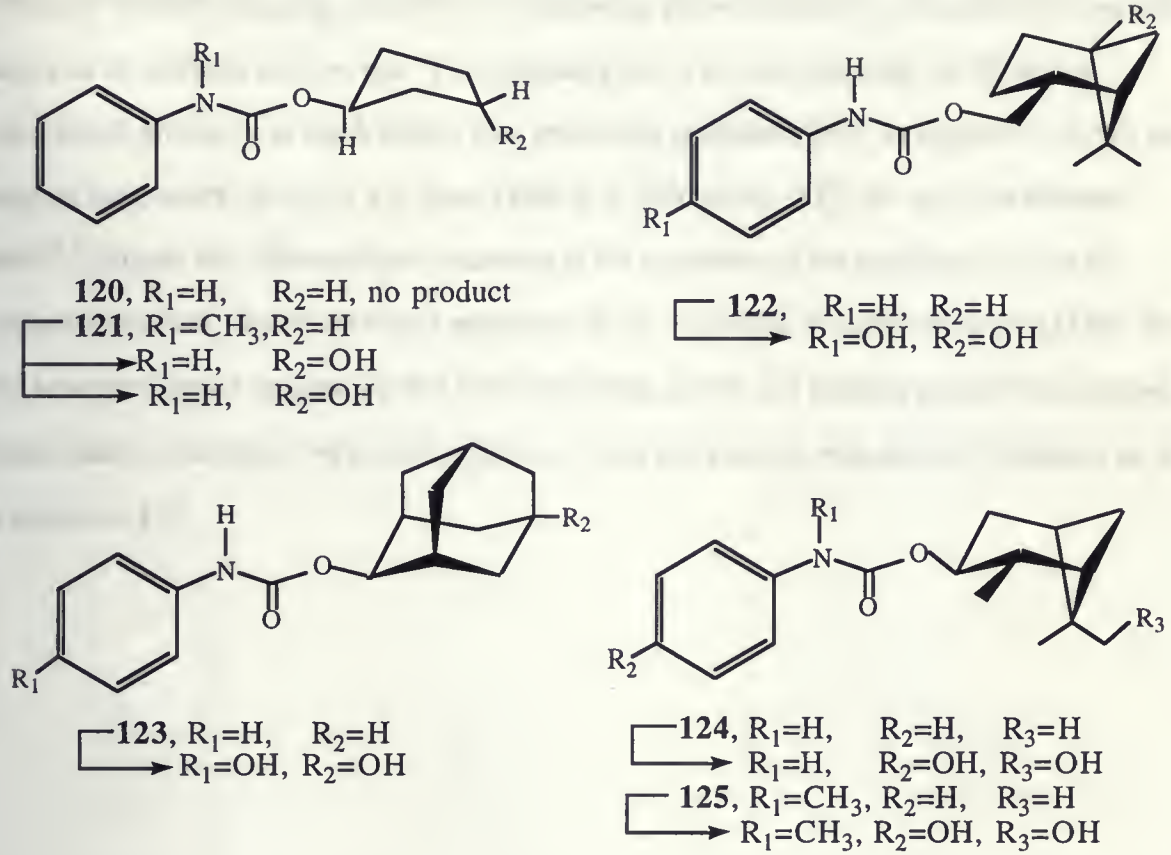

**116**

**117** $R_1$=OH, $R_2$=H, $R_3$=H
**118** $R_1$=H, $R_2$=OH, $R_3$=H
**119** $R_1$=OH, $R_2$=H, $R_3$=OH

This fact can be explained by either the higher lipophilic character of the cyclohexyl derivative compared to isopropyl (better enzyme-substrate interaction) or by a variation of the alkyl group size, leading to a better fit for the required distance between the carbonyl oxygen and the hydroxylated carbon. The kinetic study of the reaction suggested that the double hydroxylation process observed in product **119** occurred *via* two successive steps: first hydroxylation of the cyclohexane moiety, followed by hydroxylation on the aromatic ring.

Since the aromatic ring of the corresponding amides (Ph-CO-NR) was not hydroxylated using the same fungus, it appeared that the presence of a urethane moiety is necessary in order for this reaction to occur. A more systematic study was carried out in order to study the effect of lipophilicity and bulkiness of the substrates on the result of biotransformation.[52] (Figure 53)

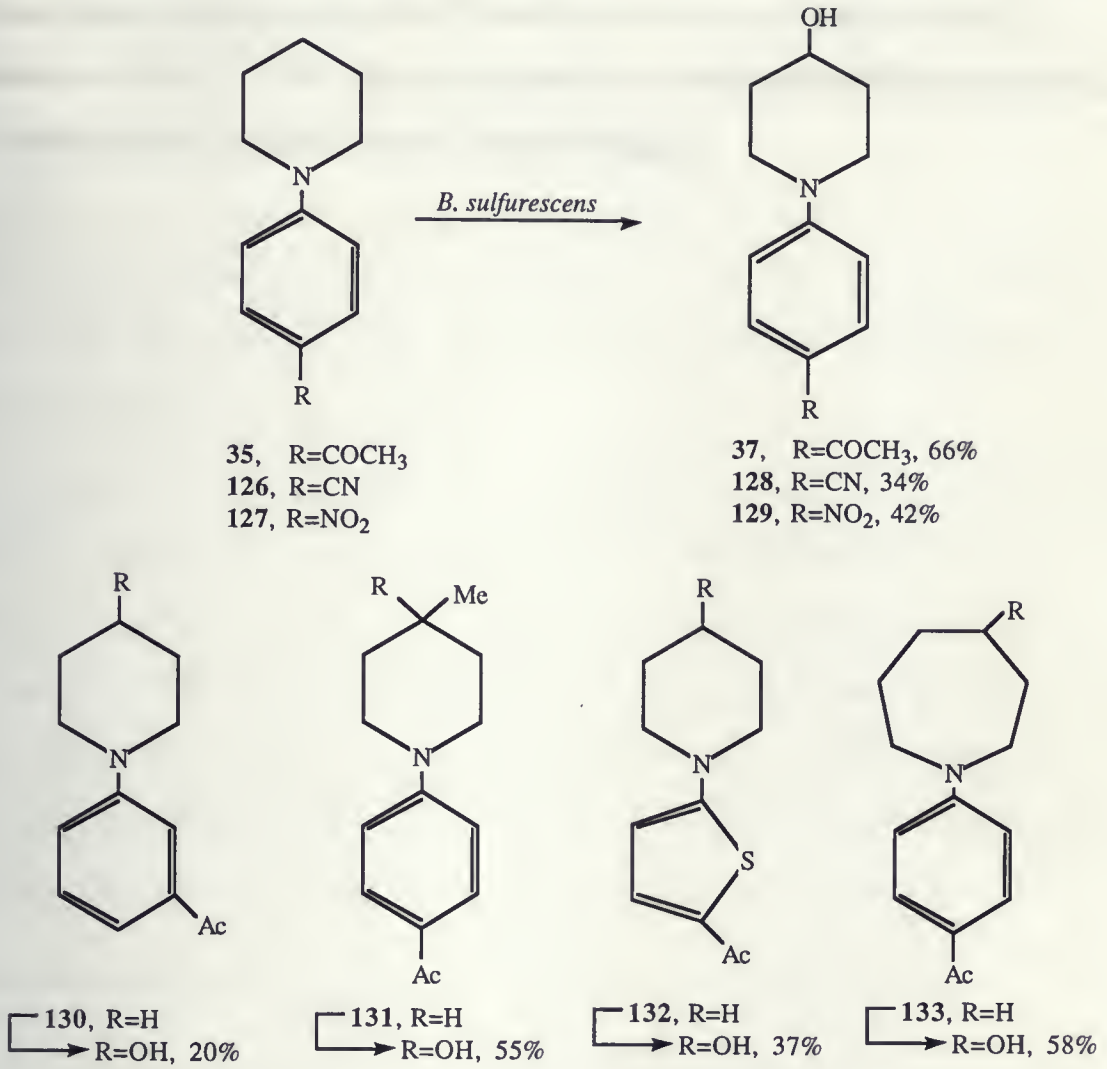
Figure 53: Microbial transformation of aromatic carbamates




All substrates possessing an alkyl N-phenyl carbamate structure were biotransformed. When the alkyl substituent is relatively small (R= methyl, isopropyl), the only observed hydroxylation occurs at the aromatic ring in highly regiospecific manner. This could be explained with lower lipophilic character of the substrates or because of geometric factors (distance between the oxygen and/or nitrogen atom and the site of hydroxylation). Going from isopropyl to cyclopentyl N-methyl carbamate **121**, aromatic hydroxylation is replaced by alkyl hydroxylation. And finally, substrates with highly lipophilic groups (**122, 123, 124** and **125**) undergo both alkyl and aromatic hydroxylation. The previous observation that changing the position of the amide moiety modifies the stereoselectivity of hydroxylation was confirmed in the case of urethane substrates with pinanyl derivatives (**122, 124** and **125**).

A more recent paper reported bioconversion of several piperidine derivatives.[53] The idea was to investigate whether changing the electron-withdrawing para-substituent on the aromatic ring affects binding in the enzyme's active site. The reported yield of the bioconversion of **35** to its 4-hydroxylated product was much higher than previously published (66% as opposed to 20%), and changing the p-acetyl group to a p-cyano (**126**) or p-nitro group (**127**) did not give different results.[53] (Figure 54) Hydroxylation occurred at the 4-position of the piperidine ring in all compounds studied. When the acetyl group was at the 3-position in the benzene ring (**130**), the yield was much lower, suggesting that the acetyl group serves as a binding point to the enzyme. 4-Hydroxylation occurred in very good yield even when this position was sterically hindered as in the substrate **131**.

Figure 54: Hydroxylation of piperidine derivatives




From the examples shown in the literature review, we can see that whole cell biotransformation using *B. sulfurescens* can provide easy access to products hydroxylated at an unactivated carbon, products otherwise hard or impossible to achieve. Yields are relatively good and the hydroxylated products often optically active.

Since no isolated hydroxylase enzymes are available from *B. sulfurescens*, a large number of substrates was used during 30 years of study in order to develop a model for hydroxylation. However, the predictability of this reaction has not improved, although the functional requirements of the substrate were defined many times and at least two models were proposed. In order to test

the proposed models, and to investigate whether the suggested requirements for electron-rich functional group are really necessary for bioconversion to occur, the bioconversion by *B. sulfurescens* of a series of inter-related compounds (i.e. amides, amines and hydrocarbons) has been studied, and the results are presented in the next section.

# EXPERIMENTAL

*Apparatus, materials and methods*

$^{1}H$ and $^{13}C$ NMR spectra were recorded at 300 MHz with a Bruker Avance DPX 300 spectrometer using deuterochloroform as a solvent. Mass spectra were recorded with a Kratos Concept 1S spectrometer operating in EI or FAB mode. Infrared spectra were obtained using a Mattson spectrometer Research Series I, interfaced to Dell Pentium PC, or a Bomem FTIR spectrometer (MB series).

Flash column chromatography was performed using Inter Science silica gel (70-230 mesh). Thin layer chromatography (TLC) was done on Macherey-Nagel Polygram Sil G/$UV_{254}$ plates (0.25 mm thick). The optical rotation measurements were performed with a Rudolph Autopol III automatic polarimeter at ambient temperature. The enantiomeric ratios were examined by $^{1}H$ NMR analysis at 300 MHz in the presence of tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium (III) derivative. The melting points were determined on a Kofler hot stage apparatus.

*Maintenance and growth conditions of microorganisms*

*Beauveria sulfurescens* ATCC 7159 was obtained from the American Type Culture Collection, and was used to inoculate slopes (made of 20g corn steep liquor, 10g glucose, 20g of bactoagar per 1L of distilled water) and incubated for three days at $27^{0}C$. The slopes were stored at $4^{0}C$ and subcultured periodically.

Sterilized culture media, 1L (20 g corn steep liquor, 10g glucose in 1L tap water) placed in five 1L Erlenmeyer flasks were inoculated with one slope of the fungus, using sterile techniques. After 72 h growth at $27^{0}C$ (180 rpm) the mycelium was harvested by filtration and washed with water. The fungal cake obtained in this way was used to carry out biotransformations (see section on biotransformation).

*Aspergillus niger* ATCC 9142 was obtained from the American Type Culture Collection, and was maintained on malt agar slopes, grown at 28$^0$C, and stored refrigerated at 4$^0$C. The growth conditions were the same as for *B. sulfurescens* ATCC 7159.

*Aspergillus niger* LCP 521 was obtained from R. Furstoss (Faculte des Sciences de Luminy, Marseille, France) and it was maintained on slopes made of 20g corn steep liquor, 10g glucose, 20g of bactoagar per 1L of distilled water. Slopes were stored refrigerated at 4$^0$C. The growth conditions were the same as for *B. sulfurescens* ATCC 7159.

*Rhizopus arrhizus* ATCC 11145 was obtained from the American Type Culture Collection, and was maintained on malt agar slopes, grown at 28$^0$C, and stored refrigerated at 4$^0$C.

*Diplodia gossypina* ATCC 10936 was obtained from the American Type Culture Collection, and was maintained on potato dextrose agar slopes, grown at 24$^0$C, and stored refrigerated at 4$^0$C. The growth liquid medium[54] was made of peptone (10g/L), glucose (10g/L), malt extract (20g/L) and yeast extract (2g/L). The prepared medium (500 mL) was equally distributed between two 1L Erlenmeyer flasks, sterilized, inoculated with the fungus and left on the shaker (80 rpm) for three days at 28$^0$C.

PART ONE: PREPARATION OF SUBSTRATES

*1-Methyl-2-phenyl aziridine* **136**[55,56] (Figure 55)

(±)-Styrene oxide (60.0g, 0.5 mole)[55] and 40% aqueous methylamine (172.6 mL, 2.0 mole) were heated in the steel bomb at 95-100$^0$C for 5.5 h with constant stirring. The resulting mixture was cooled to a room temperature and the excess methylamine and water distilled off at atmospheric pressure. The product **134** was distilled at 97-105$^0$C, 0.6 mmHg (white semisolid). Yield: 47.8g(63%); $^1$Hnmr δ 2.4(3H, s), 2.7(2H, d), 3.1-3.4(1H, OH), 3.5-3.7(1H, NH), 4.7(1H, t), 7.3(5H, s); $^{13}$Cnmr δ 34.5, 59.7, 72.0, 126.2, 127.8, 128.7; IR cm$^{-1}$ 2960, 2940, 2860(CH); 2760(N-alkyl); 1110(C-N); ms m/z(%) 120(100), 77(23.5), 79(11.7), 105(11.6), 151(2.1).

To $PBr_3$ (33.7g. 0.122 mole)[57] dissolved in dry chloroform (112 mL) at 0$^0$C was added dropwise with stirring aminoalcohol **134** (17.0g, 0.112 mole) dissolved in dry chloroform (112 mL) during 50 minutes. The mixture was then heated under reflux until no more hydrogen bromide was evolved (overnight). Chloroform was then removed under reduced pressure, dry ethanol was added to the residue (130 mL) and the solution thus formed was filtered through celite. Addition of dry ether precipitated the product **135** which was then filtered off. White solid; Yield: 18.6g(56%); $^1$Hnmr δ 2.8(3H, t), 3.5-3.8(2H, m), 5.6-5.8(1H, m), 7.3-7.5(5H, m), 9.1-9.7(2H, m).

Cyclization to the aziridine **136**[56] was carried out in a flame-dried round-bottomed flask fitted with a pressure compensated dropping funnel, magnetic stirrer and a reflux condenser. Under argon, the flame-dried flask was charged with amine hydrobromide (4.7g, 0.016 mole) in 21 mL of dry ether and the dropping funnel with 20 mL of 1.4M solution of methyllithium in diethyl ether. The methyllithium solution was added dropwise over a period of 45 minutes at 0$^0$C, after which the reaction mixture was heated under gentle ether reflux for 1.5 h. The reaction mixture was filtered and the separated ether layer dried over anhydrous magnesium sulfate. Ether was evaporated and the crude yellow oil was distilled using Kugelrohr apparatus (at 120$^0$C, 16 mmHg)

to give 1-methyl-2-phenylaziridine **136**. Yield: 1.05g(49%); $^1$Hnmr δ 1.53 ($H_A$ of ABX pattern), 1.7($H_B$), 2.22($H_X$), 2.51(3H, s), 7.42(5H, s).

*2-Phenylaziridine* **138**[58,59] (Figure 56)

Method I

To a stirred solution of styrene oxide (5.0g, 0.04 mole)[60] in anhydrous methanol (20 mL) under argon was added sodium azide (2.8g, 0.04 mole). The mixture was refluxed for 4.5 h, the solvent was then evaporated and the residue partitioned between water (150 mL) and chloroform (100mL). The aqueous layer was extracted with chloroform (2X100 mL) and combined organic phase was washed with water (100 mL), dried with anh. magnesium sulfate and solvent evaporated. The product **137** was obtained in a quantitative yield. $^1$Hnmr δ 3.2(1H, t), 3.7(2H, d), 4.6(1H, t), 7.3(5H, m).

2-Azido-2-phenylethanol **137** was also prepared by the following procedure:

To a refluxing solution of styrene oxide (15.0g, 0.125 mole)[61] in dioxane (125 mL) a solution of sodium azide (10.2g, 0.157 mole) in water (26 mL) was added dropwise. Refluxing was continued for 20 h, after which the two layers were separated. The organic layer was then distilled; dioxane distilled off at 25$^0$C, 16 mmHg and the distillation then continued at 85-86$^0$C, 0.2 mmHg to give a product **137** in a quantitative yield.

The ring closure to the aziridine **138** was attempted using three different procedures, notably Ia, Ib and Ic:

Ia: To a stirred solution of triphenylphosphine (9.6g, 0.036 mole)[60] in acetonitrile (192 mL) was added carbon tetrachloride (55 mL). The solution turned yellow over a period of 30 minutes at which time 2-azido-2phenylethanol **137** (2.0g, 12.3 mole) in a solution of triethylamine (5.5 mL) and acetonitrile (82 mL) was added dropwise. The reaction mixture was stirred for 16 h at room

temperature. Solvent was removed by rotary evaporation. The desired product **138** was not obtained.

Ib: Triphenylphosphine (0.84g, 0.003 mole)[62] was added to a stirred azidoalcohol **137** (0.5g, 3.06 mole) in acetonitrile (14 mL). The reaction mixture was stirred for one hour (until nitrogen evolution had ceased) and then heated at reflux for 9 h. The solvent was then evaporated, triphenylphosphine oxide was partly removed by filtration after addition hexane-ether and the crude product was passed through silica gel column (hexane 50%-ether 50% solvent). The product **138** was not isolated.

Ic: A solution of azidoalcohol **137** (3.15g, 0.02 mole)[63] and triphenylphosphine (5.05g, 0.02 mole) in dry ether (96 mL) was stirred at room temperature. The evolution of nitrogen and precipitation of triphenylphosphine oxide started after 90 minutes. When gas evolution had ceased, the $Ph_3P=O$ was filtered off and the ether removed in vacuum. The product **138** was distilled by Kugelrohr apparatus ($85^0$C, 16 mmHg). Yield: 0.25g (10%), but the product polymerized rapidly.

Method II

2-Bromo-2-phenylethylamine hydrobromide **139** was prepared using the same procedure as for 1-methyl-2-phenyl aziridine **136**, starting with 1-phenyl-2-amino-1-ethanol (4.5g, 0.032 mole)[57]. Yield: 5.75g(62%); M.p.158-$160^0$C; $^1$Hnmr δ 2.1-2.5(3H, s), 3.6(2H, d), 5.5(1H, t), 7.3-7.5(5H, m); $^{13}$Cnmr δ 51.7, 54.4, 132.9, 134.2, 134.6, 142.8; IR $cm^{-1}$ 3000-3200($NH_3^+$), 1600(aromatic ring); ms m/z(%) 200(100), 202(98), 120(86.4), 104(56), 121(38.8).

The ring closure to the aziridine **138** was attempted using three different procedures, IIa, IIb and IIc.

IIa: The same procedure was used as for 1-methyl-2-phenyl aziridine **136**[56], starting with 2-bromo-2-phenylethylamine hydrobromide **139** (3.75g, 0.013 mole). Yield: 1.5g (94%), but the

product polymerized quickly. Attempts to prevent polymerization by cooling, keeping it under argon were not successful.

IIb: The mixture of 2-bromo-2-phenylethylamine hydrobromide **139** (11.4g, 0.04 mole)[59] and 10% solution of sodium hydroxide (72 mL) was heated at 60-70$^0$C for 2 h. The excess solid potassium hydroxide was added and the product distilled with steam. The distillate was saturated with KOH and extracted with ether. After ether was evaporated, the product polymerized.

IIc: Since the aziridine **138** was highly unstable, making a stable tartrate salt **140** was attempted, while the aziridine **138** is still in ether solution (procedure IIa, the same amounts used). Once the reflux was finished and the product was still in ether, the reaction flask was placed in the ice bath and a solution of dibenzoyl-L-tartaric acid monohydrate (4.7g, 0.012 mole) dissolved in ether (10 mL) was added dropwise. The resulting white precipitate was filtered off. Produced tartrate salt was stable for 12 months stored at 4$^0$C. Yield: 4.56g(69%). M.p.136-138$^0$C; $^1$Hnmr δ 1.9(1H, d), 2.3(1H, d), 3.2(1H, m), 4.0-4.5($NH_2$, broad), 5.9(2H, s), 7.2-7.6(11H, m), 8.1(4H, d); IR $cm^{-1}$ 2700-3500(OH), 1700(C=O); ms m/z(%) 105(100), 122(79.8), 77(61.1), 51(32.1).

*N-methyl-7-aza bicyclo[4.1.0] heptane* **141** (Figure 57)

Chlorosulfonic acid (4.2 mL, 0.06 mole) was added dropwise during 60 minutes to a solution of *trans*-2-methylamino cyclohexanol (8.0g, 0.06 mole) in 50 mL of anhydrous ether, keeping the temperature at 5-10$^0$C. The thick mixture was stirred mechanically for 1.5 h at room temperature. Ether was decanted and the product washed once with 50 mL of ether by decantation. It was then cooled on ice, treated with a solution of 9.2g of sodium hydroxide in 44 mL of water (cautiously at first). The mixture was then distilled while adding water from a dropping funnel to keep the volume constant. Distillation temperature was 95$^0$C. The distillate was saturated with solid sodium hydroxide and extracted with ether (3X50 mL). The extract was dried with anhydrous magnesium sulfate and the ether removed using a Vigreux column at atmospheric pressure and 35$^0$C. The pure product **141** distilled at 45$^0$C, 30 mmHg. Clear oil; Yield: 3.0g(45%); $^1$Hnmr δ

1.0-1.5(6H, m), 1.6-1.9(4H, m), 2.3(3H, s); $^{13}$Cnmr δ 20.4, 24.1, 39.2, 47.3; IR cm$^{-1}$ 2960, 2940, 2860(CH); 2760(N-alkyl); 1110(C-N); ms m/z(%) 82(100), 96(88.7), 83(74.3), 111(73.4), 110(62.8).

*Styrene sulfide* **142**[64] (Figure 58)

Formation of thiuronium salt:

Thiourea (4.6g, 0.06 mole) was added to a solution of 1.8 mL of conc. sulfuric acid in 21 mL of water, and the mixture was cooled to 0-5$^0$C. Styrene oxide (6.9 mL, 0.06 mole) was added during 2 h to the stirred solution, still keeping the temperature 0-5$^0$C. After the addition was completed, stirring and cooling were continued for further 10 minutes, then the mixture was allowed to warm up to room temperature in the course of 2 h.

Episulfide formation:

To a previously prepared mixture was added a solution of sodium carbonate (6.4g, 0.06 mole) in 30 mL of water. The addition lasted for 30 minutes, at room temperature. The stirring was continued for a further 2 h at room temperature. Water (60 mL) was added and the resulting mixture was extracted with ether (3X50 mL), the ethereal solution dried over anhydrous magnesium sulfate and ether then evaporated. The crude product was purified by passing through silica gel column with 50% ethyl acetate-50% hexane solvent. Clear oil; Yield: 4g(49%); $^1$Hnmr δ 2.6(1H, d), 2.8(1H, d), 3.8(1H, t), 7.3(5H, s); $^{13}$Cnmr δ 27.6, 36.4, 127.1, 127.9, 128.9, 139.5; IR cm$^{-1}$ 1580(C=C), 680(C-S stretch); ms m/z(%) 104(100), 136(36.7), 78(33.8), 91(33.3), 103(32.7).

BIOHYDROLYSIS OF AZIRIDINES AND STYRENE SULFIDE

*1-Methyl-2-phenyl aziridine* **136** (Figures 59, 60, 61)

Biotransformation using *Aspergillus niger* LCP 521:

The wet fungal cake (53.2g) obtained from 1L of the fungal growth (as described on p. 55) was transferred into a 2L Erlenmeyer flask which contained 1L of phosphate buffer (0.1M, pH 8). The substrate (1g) was dissolved in 5mL of 95% ethanol and poured into the biotransformation flask, which was then placed on the rotary shaker (200 rpm, 28$^0$C). The course of the biotransformation was monitored by TLC (sampled after 1h, 2h and 23h). The biotransformation was stopped after 24 h, the fungal material then filtered and washed with 250 mL of hexane. After separation, the aqueous phase was saturated with sodium chloride and extracted with another 250 mL of hexane. The combined organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated. The starting material (0.1 g)was recovered, with $[\alpha]_D$= +0.46 (c=4.97, chloroform). The remaining aqueous phase was put on the continuous extractor for the extraction with dichloromethane for two days. After the extraction, the solvent was evaporated and the residue distilled by Kugelrohr distillation. The distilled product was 1-phenyl-2-methylamino-1-ethanol **134** (0.1g), $[\alpha]_D$= +0.9 (c=0.78, chloroform).

Biotransformation of **136** using *Beauveria sulfurescens* ATCC 7159:

The biotransformation using *B. sulfurescens* was performed in the same way as biotransformation using *A. niger*, starting with 0.5g of the substrate and 10g of the fungal cake (for growing conditions see p.55). After 24h biotransformation, the hexane extract yielded 0.23g of unreacted starting material; $[\alpha]_D$= +0.16 (c=5.7, chloroform). After the extraction of the aqueous phase with dichloromethane, followed by Kugelrohr distillation, 1-phenyl-2-methylamino-1-ethanol **134** (0.02g) was obtained; $[\alpha]_D$= -0.94 (c=0.32, chloroform).

Biotransformation of **136** using *Diplodia gossypina* ATCC 10936:[54]

The biotransformation of 1-methyl-2-phenyl aziridine **136** was performed in the following way: The fungal growth (250 mL;for growth conditions see p.55) was filtered, the fungal cake blended and resuspended 0.1N Tris-buffer (30 mL), pH 7. The substrate (0.07g) was dissolved in 95% ethanol and poured into the prepared buffered fungal material, and the flask then placed on the rotary shaker (180 rpm, 28$^0$C). The biohydrolysis was followed by TLC during first few hours (after 3 h the starting material was the only detected compound), and it was stopped after 24 h. The fungal growth was then filtered off and the filtrate (first saturated with sodium chloride) was extracted with hexane (3X30 mL), and then with dichloromethane (3X30 mL). After evaporation of the solvents, 2-formyloxy-2-phenylethylamine **143** (Figure 61) was recovered from both extracts in 11% total yield (10 mg); $^1$Hnmr δ 2.9(3H, s), 3.4(1H, t), 3.9(2H, m), 5.5(1H, t), 7.4(5H, m), 7.6(1H, formate); $^{13}$Cnmr δ 31.1, 54.5, 74.1, 125.5, 125.9, 128.9, 158.0 (formate); IR cm$^{-1}$ 1710 (formate C=O); ms m/z(%) 177(100), 132(85.4), 91(43.3), 77(26.0), 58(42.4); $[\alpha]_D$= -11.0 (c=0.2, chloroform).

*2-Phenylaziridine* **138**

The biotransformation of 2-phenylaziridine, tartrate salt **140** was attempted using *Beauveria sulfurescens* ATCC 7159 and *Aspergillus niger* LCP 521, but the recovered products were just polymers/natural products in both cases. The procedures followed were identical to those used for the biotransformation of 1-methyl-2-phenyl aziridine **136** (with different time periods 6h, 24h and 36h).

*N-methyl-7-aza-bicyclo[4.1.0] heptane* **141**

Biotransformation using *Aspergillus niger* ATCC 9142:

The washed fungal cake (62.7g of wet weight; see p. 55 for growing conditions) of *A. niger* cells was transferred into a 2L flask containing 1L of the phosphate buffer (0.1M, pH 8). A solution of

1g of the substrate dissolved in 5 mL 95% ethanol was poured into the medium and the flask was placed on the rotary shaker (180 rpm, 28$^0$C) and stoppered to avoid loss of substrate by evaporation. The biotransformation was allowed to proceed for 24 h. The fungal material was then filtered off, the filtrate saturated with sodium chloride and extracted with dichloromethane (continuous extractor, 72 h). The solvent was evaporated, and the residue distilled by Kugelrohr apparatus (108$^0$C, 16 mmHg). The only recovered compound was starting material (0.43g).

Biotransformation using *Beauveria sulfurescens* ATCC 7159:

The biotransformation of **141** with *B. sulfurescens* was carried out for 24 h in the same way as with *A niger*, starting with 31.2g of the wet fungal cake and 1g of the substrate. After Kugel-Rohr distillation 0.34g of the starting material was recovered.

Biotransformation using *Diplodia gossypina* ATCC 10936:

The fungal growth was filtered, blended for 1s and 112g of the wet biomass resuspended in Tris-buffer (0.1N, pH 7; 500 mL). N-Methyl-7-aza-bicyclo[4.1.0] heptane **141** (1g) was dissolved in 95% ethanol (4 mL) and poured into a prepared fungal mixture. After 24 h of biotransformation at 28$^0$C, 180 rpm (the flask was plugged with a rubber stopper), the fungal material was separated by centrifugation, the filtrate saturated with sodium chloride and extracted for 72 h with dichloromethane. The solvent was then evaporated, and the crude product purified by column chromatography (chloroform-methanol). Only starting material was recovered.

*Styrene sulfide* **142** (Figures 62, 63)

The biotransformation of the styrene sulfide was done using *Beauveria sulfurescens* ATCC 7159 and *Aspergillus niger* LCP 521 in the same way as already described before. In both cases three separate biotransformations were performed, lasting for 2, 4 and 6 hours.

For biotransformation with *B. sulfurescens* 13.6g of the wet fungal cake was resuspended in 341 mL of the phosphate buffer (0.1M, pH 8) and 0.85g of the substrate dissolved in 95% ethanol was

fed to the fungus. After the extraction with hexane, the aqueous phase from all three biotransformations was combined and extracted with dichloromethane.

| Hexane extracts | Yield (g) | $[\alpha]_D$ |
|---|---|---|
| 2 h biotransformation | 0.23 | +1.2 (c=1.14, $CHCl_3$) |
| 4 h biotransformation | 0.25 | +0.85 (c=0.94, $CHCl_3$) |
| 6 h biotransformation | 0.10 | +0.57 (c=1.39, $CHCl_3$) |
| Combined aq. phase ($CH_2Cl_2$ extract) | 0.49 | -28.4 (c=0.46, $CHCl_3$) |

For the biotransformation of styrene sulfide **142** with *A. niger* LCP 521 in each case (2, 4, 6 h biotransformation) 13.0g of the wet fungal cake was resuspended in 300 mL of phosphate buffer (0.1M, pH 8), and 0.58g of the substrate dissolved in 95% ethanol was added.

| Hexane extracts | Yield (g) | $[\alpha]_D$ |
|---|---|---|
| 2 h biotransformation | 0.14 | +0.67 (c=1.49, $CHCl_3$) |
| 4 h biotransformation | 0.20 | -0.96 (c=2.27, $CHCl_3$) |
| 6 h biotransformation | 0.30 | 0 |
| Combined aq. phase ($CH_2Cl_2$ extract) | 0.12 | -1.34 (c=2.61, $CHCl_3$) |

In both cases (using *A niger* and *B. sulfurescens*), the only product of the extraction with hexane was recovered starting material. Also, in both cases, the product of the extraction of the combined aqueous phase with dichloromethane was compound **144**. (Figure 62)

$^1$Hnmr δ 3.6-3.9(2H, m), 4.8-4.9(1H, m), 7.1-7.5(5H, m); $^{13}$Cnmr δ 68.5, 75.1,126.5, 128.4, 128.9, 140.9; IR $cm^{-1}$ 3100-3600(OH), 2800-3000(CH), 1600(arom. ring); ms m/z(%) CI

121(100), 107(89.2), 79(86.5), 77(85.0), 91(44.8); FAB 43(100), 55(95.2), 121(72.2), 149(54.2), 281(41.9); EI 107(100), 79(56.7), 77(48.7), 104(33.4), 121(24.1).

The enantiomeric excess of **144** was determined to be e.e.=64% (*B. sulfurescens)* using chiral shift reagent tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium (III) derivative.

PART TWO: PREPARATION OF SUBSTRATES

Following substrates were purchased from Sigma-Aldrich Canada Ltd (Figure 64):

4-morpholino acetophenone **145**, 1-benzyl-4-piperidone **146**, 1-phenethyl-4-piperidone **147**, 1,1'-carbonyldipiperidine **148**, dipiperidinomethane **149**, 1,1'-ethylenedipiperidine dihydrochloride **150**, bicyclohexyl **151**, phenylcyclohexane **152**, 4'-cyclohexylacetophenone **153**, 4'-piperidinoacetophenone **35**, 1-benzyl-4-piperidinol **154**, 1-phenylpiperidine **155**, 1-benzoyl-4-piperidone **156**, 2-benzoylthiophene **157**.

*1-Benzylpiperidine* **158** (Figure 65)

Piperidine (5.0g, 0.06 mole) was dissolved in acetonitrile. Benzyl chloride (7.3g, 0.06 mole) was added with stirring. Potassium carbonate (16.0g, 0.12 mole) was then added and mixture refluxed for 1 hour. After cooling, precipitate was filtered, washed with acetonitrile and filtrate was evaporated to give a pale yellow liquid. Yield: 9.5g (93%); $^{1}$Hnmr δ 1.4-1.6(2H, m), 1.6-1.7(4H, m), 2.3-2.5(4H, m), 3.5(2H, s), 7.3-7.5(5H, m); $^{13}$Cnmr δ 22.7, 24.3, 52.8, 62.2, 125.1, 126.3, 127.5, 137.0; IR cm$^{-1}$ 2700-3000(CH), 1500(arom. ring); ms m/z(%) 91(100), 174(90.2), 175(79.6), 98(68.2), 84(47.5).

*1-Benzylpyrrolidine* **159** (Figure 65)

1-Benzylpyrrolidine **159** was prepared using the above procedure starting with pyrrolidine (4.1g, 0.06 mole), with the following modification: the reaction mixture was refluxing for 2 hours.

Yellow oil; Yield: 9.3g (100%); $^{1}$Hnmr δ 1.8-1.9(4H, m), 2.5-2.7(4H, m), 3.7(2H, s), 7.3-7.5(5H, m); $^{13}$Cnmr δ 23.9, 54.6, 61.2, 127.5, 128.6, 129.0, 139.9; IR $cm^{-1}$ 2700-3000(CH), 1500(arom. ring); ms m/z(%) 91(100), 160(80.4), 84(75.3), 161(65.6), 70(41.4).

*1-(p-Ethylphenyl) piperidine* **160** (Figure 66)

1-(p-Ethylphenyl) piperidine **160** was prepared by Huang-Minlon modification of Wolf-Kishner reduction[65]: Into two-necked round-bottomed flask equipped with a reflux condenser and a thermometer were added 4'-piperidino acetophenone **35** (5.0g, 0.02 mole), dipropylene glycol (50 mL), 90% hydrazine hydrate (2.5mL, 0.08 mole) and potassium hydroxide pellets (3.3g). The mixture was warmed on a boiling water bath until most of KOH dissolved and then heated under reflux for 5.5 h (kept under argon). After cooling, water was added (50 mL) and the mixture was extracted with ether (3X50 mL). The ethereal extract was washed with water, dried and the ether evaporated. The crude product was purified by column chromatography (silica gel; hexane-ethylacetate 10% stepwise gradient) to give a yellow oil. Yield: 1.5g(34%); $^{1}$Hnmr δ 1.3(3H, t), 1.6-1.7(2H, m), 1.7-1.9(4H, m), 2.7(2H, q), 3.1-3.3(4H, m), 7.0(2H, d), 7.2(2H, d); $^{13}$Cnmr δ 16.2, 24.8, 26.4, 28.4, 51.7, 117.3, 128.7, 135.6, 150.9; IR $cm^{-1}$ 2750-3000(CH), 1600(arom. ring); ms m/z(%) 189(100), 188(79), 174(76.4), 118(15.6), 190(14.4).

*p-Ethylphenyl cyclohexane* **161** (Figure 66)

p-Ethylphenyl cyclohexane **161** was prepared using the same procedure as for synthesis of 1-(p-ethylphenyl) piperidine **160**, starting with 4'-cyclohexylacetophenone **153** (4.8g, 0.024 mole), 90% hydrazine hydrate (2.5mL, 0.08 mole), dipropylene glycol (25 mL) and KOH pellets (3.3g) in 86.5% yield (crude product). After cleaning of the crude product (column chromatography, hexane-ethylacetate 10% stepwise gradient). The pure product was a clear oil. Yield: 2.1g (46%); $^{1}$Hnmr δ 1.3(3H, t), 1.5-1.7(5H, m), 1.8-2.0(5H, m), 2.5-2.6(1H, m), 2.7(2H, q), 7.2(4H, d); $^{13}$Cnmr δ 16.0, 26.7, 27.4, 28.8, 35.0, 44.6, 127.1, 127.9, 141.9, 145.8; IR cm-1 2800-3000(CH), 1500(arom. ring); ms m/z(%) 188(100), 145(68.8), 117(44.7), 119(35.9), 159(35.6).

*1-(p-Ethylbenzoyl) piperidine* **162** (Figure 67)

Piperidine (1.7 mL, 0.017 mole) was mixed with 10% solution of sodium hydroxide (29 mL). 4-Ethylbenzoyl chloride (2.8g, 0.02 mole) was added in small portions, with stirring vigorously between additions and stirring continued for five minutes more after addition was completed. The reaction mixture was extracted with ether (3X30 mL) and ethereal extract was washed well with water, then with 5% hydrochloric acid and again with water. The washed extract was dried over anhydrous magnesium sulfate and ether evaporated. Clear oil; Yield: 3.5g (96%); $^{1}$Hnmr δ 1.3(3H, t), 1.4-1.8(6H, m), 2.7(2H, q), 3.2-3.5(2H, t), 3.5-3.9(2H, t), 7.2(2H, d), 7.3(2H, d); $^{13}$Cnmr δ 15.7, 24.9, 26.5, 29.0, 43.6, 49.0, 127.3, 128.1, 134.1, 145.9, 170.8; IR cm$^{-1}$ 2800-3000(CH), 1650(C=O); ms m/z(%) 216(100), 133(87.5), 217(43.5), 134(10.4), 77(9.0).

*1-(p-Ethylbenzoyl) pyrrolidine* **163** (Figure 67)

1-(p-Ethylbenzoyl) pyrrolidine **163** was prepared using the above procedure, starting with pyrrolidine (1.4 mL, 0.017 mole) and 4-ethylbenzoyl chloride (2.8g, 0.02 mole). White solid; Yield:2.8g (80%); M.p.=85-86$^{0}$C; $^{1}$Hnmr δ 1.3(3H, t), 1.9-2.1(4H, m), 2.7(2H, q), 3.5(2H, t), 3.7(2H, t), 7.2(2H, d), 7.5(2H, d); $^{13}$Cnmr δ 15.4, 24.5, 26.4, 28.7, 46.1, 49.6, 127.3, 127.6, 134.6, 146.2, 169.8; IR cm$^{-1}$ 2800-3000(CH), 1630(C=O); ms m/z(%) 133(100), 203(46.9), 202(19.8), 146(11.8), 134(10.7).

*2-(4'-Piperidino)-phenyl-2-propanol* **164** (Figure 68)

4'-Piperidinoacetophenone **35** (3.0g, 0.015 mole) was added under argon to a flame dried, two-necked round-bottomed flask fitted with a reflux condenser and dissolved in anhydrous ether. A 3M solution of methylmagnesium bromide in diethyl ether (4.8 mL) was added slowly with cooling in the ice bath. After stirring overnight at room temperature, the reaction mixture was refluxed for 1 hour, then cooled. The saturated solution of ammonium chloride was added slowly with cooling in the ice bath. The ether and aqueous layers were separated and the aqueous layer then extracted with ether. The combined ethereal solution was dried and the ether evaporated to

give a yellow solid. M.p.60-62$^0$C; Yield: 2.8g (86%); $^1$Hnmr δ 1.6(6H, s), 1.7-1.8(6H, m), 3.1-3.2(4H, m), 6.9(2H, d), 7.4(2H, d); $^{13}$Cnmr δ 24.7, 26.2, 32.0, 51.0, 72.5, 116.4, 125.5, 140.0, 151.3, IR cm$^{-1}$ 3100-3400(OH), 1600(arom. ring); ms m/z(%) 201(100), 200(83.2), 204(24.8), 202(17.7), 219(10.0).

*1-(p-Isopropenylphenyl) piperidine* **165** (Figure 68)

2-(4'-Piperidino)-phenyl-2-propanol **164** (2.0g, 0.009 mole) was dissolved in acetone (23 mL). Water (1.35 mL) was added and then conc. hydrochloric acid (0.9 mL), and the reaction mixture refluxed for 1 hour. After cooling, water was added (23 mL) and solution was then extracted with ether. The ethereal solution was washed with saturated sodium bicarbonate, then with water; dried and evaporated to give a yellow solid. Yield: 1.1g (43%); M.p.36-38$^0$C; $^1$Hnmr δ 1.5-1.8(6H, m), 2.1(3H, s), 3.1-3.3(4H, m), 5.0(1H, d), 5.3(1H, d), 6.9(2H, d), 7.4(2H, d); $^{13}$Cnmr δ 22.1, 24.7, 25.7, 50.7, 109.9, 116.1, 126.5, 132.0, 143.0, 151.8; IR cm$^{-1}$ 1650(C=C, vinylidene), 1600(arom. ring); ms m/z(%) 201(100), 200(79.9), 202(21.2), 145(15.4), 199(10.6).

*p-1-Hydroxyisopropylphenyl cyclohexane* **166** (Figure 69)

This substrate was made by the procedure described for 2-(4'-piperidino)-phenyl-2-propanol **164**, starting with 4'-cyclohexylacetophenone **153** (5.0g, 0.02 mole) and methylmagnesium bromide (8.2 mL of 3M solution in diethylether) to give a slightly yellow solid. Yield: 4.7g (87%); M.p.67-69$^0$C; $^1$Hnmr δ 1.3-1.5(5H, m), 1.6(6H, s), 1.7-2.0(5H, m), 2.4-2.6(1H, m), 7.2(2H, d), 7.4(2H,d); $^{13}$Cnmr δ 26.5, 27.3, 32.0, 34.8, 44.5, 72.7, 124.7, 127.0, 146.8; IR cm$^{-1}$ 3100-3400(OH), 2800-3000(CH); ms m/z(%) 203(100), 204(16.1), 200(12.6), 201(10.2), 218(9.9).

*p-Isopropenylphenyl cyclohexane* **167** (Figure 69)

p-Isopropenylphenyl cyclohexane **167** was obtained by dehydration of p-1-hydroxyisopropylphenyl cyclohexane **166** (3.8g, 0.017 mole) using the same procedure as for preparation of **165**. The product was recovered as a yellow liquid. Yield: 3.4g (95%); 1Hnmr δ 1.3-1.6(5H, m), 1.7-2.0(5H, m), 2.2(3H, s), 2.5-2.6(1H, m), 5.1(1H, s), 5.4(1H, s), 7.2(2H, d), 7.5(2H, d); $^{13}$C δ 22.2, 26.6, 27.3, 32.1, 34.7, 44.5, 72.7, 112.0, 124.8, 125.8, 127.0, 143.5, 147.8; IR cm$^{-1}$2800-3000(CH), 1650(C=C, vinylidene), 1600(arom. ring); ms m/z(%) 200(100), 131(28.8), 157(26.9), 144(20.3), 201(18.3).

*1-Cyclohexylpiperidine* **168** (Figure 70)

Cyclohexyl bromide (15.0g, 0.09 mole) was dissolved in piperidine and the solution was refluxed for 4 hours. After cooling, 5% solution sodium hydroxide was added to pH 12. The solution was then extracted with ether and the extract washed with water, dried over anhydrous magnesium sulfate and evaporated. The oily crude product was distilled at 0.3 mmHg/55$^0$C to give a yellow oil. Yield: 3.2g (21%); $^1$Hnmr δ 1.0-1.3(5H, m), 1.3-1.5(2H, m), 1.5-1.7(5H, m), 2.2-2.3(1H, m), 2.4-2.6(4H, m); $^{13}$Cnmr δ 25.2, 26.3, 26.7, 26.8, 28.9, 50.2, 64.5; IR cm$^{-1}$ 2700-3000(CH); ms m/z(%) 124(100), 167(13.2), 125(10.0), 55(5.5), 110(4.9).

*2-Methyl-2-(p-cyanophenyl)-1,3-dioxolane* **169** (Figure 71)

A mixture of p-acetylbenzonitrile (5.0g, 0.034 mole), ethylene glycol (5.6.0g, 0.09mole), p-toluenesulfonic acid (0.075g) and benzene (76 mL) was refluxed overnight using a Dean-Stark trap. The reaction mixture was then cooled, washed with saturated sodium bicarbonate, water and then with saturated sodium chloride. The benzene layer was dried and evaporated to give a solid. Yield: 6.3g (96%); M.p. 61-63$^0$C; $^1$Hnmr δ 1.7(3H, s), 3.7(2H, t), 4.1(2H, t), 7.5-7.7(4H, m); $^{13}$Cnmr δ 27.7, 65.0, 108.5, 119.1, 126.5, 132.5, 149.0, 200.0; IR cm$^{-1}$ 2800-3000(CH), 2240(CN), 1600(arom. ring); ms m/z(%) 174(100), 130(41), 87(19.7), 102(16.9), 158(10).

*2-Methyl-2-(p-carboxyphenyl)-1,3-dioxolane* **170** (Figure 71)

The nitrile **169** (5.2g, 0.027 mole)[66] was hydrolyzed to acid **170** by refluxing with 10% solution of sodium hydroxide (41 mL) for 5 hours. The cooled mixture was washed with ether, then acidified with concentrated hydrochloric acid to pH 3 and extracted with ether. The ethereal solution was dried and evaporated to give a white solid. Yield: 5.7g (100%); M.p.158-161$^0$C; $^1$Hnmr δ 1.7(3H, s), 3.8(2H, t), 4.1(2H, t), 7.6(2H, d), 8.1(2H,d); $^{13}$Cnmr δ 27.7, 64.9, 108.9, 125.9, 128.6, 130.6, 149.7, 172.0; IR cm$^{-1}$2500-3100(OH), 1700(C=O); ms m/z(%) 193(100), 149(43.9), 87(22.9), 194(11.4), 65(7.4).

*p-Acetylbenzoic acid* **171** (Figure 71)

Deprotection of **170** was done in 2-necked round-bottom flask fitted with a reflux condenser and a drying tube. 2-Methyl-2-(p-carboxyphenyl)-1,3-dioxolane **170** (11.95g, 0.05mole) was dissolved in dry acetone, anhydrous hydrochloric acid (4 mL of 1M solution in diethylether) was added and the reaction mixture was refluxed for 5 hours. The acetone was evaporated and crude product was washed with cold acetone to give a white solid. Yield: 9.4g (100%); M.p.208-210$^0$C; $^1$Hnmr δ 2.7(3H, s), 8.0(2H, d), 8.2(2H, d); $^{13}$Cnmr δ 27.2, 128.5, 130.4, 135.2, 140.4, 168.1, 198.1; IR cm$^{-1}$ 2500-3100(OH), 1750(C=O, doublet); ms m/z(%) 149(100), 164(21.2), 121(14.0), 65(12.4), 150(9.2).

*p-Acetylbenzoyl chloride* **172** (Figure 71)

p-Acetylbenzoic acid **171** (2.6g, 0.015 mole) was dissolved in thionyl chloride (30 mL) in a two-necked round-bottom flask equipped with a condenser and drying tube. After refluxing for two hours, thionyl chloride was removed by distillation and **172** was recovered in a quantitative yield as a yellow semisolid. $^1$Hnmr δ 2.7(3H, s), 8.1(2H, d), 8.2(2H, d); $^{13}$Cnmr δ 27.3, 128.9, 131.9, 136.9, 142.1, 197.3; IR cm$^{-1}$ 1750(C=O, doublet), 1650(arom. ring); ms m/z(%) 147(100), 167(29), 76(13), 104(12), 182(11), 169(10).

*1-(p-Acetylbenzoyl) piperidine* **173** (Figure 72)

Piperidine (8.85g, 0.104 mole) was dissolved in dry tetrahydrofuran in a flame-dried two-necked round-bottomed flask under argon. p-Acetylbenzoyl chloride (4.7g, 0.026 mole) dissolved in THF was added slowly with stirring and the reaction mixture was refluxing for an hour. After the solvent was evaporated the residue was dissolved in ether, and the ethereal solution washed with 5% hydrochloric acid, then 10% sodium hydroxide and finally with water. After the extract was dried,evaporation gave 6.6g of crude material. It was purified by passing through silica gel column with a solvent 50% benzene-50% ethylacetate to give a yellow oil; Yield: 3.6g (69%); $^{1}$Hnmr δ 1.4-1.8(6H, m), 2.6(3H, s), 3.3(2H, t), 3.7(2H, t), 7.5(2H, d), 8.0(2H,d); $^{13}$Cnmr δ 24.9, 26.8, 43.5, 49.0, 127.3, 128.7, 130.4, 137.9, 169.5, 197.0; IR cm$^{-1}$ 1650(C=O, doublet), 1600(arom. ring); ms m/z(%) 181(100), 182(75), 98(54), 183(14).

*1-(p-Acetylbenzoyl) pyrrolidine* **174** (Figure 72)

The procedure for preparation of **174**, starting with pyrrolidine (1.4g, 0.02 mole) was the same as for preparation of 1-(p-acetylbenzoyl) piperidine **173** with the following modifications. After the addition of the acid chloride was completed, the reaction mixture was stirred at room temperature overnight . The extract was washed with 5% sodium hydroxide and then with water to give a yellow oil. Yield: 0.45g (41%); $^{1}$Hnmr δ 1.9-2.1(4H, m), 2.6(3H, s), 3.4(2H, t), 3.7(2H, t), 7.6(2H, d), 8.0(2H,d); $^{13}$Cnmr δ 24.7, 26.6, 27.0, 46.5, 49.7, 127.6, 128.6, 138.1, 141.7, 168.8, 197.8; IR cm$^{-1}$ 1650(C=O, doublet), 1570(arom. ring); ms m/z(%) 147(100), 217(70.0), 216(24.8), 148(10.9), 218(10.6).

*1-(p-Ethylbenzyl) piperidine* **175** (Figure 73)

1-(p-Ethylbenzoyl) piperidine **162** (4.8g, 0.022 mole) was dissolved in dry tetrahydrofuran in a flame dried two-necked round-bottomed flask fitted with a reflux condenser under argon. Lithium aluminum hydride (1.7g, 0.04 mole) was added (the reaction flask was kept in the ice bath while adding) and the reaction mixture was refluxed for 48 hours. Decomposition of the lithium

aluminum hydride was done by subsequent addition of 1.7 mL of water, 1.7 mL of 15% sodium hydroxide and 5.1 mL of water. The precipitate was filtered, washed with dry THF, and the filtrate then dried over anhydrous magnesium sulfate and evaporated to give a yellow oil. Yield: 3.7g (84%); $^{1}$Hnmr δ 1.2(3H, t), 1.4-1.5(2H, m), 1.5-1.7(4H, m), 2.3-2.4(4H, t), 2.6(2H, q), 3.5(2H, s), 7.2(2H, d), 7.3(2H,d); $^{13}$Cnmr δ 16.3, 25.0, 26.5, 29.0, 54.9, 64.1, 128.0, 129.7, 136.2, 143.1; IR cm$^{-1}$ 2800-3000(CH), 1550(arom. ring); ms m/z(%) 202(100), 119(99.4), 203(92.1), 84(89.1), 98(67.8).

*1-(p-Ethylbenzyl) pyrrolidine* **176** (Figure 73)

1-(p-Ethylbenzyl) pyrrolidine **176** was prepared using the same procedure as for 1-(p-ethylbenzyl) piperidine **175**, starting with 1-(p-ethylbenzoyl) pyrrolidine **163** (4.1g, 0.02 mole) and lithium aluminium hydride (1.5g, 0.04 mole). Yellow oil; Yield: 3.2g (85%); $^{1}$Hnmr δ 1.3(3H, t), 1.7-2.0(4H, m), 2.4-2.6(4H, t), 2.7(2H, q), 3.6(2H, s), 7.2(2H, d), 7.3(2H,d); $^{13}$Cnmr δ 16.1, 23.9, 29.0, 54.6, 60.9, 128.1, 129.3, 137.0, 143.1; IR cm$^{-1}$ 2800-3000(CH), 1550(arom. ring); ms m/z(%) 119(100), 188(97.7), 189(83.1), 84(62.0), 70(56.7).

*1-(p-Hydroxyethylbenzyl) piperidine* **177** (Figure 73)

The procedure used for preparation of **177** was the same as for synthesis of 1-(p-ethylbenzyl) piperidine **175**, starting with 1-(p-acetylbenzoyl) piperidine **173** (4.0g, 0.017 mole) and lithium aluminium hydride (2.0g, 0.05 mole) to obtain 3.56g of the crude product (96% yield). The crude product was purified by column chromatography using ethylacetate and a mixture of methanol/ammonium hydroxide 9:1 in 5%stepwise gradient. Yield: 3.4g (94%); $^{1}$Hnmr δ 1.3-1.5(2H, m), 1.5(3H, d), 1.5-1.7(4H, m), 2.4(4H, t), 3.5(2H, s), 4.9(1H, q), 7.3(4H,m); $^{13}$Cnmr δ 23.8, 24.5, 25.4, 53.9, 63.0, 69.7, 124.6, 128.8, 137.3, 143.8; IR cm$^{-1}$ 3100-3500(OH), 2800-3000(CH); ms m/z(%) 84(100), 98(91.8), 218(82.1), 219(72.4),135(22.3).

*1-(p-1-Hydroxyethylbenzyl) pyrrolidine* **178** (Figure 73)

The synthesis was performed as for 1-(p-ethylbenzyl) pyrrolidine **176**, starting with 1-(p-acetylbenzoyl) pyrrolidine **174** (2.3g, 0.01 mole) to give 2.0g of the crude product. The purification was done by column chromatography using ethylacetate and a mixture of methanol/ammonium hydroxyde 9:1 in 5%stepwise gradient. Yield: 1.5g (69%); $^{1}$Hnmr δ 1.5(3H, d), 1.6-1.9(4H, m), 2.5(4H, t), 3.6(2H, s), 4.9(1H, q), 7.3(4H, m); $^{13}$Cnmr δ 23.8, 25.5, 54.5, 60.7, 70.5, 125.7, 129.5, 138.6, 145.0; IR cm$^{-1}$ 3100-3500(OH), 2800-3000(CH); ms m/z(%) 84(100), 204(74), 70(69.1), 205(65.5), 92(26).

*1-(p-Acetylbenzyl) piperidine* **179** (Figure 74)

1-(p-1-Hydroxyethylbenzyl) piperidine **177** (0.1g, 0.45 mmole) was dissolved in acetone. Jones' reagent (20 μL) was added with stirring. After 10 minutes of stirring at room temperature the acetone was evaporated, dichloromethane added, and the mixture basified with 5% potassium hydroxide and the two layers separated. The dichloromethane layer was washed with saturated sodium bicarbonate, saturated sodium chloride, then dried and evaporated to yield 0.07g of **179** (74%); $^{1}$Hnmr δ 1.4-1.7(6H, m), 2.3-2.5(4H, t), 2.6(3H, s), 3.5(2H, s), 7.4(2H, d), 7.9(2H,d); $^{13}$Cnmr δ 22.9, 23.9, 25.2, 53.2, 62.0, 126.9, 127.8, 134.6, 143.1, 196.6; IR cm$^{-1}$ 1700(C=O), 1600(arom. ring); ms m/z(%) 216(100), 217(90.7), 98(78.5), 84(52.3), 133(43.5).

*1-(p-Acetylbenzyl) pyrrolidine* **180** (Figure 74)

1-(p-Acetylbenzyl) pyrrolidine **180** was prepared using the same procedure as for 1-(p-acetylbenzyl) piperidine **179**, starting with 1-(p-1-hydroxyethylbenzyl) pyrrolidine **178** (1.0g, 0.005 mole) and Jones' reagent (0.87 mL). Yellow oil; Yield: 0.45g (45%); $^{1}$Hnmr δ 1.7-1.9(4H, m), 2.4-2.6(4H, m), 2.6(3H, s), 3.6(2H, s), 7.5(2H, d), 7.9(2H,d); $^{13}$Cnmr δ 23.9, 27.0, 54.6, 60.7, 128.8, 129.3, 136.3, 145.4, 198.3; IR cm$^{-1}$ 1700(C=O), 1600(arom. ring); ms m/z(%) 202(100), 84(96.4), 203(92.4), 70(55.4), 133(42.4).

PART TWO : BIOTRANSFORMATIONS using *Beauveria sulfurescens* ATCC 7159

The growth medium used in cultivation of the microorganism *Beauveria sulfurescens* ATCC 7159 consisted of 10g of glucose and 20g of corn steep liquor per L of distilled water, pH adjusted to pH 4.85 with 1M NaOH . This medium (3L) was distributed among 15 1L Erlenmeyer flasks which were stoppered with sponge plugs and sterilized in the autoclave at 20 psi/121$^0$C for 20 mins. After cooling, the growth medium was inoculated under sterile conditions with one slope of the fungus. The flasks were allowed to stand over night at 27$^0$C (stationary growth), then placed on a rotary shaker at 180 rpm, 27$^0$ for further 48 hours. After three days the substrate (0.75g) dissolved in 95% ethanol (30 mL) was distributed among the flasks (added directly to the flasks with a syringe), which were stoppered with foam plugs and placed on a rotary shaker at 200 rpm/27$^0$C for further 72 hours. The fungal material was removed by filtration, and the filtrate was extracted on the continuous extractor for three days with dichloromethane. Dichloromethane was evaporated using the rotary evaporator, the extract was submitted to a flash chromatography.

1. THE AMIDE SUBSTRATES

*1-(p-Ethylbenzoyl) piperidine* **162** (Figure 75)

The crude extraction product (1.1g from 0.37g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **181** was a yellow liquid; Yield: 78%; $^1$Hnmr $\delta$ 1.4(3H, d), 1.6-1.8(6H, m), 3.4(2H, t), 3.7(2H, t), 5.0(1H, q), 7.3-7.5(4H, m); $^{13}$C $\delta$ 26.6, 27.3, 28.6, 45.2, 50.8, 72.1, 127.4, 129.1, 137.5, 149.2, 172.3; IR cm$^{-1}$ 3100-3600(OH), 1650(C=O), 1600(arom. ring); ms m/z(%) 55(100), 127(96.3), 232(93.2), 149(60.6), 233(54.3); $[\alpha]_D$= -6.9 (c=1.28, chloroform). The enantiomeric purity was examined using tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium(III) derivative as a chiral shift reagent, e.e.=21±5%.

*1-(p-Ethylbenzoyl) pyrrolidine* **163** (Figure 76)

The crude extraction product (1.4g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **182** was a yellow liquid; Yield: 83%; $^{1}$Hnmr δ 1.4(3H, d), 1.7-2.0(4H, m), 3.3(2H, t), 3.5(2H, t), 4.8(1H, q), 7.2-7.5(4H, m); $^{13}$C δ 24.7, 25.6, 26.7, 46.6, 50.0, 69.7, 125.6, 127.5, 135.8, 148.7, 170.2; IR cm$^{-1}$ 3100-3700(OH), 1650(C=O), 1600(arom. ring); ms m/z(%) 43(100), 126(73.2), 85(56.4), 149(45.9), 219(41.5), $[\alpha]_D$= -3.5 (c=2.38, chloroform). The enantiomeric purity of **182** was e.e.=20±5%, determined using tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium(III) derivative as a chiral shift reagent.

*1-(p-Acetylbenzoyl) piperidine* **173** (Figure 77)

The crude extraction product (1.7g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient, followed by an ether-methanol 5% stepwise gradient). Two products were isolated.

Product **181** was a pale yellow oil; Yield: 40%; $^{1}$Hnmr δ 1.4(3H, d), 1.6-1.8(6H, m), 3.4(2H, t), 3.7(2H, t), 5.0(1H, q), 7.3-7.5(4H, m); $^{13}$C δ 26.5, 27.2, 28.5, 45.2, 50.8, 71.6, 127.4, 128.7, 137.0, 149.5, 172.3; IR cm$^{-1}$ 3100-3600(OH), 1650(C=O); ms m/z(%) 232(100), 149(67.4), 127(61.9), 83(43.9), 233(40.2); $[\alpha]_D$= -17.0 (c=1.71, chloroform). Product was a single enantiomer, determined using tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium(III) derivative as a chiral shift reagent.

Product **183** was a yellow semisolid; Yield: 60%; $^{1}$Hnmr δ 1.5(3H, d), 1.5-1.8(5H, m), 3.4(2H, t), 3.7(2H, t), 4.1(1H, d), 4.9(1H, q), 7.2-7.4(4H, m); $^{13}$C δ 24.6, 24.9, 45.6, 49.2, 74.4, 75.5, 127.3, 127.6, 135.2, 144.2, 170.6; IR cm$^{-1}$ 3100-3700(OH), 1650(C=O); ms m/z(%) 84(100), 149(39.1), 248(32.1), 249(17.9), 128(12.2); $[\alpha]_D$= -61.97(c=1.06, chloroform).

*1-(p-Acetylbenzoyl) pyrrolidine* **174** (Figure 78)

The crude extraction product (1.2g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient, followed by an ether-methanol 5% stepwise gradient). Product **182** was a yellow semisolid; Yield: 54%; $^{1}$Hnmr δ 1.4(3H, d), 1.6-2.0(4H, m), 3.4(2H, t), 3.6(2H,t), 4.8(1H, q), 7.2-7.5(4H, m); $^{13}$C δ 24.7, 25.6, 26.7, 46.6, 49.8, 69.7, 125.5, 127.5, 135.8, 148.7, 170.1; IR cm$^{-1}$ 3100-3600(OH), 1650(C=O); ms m/z(%) 149(100), 219(56.8), 55(51.7), 218(33.9); $[\alpha]_D$= -32.55 (c=1.15, chloroform). The product was a single enantiomer, determined using tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato], Europium(III) derivative as a chiral shift reagent.

*1-Benzoyl-4-piperidone* **156** (Figure 79)

The crude extraction product (1.5g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient, followed by an ether-methanol 5% stepwise gradient). The product **184** was a yellow liquid; $^{1}$Hnmr δ 1.2-1.5(2H, m), 1.6-1.9(2H, m), 3.0-3.3(2H, m), 3.4-3.7(2H, m), 3.8-4.1(2H, m), 7.1-7.4(5H, s); $^{13}$C δ 34.0, 34.7, 40.0, 45.3, 66.9, 127.0, 128.8, 129.7, 136.3, 170.8; IR cm$^{-1}$ 1600-1650(C=O, amide), 3150-3500(OH); ms m/z(%) 105(100), 204(66.8), 77(44.1), 205(31.6), 51(10.0).

*1,1'-Carbonyldipiperidine* **148** (Figure 80)

The crude extraction product (1.6g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Amide **148** was not transformed by *B. sulfurescens*; pure starting material was recovered.

2. THE AMINE SUBSTRATES

*1-Phenyl iperidine* **155** (Figure 82)

The crude extraction product (1.9g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient, followed by an ether-methanol 5% stepwise gradient). The biotransformation of 1-phenylpiperidine **155** gave **185** in 7% yield. Pale yellow solid; M.p.125-127$^0$C; $^1$Hnmr δ 1.4-1.6(2H, m), 1.6-1.8(4H, m), 2.9-3.1(4H, t), 6.6-6.8(2H, m), 6.8-6.9(2H, m); $^{13}$C δ 24.5, 26.4, 53.1, 116.2, 119.6, 129.4, 151.1; ms m/z(%) 66(100), 176(89.4), 84(84.5), 177(76.0), 121(27.1).

*1-(p-Ethylphenyl) piperidine* **160** (Figure 83)

The crude extraction product (1.7g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **186** was a yellow liquid; Yield: 39%; $^1$Hnmr δ 1.5-1.6(2H, m), 1.7-1.9(4H, m), 2.8(2H, t), 3.2(4H, t), 3.4(1H, s), 3.8(2H, t), 6.9(2H, d), 7.1(2H, d); $^{13}$C δ 24.6, 26.2, 38.6, 51.5, 64.2, 117.4, 129.6, 129.9, 151.2; IR cm$^{-1}$ 3100-3600(OH), 1600(arom. ring); ms m/z(%) 174(100), 205(29.7), 175(14.0), 118(8.2), 91(4.7).

*1-(p-Isopropenylphenyl) piperidine* **165** (Figure 84)

The crude extraction product (0.79g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). 1-(p-Isopropenylphenyl) piperidine **165** was converted to **35** in 12% yield. M.p.203$^0$C; $^1$Hnmr δ 1.5-1.8(6H, m), 2.5(3H, s), 3.1-3.3(4H, m), 6.9(2H, d), 7.9(2H, d); $^{13}$C δ 24.7, 25.7, 26.4, 49.0, 113.6, 127.1, 130.8, 154.7, 196.6; IR cm$^{-1}$ 1600, 1650(C=O), 2800-3000; ms m/z(%) 203(100), 202(78.4), 188(71.6), 132(21.3), 204(14.2).

3. NEUTRAL SUBSTRATES

*Phenylcyclohexane* **152** (Figure 89)

The crude extraction product (0.8g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient, followed by an ether-methanol 5% stepwise gradient). Products **187** and **188** were recovered as a pale yellow crystals; Yield: 14%; M.p.91-95$^0$C; $^1$Hnmr δ 1.3-1.7(8H, m), 1.8-2.0(4H, m), 2.0-2.3(4H, m), 2.5-2.7(2H, m), 3.1-3.4(2H, m), 3.6-3.9(2H, m), 7.2-7.5(10H, m); $^{13}$C δ **187**: 32.7, 36.2, 43.8, 70.9, 126.5, 127.4, 128.8,147.0; $^{13}$C **188**: 24.9, 33.9, 35.6, 43.2, 43.5, 71.3, 126.5, 127.4, 128.8, 146.7; IR cm$^{-1}$ 3100-3400(OH), 2800-3000(CH); ms m/z(%) 91(100), 104(80), 158(69.9), 176(52.7), 117(47.2).

*4'-Cyclohexylacetophenone* **153** (Figure 90)

The crude extraction product (1.3g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Products **189** and **190** were recovered as a yellow solid, M.p.118-120$^0$C; Yield: 57%; $^1$Hnmr δ 1.3-2.2(8H, m), 2.5-2.7(1H, m), 2.6(3H, s), 3.6-3.8(1H, m), 7.3(2H, d), 7.9(2H, d); $^{13}$C δ **189**: 26.9, 32.5, 36.1, 43.9, 70.7, 127.4, 129.1, 135.6, 152.7, 198.3; $^{13}$C **190**: 24.8, 27.8, 33.3, 35.6, 43.1, 43.2, 71.1, 127.4, 129.1, 135.6, 152.3, 198.3; IR cm$^{-1}$ 3200-3500(OH), 1700(C=O), 1600(arom. ring); ms m/z(%) 203(100), 200(93.5), 185(90.8), 131(87.5), 218(29.6).

*p-Isopropenylphenyl cyclohexane* **167** (Figure 91)

The crude extraction product (0.80g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Four different products were recovered from biotransformation of **167**.

Product **166** was a clear liquid; Yield 13%; $^1$Hnmr δ 1.2-1.5(5H, m), 1.6(6H, s), 1.7-1.9(5H, m), 2.4-2.6(1H, m), 7.2(2H, d), 7.4(2H, d); $^{13}$C δ 24.5, 25.2, 30.0, 32.8, 42.4, 70.7, 122.6,

125.3, 144.7; IR $cm^{-1}$ 3100-3650(OH); ms m/z(%) 201(100), 203(30.1), 55(26.0), 202(19.7), 218(5.3).

Product **153** was a yellow liquid; Yield 23%; $^{1}$Hnmr δ 1.3-1.6(6H, m), 1.7-2.0(4H, m), 2.4-2.5(1H, m), 2.6(3H, s), 7.3(2H, d), 7.9(2H, d); $^{13}$C δ 26.4, 26.8, 27.1, 45.0, 127.7, 128.9, 135.5, 154.1, 198.1; IR $cm^{-1}$ 1650(C=O); ms m/z(%) 43(100), 135(65.0), 100(55.6), 83(45.5), 55(42.0), 202(19.6).

Product **191** was a yellow liquid; Yield 28%; $^{1}$Hnmr δ 1.3-1.5(4H, m), 1.6(6H, s), 1.7-2.2(4H, m), 2.4-2.6(1H, m), 3.6-3.8(1H, m), 7.2(2H, d), 7.4(2H, d); $^{13}$C δ 24.8, 31.3, 32.8, 36.1, 42.7, 43.3, 70.9, 72.8, 124.8, 126.9, 145.4, 147.2; IR $cm^{-1}$ 3100-3650(OH); ms m/z(%) 217(100), 219(20.4), 43(18.7), 218(18.4), 234(5.6); $[\alpha]_D$= +5.3 (c=0.655, chloroform).

Product **192** was a white semisolid; Yield 34%;$^{1}$Hnmr δ 1.2-1.4(6H, m), 1.5(3H, s), 1.7-2.0(4H, m), 2.2-2.6(1H+2OH, m), 3.6(1H, d), 3.8(1H, d), 7.2(2H, d), 7.4(2H, d); $^{13}$C δ 23.6, 26.4, 34.6, 42.7, 43.3, 71.3, 75.1, 125.6, 127.1, 143.2, 145.4; IR $cm^{-1}$ 3100-3600(OH), 2800-2900(CH), 1600(arom. ring); ms m/z(%) 219(100), 210(16.7), 220(15.9), 91(12.3), 235(3.8); $[\alpha]_D$= -13.9 (c=1.355, chloroform).

*p-1-Hydroxyisopropylphenyl cyclohexane* **166** (Figure 93)

The crude extraction product (0.78g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **191** was a yellow semisolid; Yield: 20%; $^{1}$Hnmr δ 1.2-1.5(4H, m), 1.6(6H, s), 1.8-2.0(2H, m), 2.0-2.2(2H, m), 2.5-2.8(1H+2OH, m), 3.6-3.8(1H, m), 7.2(2H, d), 7.4(2H, d); $^{13}$C δ 24.8, 32.0, 32.8, 35.6, 36.2, 43.7, 72.8, 77.0, 124.8, 126.9, 144.9, 147.2; IR $cm^{-1}$ 3100-3500(OH), 2800-3000(CH); ms m/z(%) 219(100), 201(45.4), 216(33.2), 198(25.9), 234(11.5).

*Bicyclohexyl* **151** (Figure 94)

The crude extraction product (1.2g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **193** was a solid; Yield: 13%; M.p.115-117$^0$C; $^1$Hnmr δ 0.8-1.3(10H, m), 1.5-2.1(10H, m), 3.4-3.6(2H, m); $^{13}$C δ 22.8, 26.6, 26.8, 26.9, 27.7, 34.4, 34.5, 38.3, 39.9, 40.5, 69.7, 69.9; IR cm$^{-1}$ 3100-3500(OH), 2800-3000(CH); ms m/z(%) 81(100), 162(58), 55(42.1), 80(41.9), 97(39.2), 198(0.6).

*p-Ethylphenyl cyclohexane* **161** (Figure 95)

The crude extraction product (1.0g from 0.75g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product was a yellow semisolid; Yield: 12%; $^1$Hnmr δ 1.2-2.2(8H, m), 2.4-2.6(1H, m), 2.5(3H, s), 3.6-3.8(1H, m), 7.3(2H, d), 7.9(2H, d); $^{13}$C δ **189**: 26.9, 32.5, 36.0, 43.9, 70.7, 127.5, 129.0, 135.5, 152.7, 198.4; $^{13}$C **190**: 24.7, 27.8, 33.5, 35.5, 43.0, 43.2, 71.0, 127.5, 129.0, 135.5, 152.3, 198.4; IR cm$^{-1}$ 1680(C=O), 3150-3600(OH); ms m/z(%) 203(100), 200(40.0), 185(37.3), 218(27.7), 131(20.7).

4. OTHER SUBSTRATES:

*2-Benzoylthiophene* **157** (Figure 96)

The crude extraction product (1.2g from 1.0g) was purified by flash chromatography (silica gel; benzene-ethyl acetate 10% stepwise gradient). Product **194** was a brown oil. Yield: 49%; $^1$Hnmr δ 2.8(1H, OH), 6.0(1H, s), 6.8-7.0(2H, m), 7.2-7.5(6H, m); $^{13}$C δ 72.8, 125.3, 125.8, 126.7, 127.0, 128.4, 128.9, 143.5, 148.5; IR cm$^{-1}$ 3000-3300(OH), 1600(arom. ring); ms m/z(%) 105(100), 85(58.7), 190(47.1), 111(35.6), 84(35.1); $[\alpha]_D$= -5.07 (c=2.74, chloroform). The enantiomeric ratio examined using chiral shift reagent tris[3-heptafluoropropylhydroxy methylene)-(+)-camphorato], Europium (III) derivative, suggested that the product was a single enantiomer.

# DISCUSSION

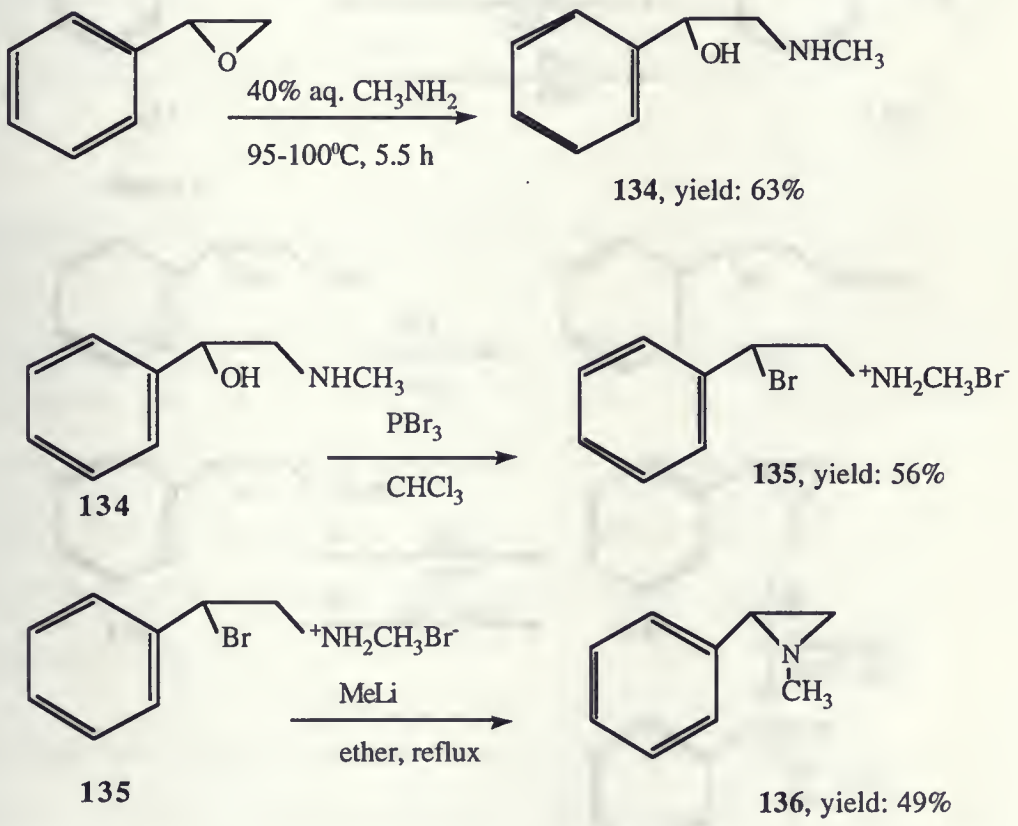
## PART ONE: MICROBIAL HYDROLYSIS OF AZIRIDINES

Preparation of the substrates is presented in the next section, followed by a discussion of the results of their microbial hydrolysis.

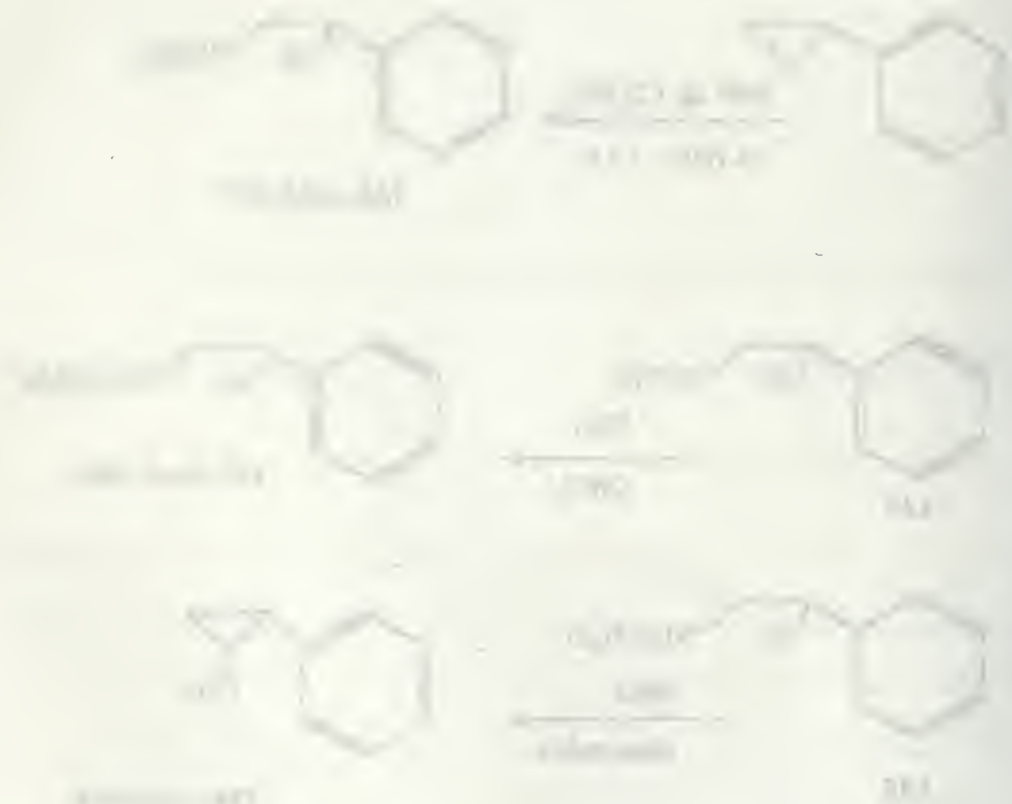
## PREPARATION OF SUBSTRATES

1-Methyl-2-phenyl aziridine **136** was prepared starting from styrene oxide as shown in Figure 55.

Figure 55: Synthesis of 1-methyl-2-phenyl aziridine **136**:

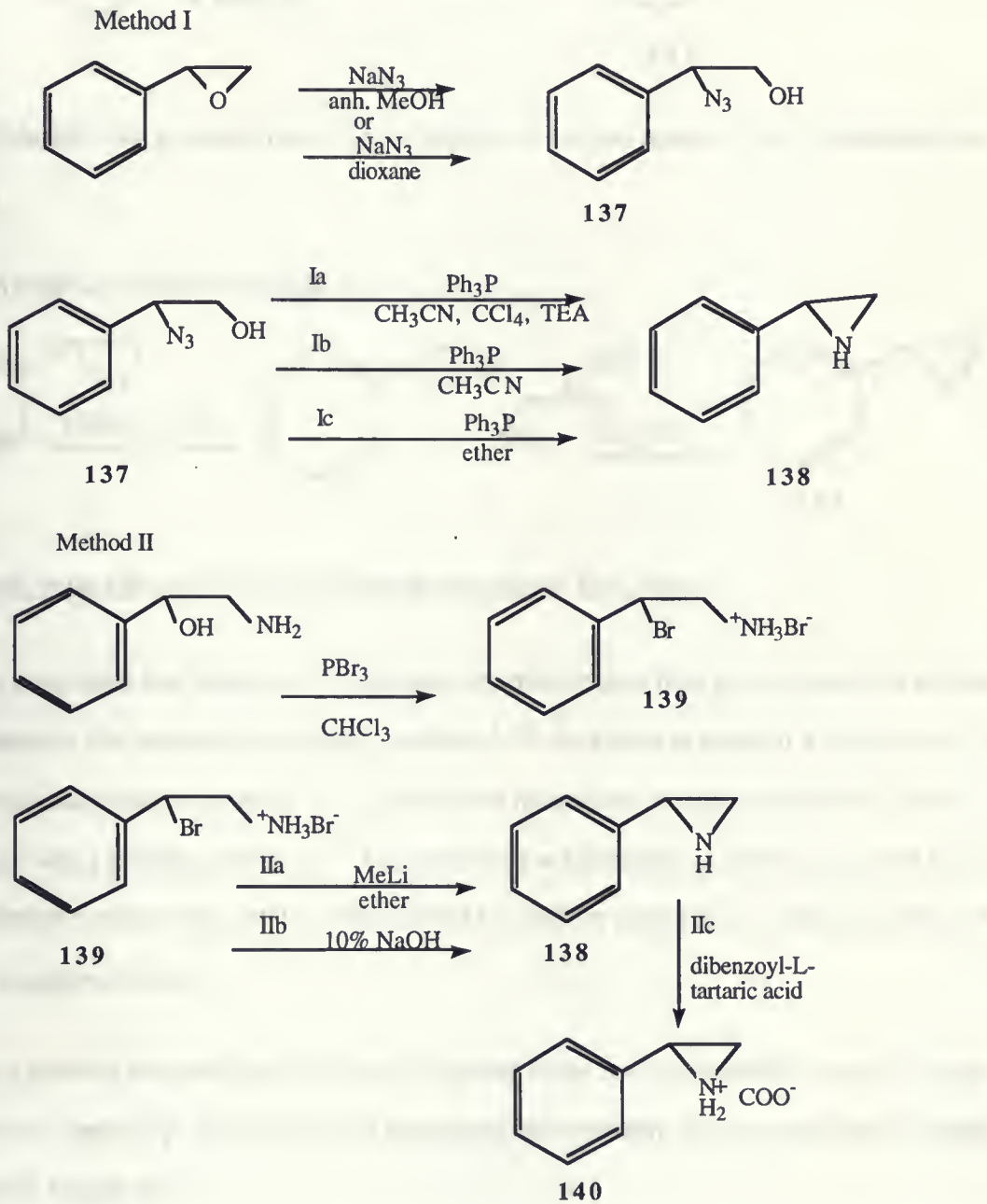



Two methods were used in the attempt to prepare 2-phenylaziridine **138**. (Figure 56) The first method involved cyclization of 2-azido-2-phenylethanol **137** (obtained from styrene oxide) using triphenylphosphine, but none of the three attempted procedures gave the desired product. Method



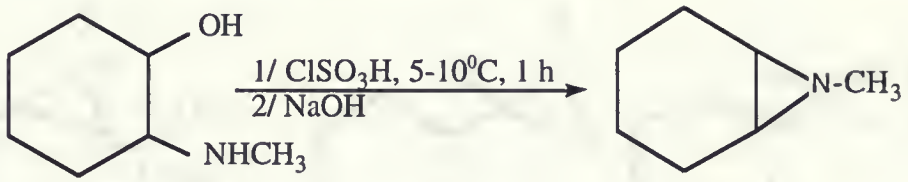
II was analogous to the synthesis of 1-methyl-2-phenylaziridine **136**, but because of the instability of 2-phenylaziridine, the tartrate salt **140** was prepared and used as a substrate.

Figure 56: Methods for the synthesis of 2-Phenylaziridine **138**




N-methyl-7-azabicyclo[4.1.0] heptane **141** was prepared by cyclization of *trans*-2-methylamino cyclohexanol. (Figure 57)

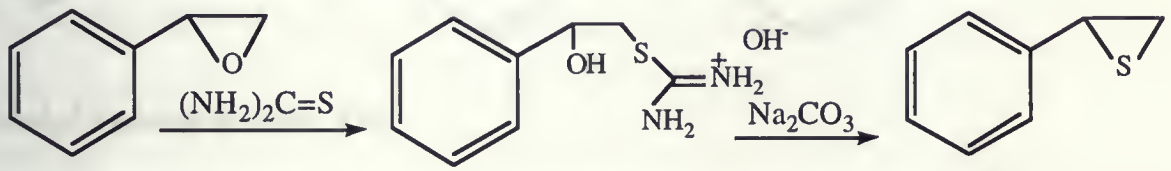
Figure 57: Synthesis of N-methyl-7-azabicyclo[4.1.0] heptane **141**




**141**

Styrene sulfide **142** was prepared from styrene oxide in a one pot reaction *via* a thiuronium salt. (Figure 58)

Figure 58: Synthesis of styrene sulfide **142**



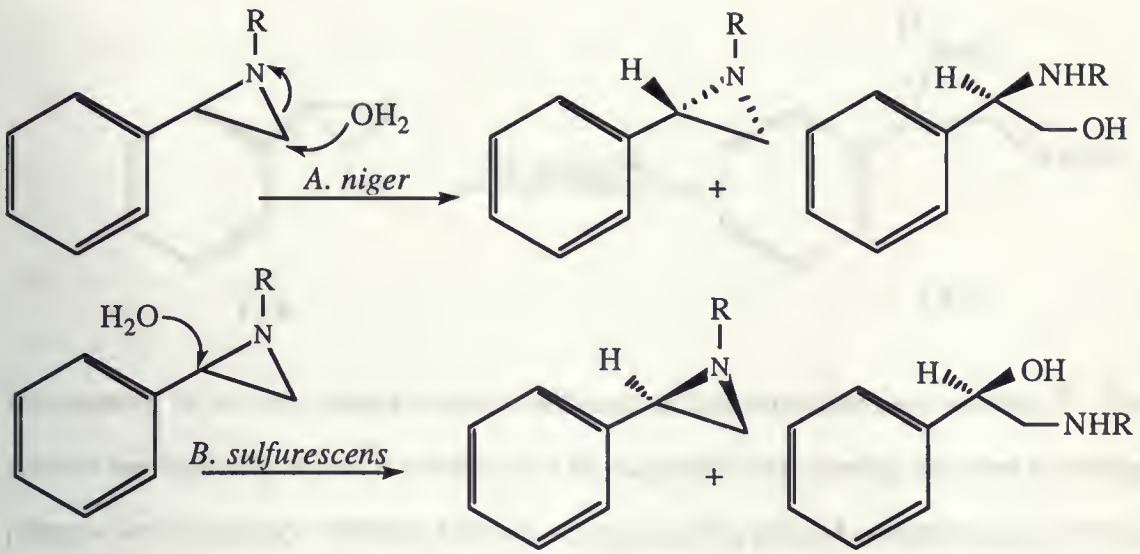

**142**

BIOHYDROLYSIS OF AZIRIDINES AND OF STYRENE SULFIDE

Since it was postulated that hydrolysis of aziridines by mammalian liver microsomes has as source of enzyme activity the microsomal epoxide hydrolase[1, 2], the idea was to test if a parallel could be drawn for their microbial hydrolysis. Enantioselective hydrolysis of styrene oxide described by Furstoss et al. was a leading reference.[23] Enantioselective hydrolysis of aziridines would lead to chiral substituted β-phenylethylamines which could be used for preparation of neuroamines such as adrenaline, noradrenaline etc.

If there was a parallel between bioconversion of styrene oxide and the aziridines using *A. niger* and *B. sulfurescens* (refer to p.10 for microbial hydrolysis of epoxides), the expected results would be as presented in Figure 59.

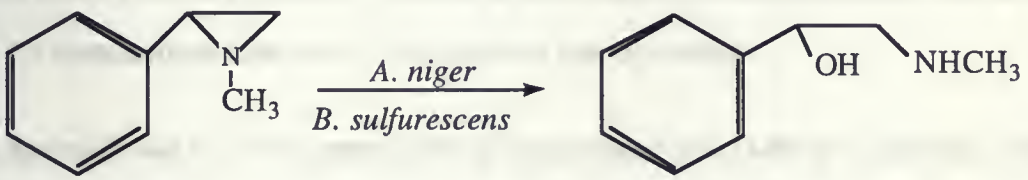
Figure 59:




*1-Methyl-2-phenyl aziridine* **136**

Biotransformation of 1-methyl-2-phenyl aziridine **136** using both *A. niger* and *B. sulfurescens* gave the same product, 1-phenyl-2-methylamino-1-ethanol **134**. (Figure 60) In both cases, however, the isolated aminoalcohol **134** was not optically active; therefore the conclusion is that the observed hydrolysis of **136** was a result of a non-enzymatic ring-opening.

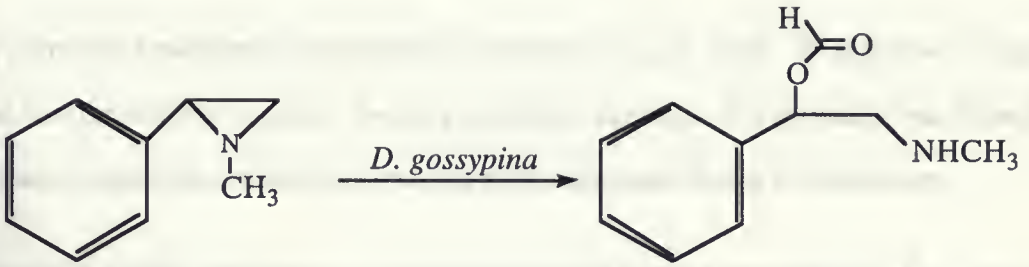
Figure 60:




**136** **134**

*Diplodia gossypina* ATCC 10936 is also known for asymmetric hydrolysis of epoxides.[54] When 1-methyl-2-phenyl aziridine **136** was submitted to the bioconversion using this fungus, the 2-formyloxy-2-phenylethylamine **143** shown in Figure 61 was recovered.

Figure 61:




**136** **143**

Formylation of primary amines in whole cell biotransformations has been reported.[67] Since the product was optically active, it could be that an enzymatic ring-opening occurred producing 1-phenyl-2-methylamino-1-ethanol **134**, which subsequently reacted with naturally occurring formic acid or formate ester. Alternatively, the formylation may be enantioselective. No literature value for specific rotation of the pure product is available for comparison with the experimental data, and the enantiomeric purity of **143** was not determined because the use of chiral shift reagent is not applicable here.

*2-Phenylaziridine* **138**

Synthesis of 2-phenylaziridine was attempted many times using several different procedures, but the desired product was not obtained. Because of its instability, it would probably not be a good substrate for biotransformation even if the synthesis was successful.

The next approach was to use the tartrate salt of 2-phenylaziridine (**140**) as a substrate, which would give free aziridine in the biotransformation media (phosphate buffer, pH 8). However, nothing but polymers and natural products was recovered from biotransformation of **140** using *A. niger* and *B. sulfurescens*, suggesting a probable polymerization of the aziridine.

*N-methyl-7-aza-bicyclo [4.1.0] heptane* **141**

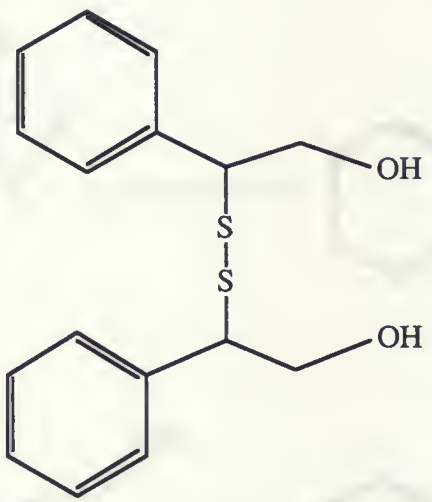
The only compound recovered from biotransformation using *A. niger* , *B. sulfurescens* and *D. gossypina* was the starting material. Being a secondary aziridine, it is probably less prone to nonenzymatic hydrolysis and the aziridine ring remained intact during the procedure.

This substrate is a meso compound, so biotransformation was not monitored by TLC over time since there was no possibility of kinetic resolution.

*Styrene sulfide* **142**

The recovered product of biotransformation of styrene sulfide **142** using *A. niger* and *B. sulfurescens* was the optically active dimer **144** (ee 64%). (Figure 62)

Figure 62:




**144**

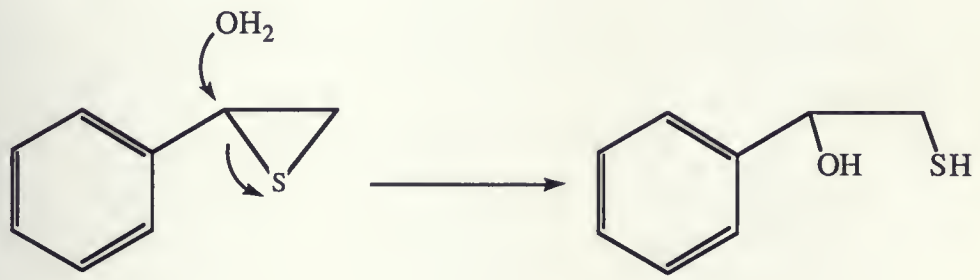
Product **144** may be the result of an enzymatic ring-opening and subsequent dimerization, since a nonenzymatic ring opening is more likely to result in water molecule attacking at the benzylic carbon (Figure 63). The monomer could have either *(R)* or *(S)* absolute configuration. That means that dimer can have (*R*,*R*), (*R*,*S*) or (*S*,*S*) configuration. Since in both cases the product

was optically active, the (*R*,*S*) combination is ruled out because it would be a meso compound, which is not optically active. Also, a meso compound may show different chemical shifts in $^{13}C$ nmr spectrum for the chiral centers compared to (*R*,*R*) or (*S*,*S*) combination. If meso compound was also present, then two sets of peaks for chiral centers would be observed in **144**, which was not the case.

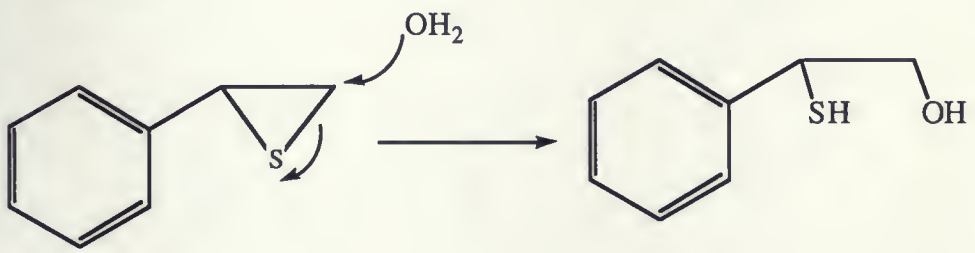
Since recovered starting material had a very low specific rotation (in 2, 4, 6 h biotransformation with both fungi), it could be speculated that both enantiomers were processed simultaneously. One of the enantiomers underwent enzymatic ring-opening and then dimerized, whereas the other one was processed in a different way or degraded.

Figure 63:

Nonenzymatic:




Enzymatic:




The project of microbial hydrolysis of aziridines was discontinued at this point since the results obtained with studied compounds were not encouraging.

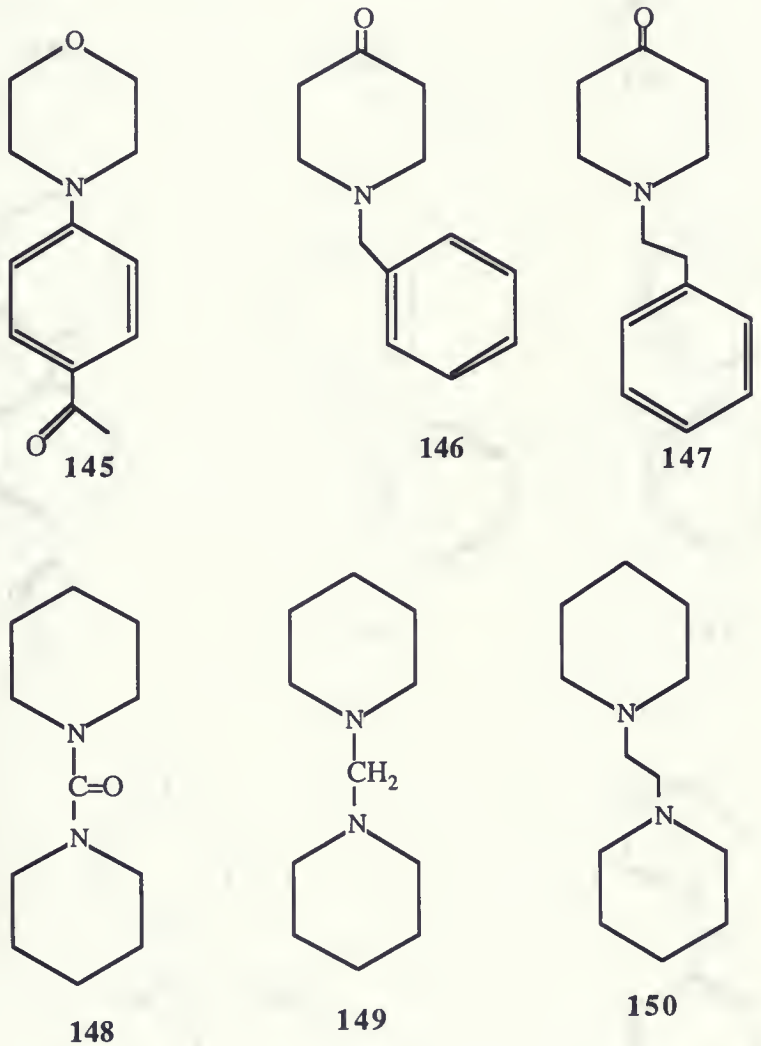
As it was noted in the introduction section, aziridines are very reactive compounds due to the reactivity of the ring nitrogen and the strained three-membered ring. Because of this reactivity, they are toxic compounds and it would be expected that the fungus would convert them into a more water soluble and chemically less reactive aminoalcohols, which can be further metabolized and excreted.

Unfortunately, synthesis of 2-phenyl aziridine, the aziridine counterpart of styrene oxide, was not reliably achieved, which also suggests that aziridines are more reactive than epoxides, and very prone to polymerization, maybe even before they reach active site of the enzyme which is responsible for epoxide hydrolysis. However, because aziridines are basic compounds, it is also reasonable to question whether they would interact with the enzyme's active site in the same way as epoxides.

PART TWO: A RATIONAL APPROACH TO THE HYDROXYLATING ENZYMES OF *Beauveria sulfurescens* ATCC 7159

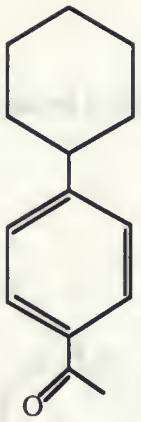
Thirty compounds overall were used as substrates. Fourteen of them were commercially available (Figure 64) and the remainder were synthesized (see section on preparation of substrates).

Figure 64: Commercially available substrates

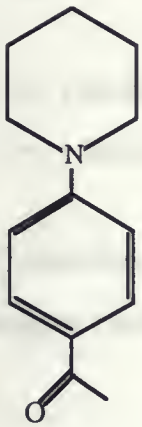


151

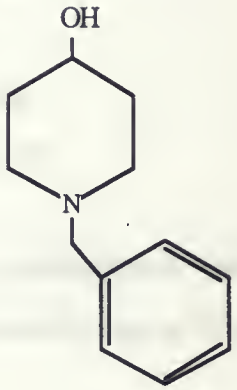
152



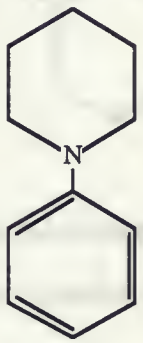

153



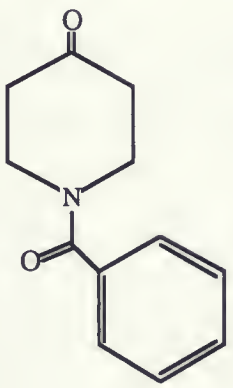

35



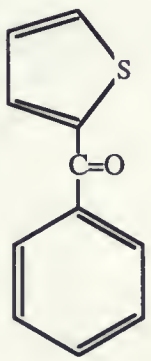

154




155




156

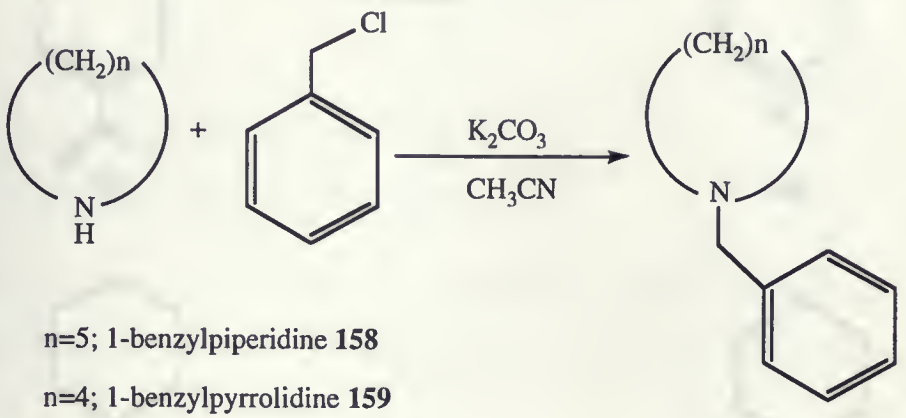



157

PREPARATION OF SUBSTRATES

1-Benzoylpiperidine **158** and 1-benzoylpyrrolidine **159** were prepared from benzyl chloride and piperidine or pyrrolidine. (Figure 65)

Figure 65:

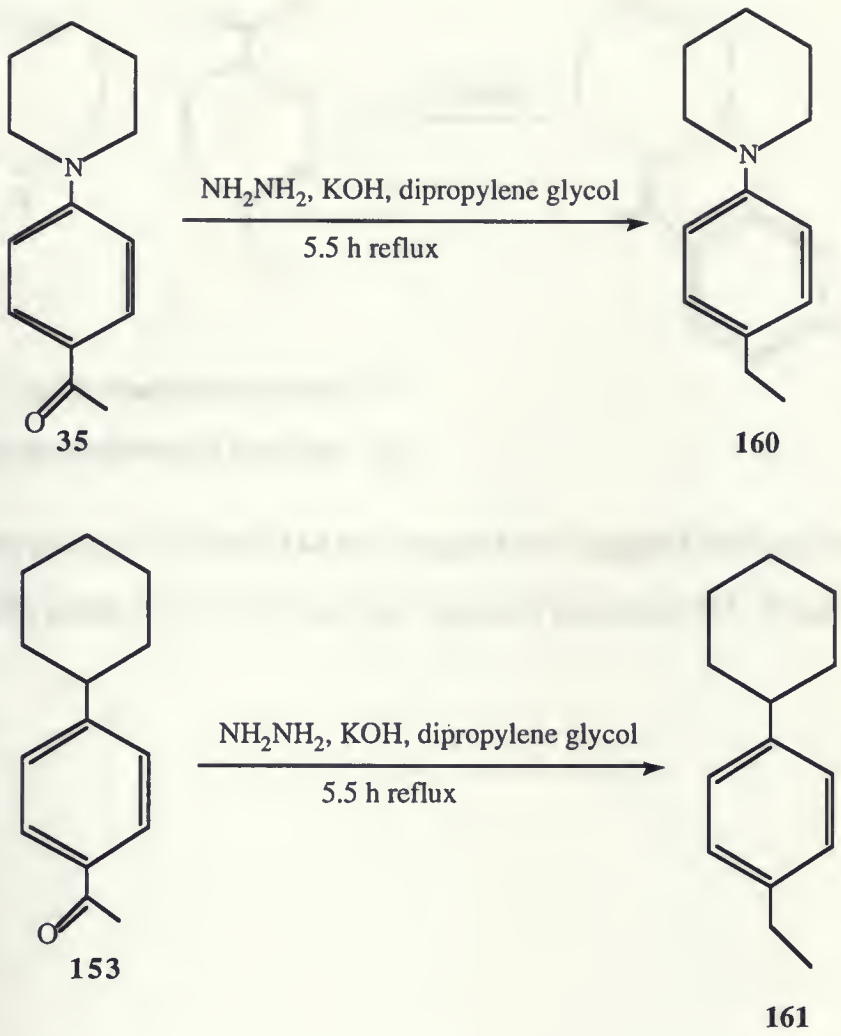



n=5; 1-benzylpiperidine **158**

n=4; 1-benzylpyrrolidine **159**

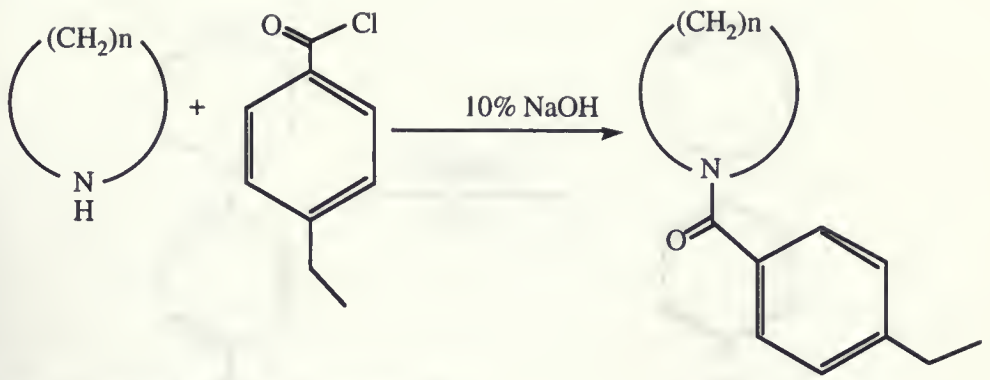
1-(p-Ethylphenyl) piperidine **160** and 1-(p-ethylphenyl) cyclohexane **161** were prepared from **35** and **153** respectively by Huang-Minlon modification of Wolf-Kishner reduction.[65] (Figure 66)

Figure 66:




1-(p-Ethylbenzoyl) piperidine **162** and 1-(p-ethylbenzoyl) pyrrolidine **163** were prepared from ethylbenzoyl chloride and piperidine or pyrrolidine. (Figure 67)

Figure 67:

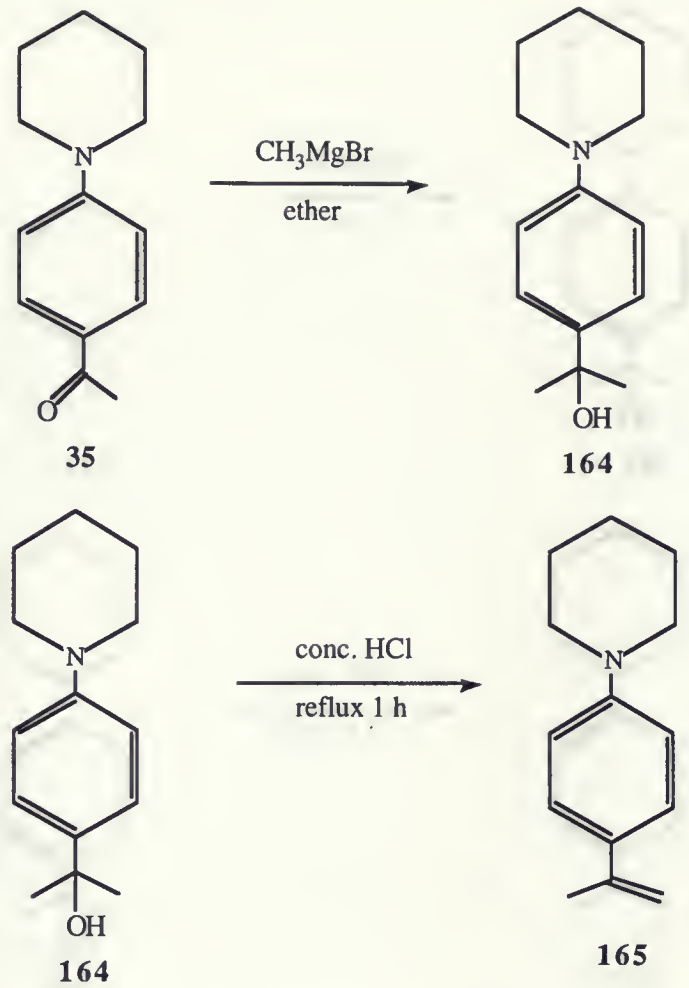



n=5; 1-(p-ethylbenzoyl) piperidine **162**

n=4; 1-(p-ethylbenzoyl) pyrrolidine **163**

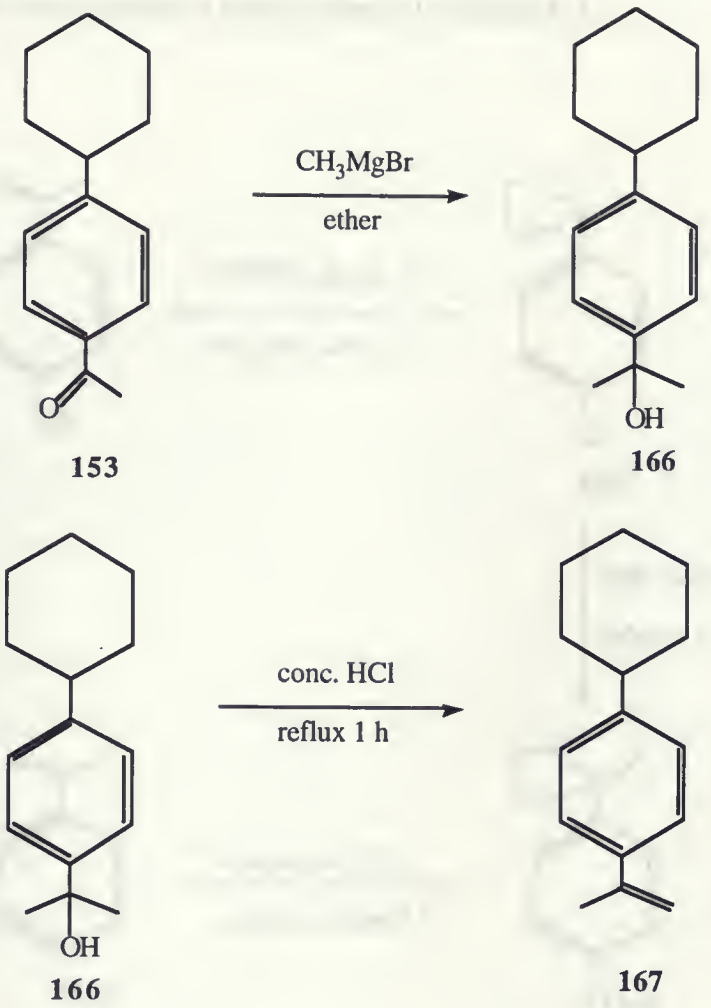
2-(4'-Piperidino)-phenyl-2-propanol **164** was prepared by a Grignard reaction from **35**, and then it was used for the preparation of 1-(p-isopropenylphenyl) piperidine **165**. (Figure 68)

Figure 68:



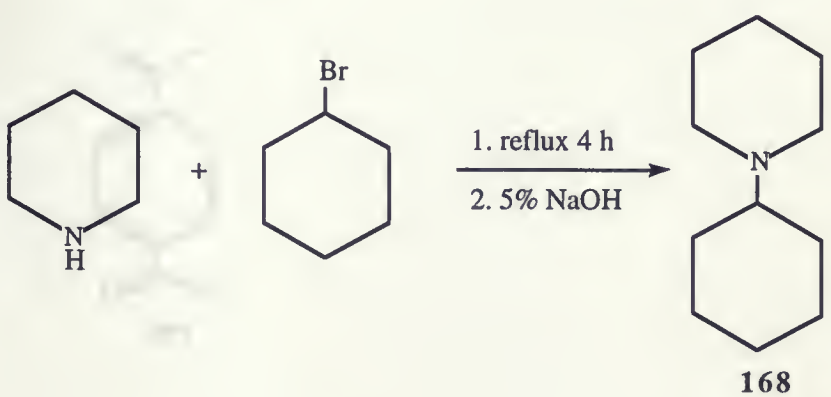

The same sequence of reactions as above was used for preparation of p-1-hydroxyisopropylphenyl cyclohexane **166** and p-isopropenylphenyl cyclohexane **167**.(Figure 69)

Figure 69:



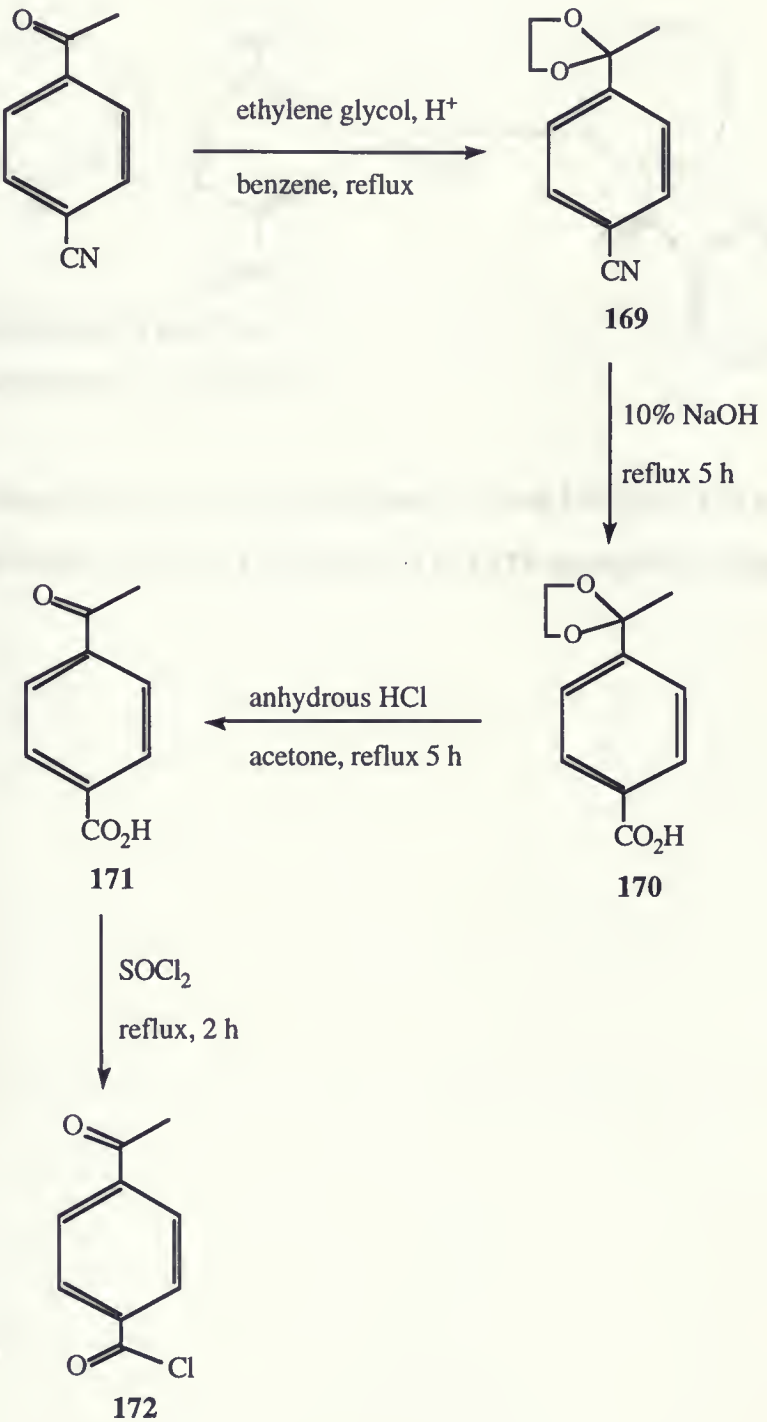

1-Cyclohexylpiperidine **168** was prepared from cyclohexyl bromide and piperidine. (Figure 70)

Figure 70:



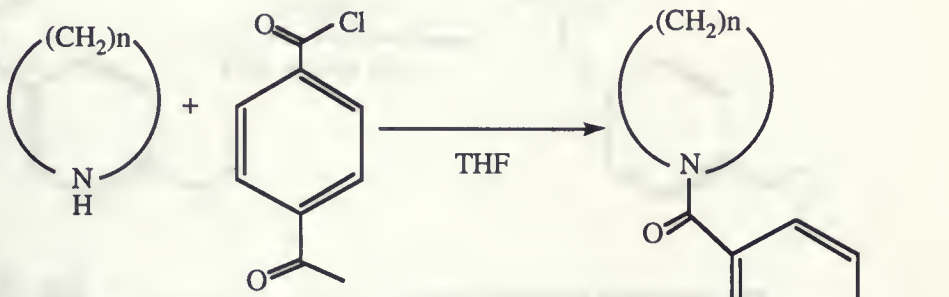
Commercially available, but expensive p-acetylbenzoic acid **171** was prepared as shown in Figure 71, and then used for the synthesis of p-acetylbenzoyl chloride **172**.

Figure 71:



1-(p-Acetylbenzoyl) piperidine **173** and 1-(p-acetylbenzoyl) pyrrolidine **174** were then prepared from p-acetylbenzoyl chloride and piperidine or pyrrolidine. (Figure 72)

Figure 72:

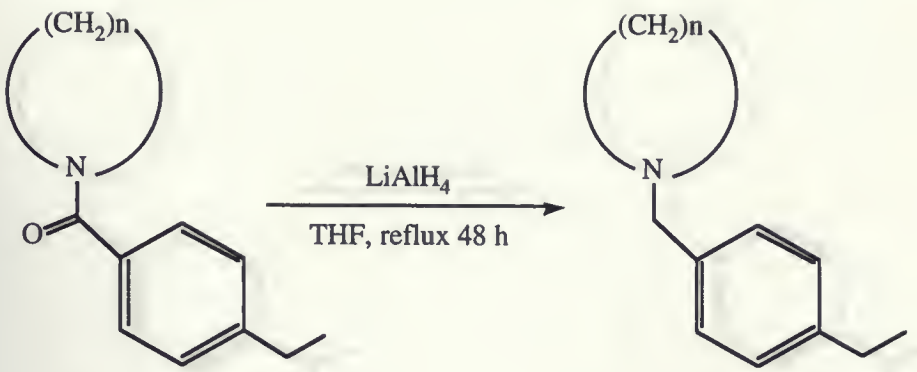



n=5; 1-(p-acetylbenzoyl) piperidine **173**

n=4; 1-(p-acetylbenzoyl) pyrrolidine **174**

Reduction using lithium aluminum hydride afforded **175** and **176** (from **162** and **163** respectively), in addition to **177** and **178** (from **173** and **174** respectively). (Figure 73)

Figure 73:

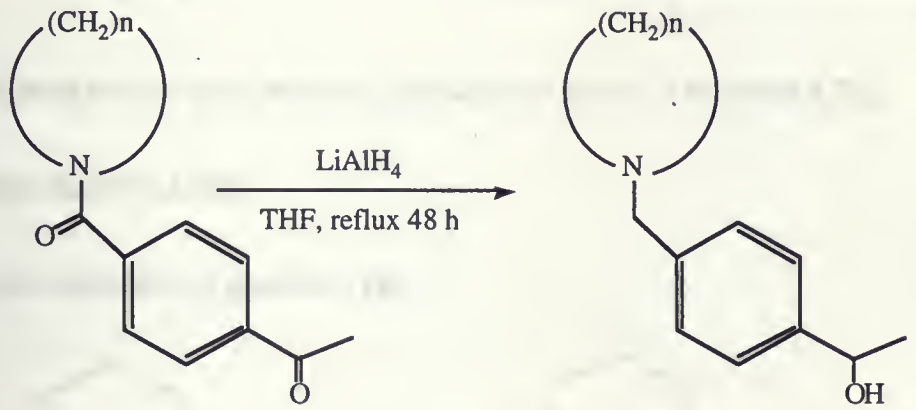



n=5; **162**

n=4; **163**

n=5; 1-(p-ethylbenzyl) piperidine **175**

n=4; 1-(p-ethylbenzyl) pyrrolidine **176**




n=5; **173**

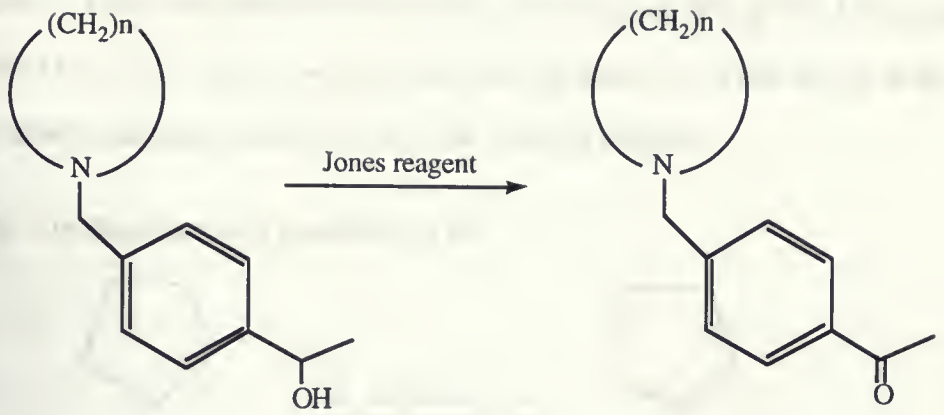
n=4; **174**

n=5; 1-(p-1-hydroxyethylbenzyl) piperidine **177**

n=4; 1-(p-hydroxyethylbenzyl) pyrollidine **178**

The oxidation of **177** and **178** using Jones' reagent afforded **179** and **180** respectively. (Figure 74)

Figure 74:




n=5; **177**

n=4; **178**

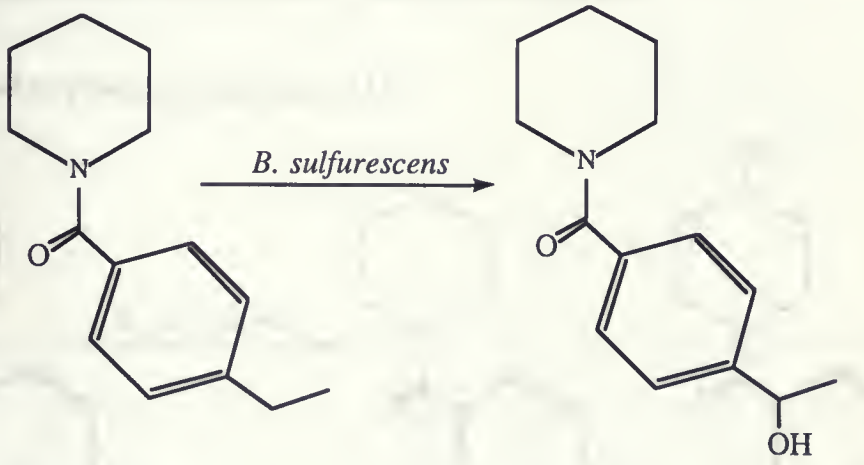
n=5; 1-(p-acetylbenzyl) piperidine **179**

n=4; 1-(p-acetylbenzyl) pyrollidine **180**

BIOTRANSFORMATION using *Beauveria sulfurescens* ATCC 7159 (RESULTS)

1. THE AMIDE SUBSTRATES

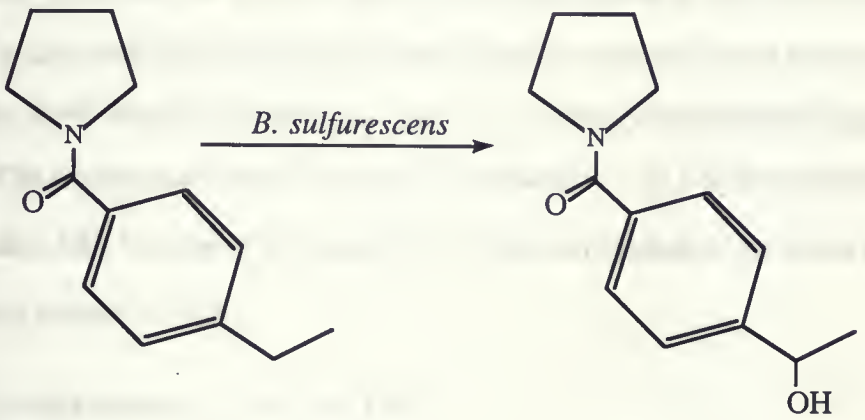
Figure 75: 1-(p-Ethylbenzoyl) piperidine **162**




**162** **181**

The product **181** was identified as 1-(p-1-hydroxyethylbenzoyl) piperidine, 78% yield, e.e=21±5%. $^1$H nmr spectrum showed a methyl group signal (3H, d) at δ 1.5 ppm and a signal for CH-OH (1H, q) at 5.0 ppm instead of ethyl group signal (3H, t and 2H, q) in the starting material, clearly indicating hydroxylation at the benzylic position.

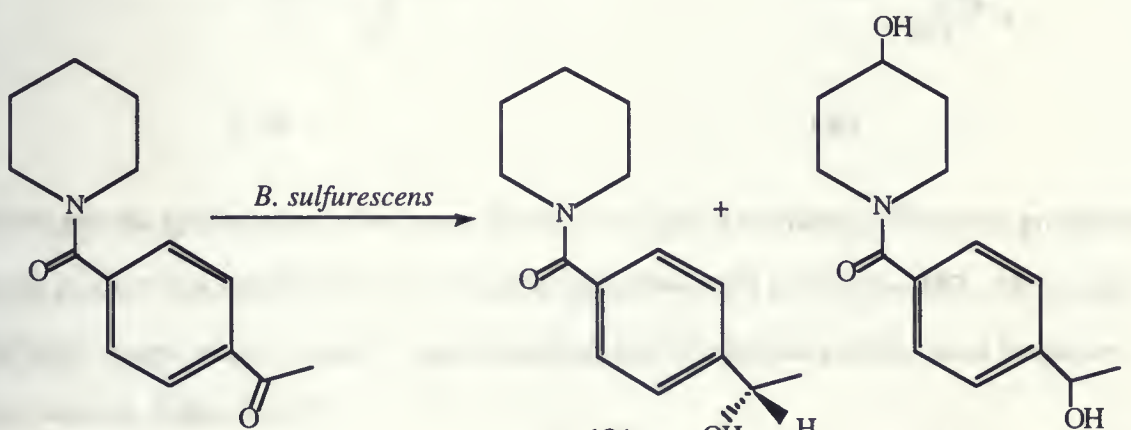
Figure 76: 1-(p-Ethylbenzoyl) pyrrolidine **163**




**163** **182**

The product **182** showed the same pattern as **181** in the $^1$H nmr spectrum and it was identified as 1-(p-1-hydroxyethylbenzoyl) pyrrolidine, 83% yield, e.e=20±5%.

Figure 77: 1-(p-Acetylbenzoyl) piperidine **173**



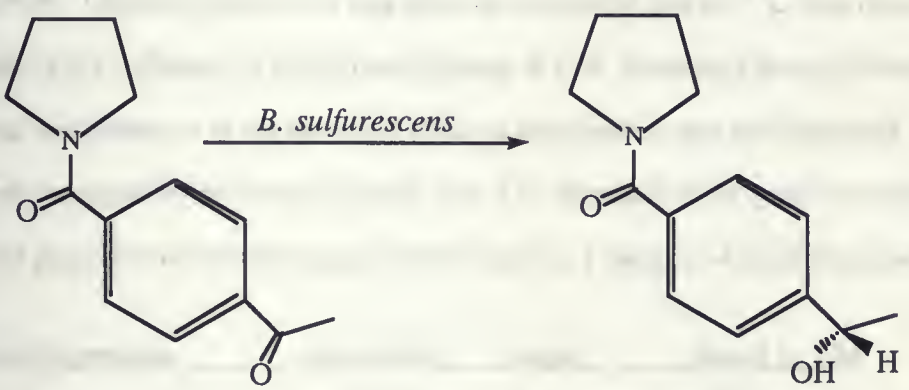

**173**

**181**
Yield: 40%

**183**
Yield: 60%

The biotransformation products were 1-(p-1-hydroxyethylbenzoyl) piperidine **181** and 1-(p-1-hydroxyethylbenzoyl)-4-hydroxy piperidine **183**. In addition to the presence of a benzylic alcohol, spectral data for **183** revealed yet another hydroxylated carbon. Chemical shifts in $^{13}C$ nmr spectrum suggested C-4 as the second site of hydroxylation. 1-(p-1-Hydroxyethylbenzoyl) piperidine **181** was a single enantiomer, *(S)* configuration. The absolute configuration was assigned by comparison with the literature data on benzylic alcohols of similar structure.[68] All reported chiral p-substituted benzylic alcohols have a negative optical rotation when their absolute configuration is *(S)*,[68] which is also the case with 1-(p-1-hydroxyethylbenzoyl) piperidine **181** ($[\alpha]_D$=-17.0). The enantiomeric excess was not determined for 1-(p-1-hydroxyethylbenzoyl)-4-hydroxy piperidine **183**, because of the complexity of the coordination of the chiral shift reagent and two hydroxy groups of **183**.

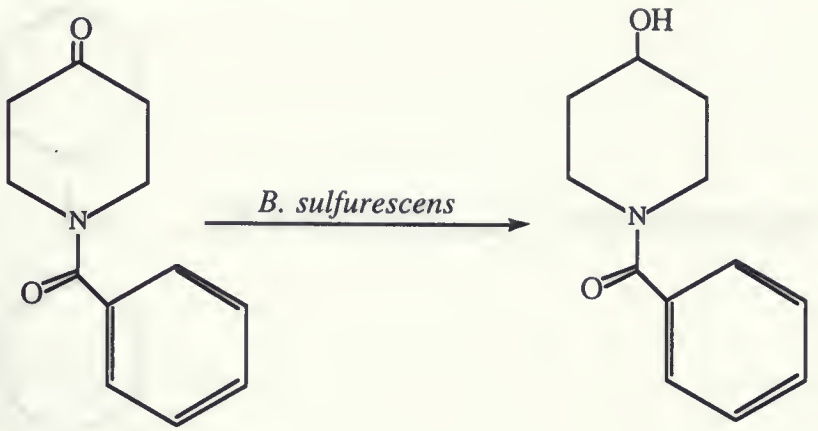
Figure 78: 1-(p-Acetylbenzoyl) pyrrolidine **174**




**174** **182**

According to the spectral data, which were identical to those of the biotransformation product of **163**, the product was identified as 1-(p-1-hydroxyethylbenzoyl) pyrrolidine **182**, 54% yield. Chiral shift reagent work revealed a single enantiomer of (*S*) absolute configuration (negative optical rotation, $[\alpha]_D$=-32.55).

Figure 79: 1-Benzoyl-4-piperidone **156**



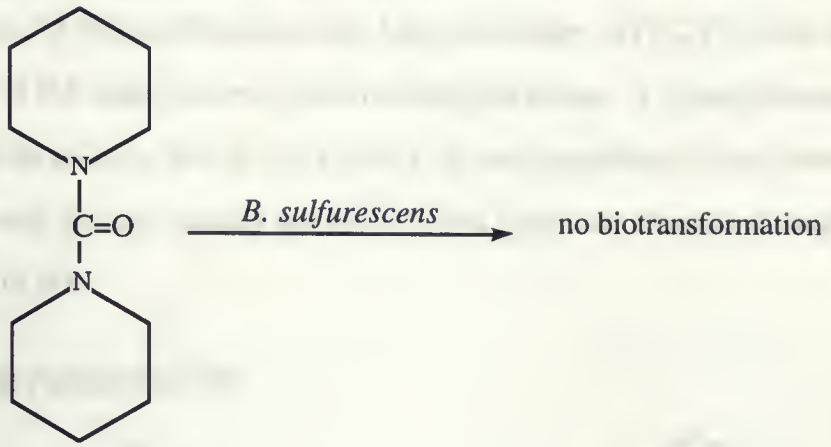

**156** **184**

1-Benzoyl-4-piperidone **156** was quantitatively reduced to 1-benzoyl-4-hydroxypiperidine **184**. The hydroxyl group was found to be in the equatorial position, according to the calculations in the $^{13}$Cnmr spectrum. 1-Benzoylpiperidine was taken as a standard and its $^{13}$C nmr chemical shifts were corrected for the influence of the hydroxyl group at C-4. Hydroxyl group influence was calculated from the difference in the chemical shifts of cyclohexane and cyclohexanol. The calculations are presented in the following table with $^{13}$C chemical shifts (ppm) calculated for equatorial/axial possible conformations and shifts found in 1-benzoyl-4-hydroxypiperidine **184**.

| | 1-benzoylpiperidine | equatorial | axial | found in **184** |
|---|---|---|---|---|
| C-2 | 43.4 / 49.0 | 40.4 / 46.0 | 36.4 / 31.8 | 40.0 / 45.3 |
| C-3 | 26.0 / 26.8 | 34.0 / 34.8 | 31.0 / 31.8 | 34.0 / 34.7 |
| C-4 | 26.9 | 67.8 | 63.8 | 66.9 |

Two ppm values are listed for each C-2 and C-3 piperidine carbons since these carbons are not equivalent because of the existence of two rotamers of the amide.

Figure 80: 1,1'-Carbonyldipiperidine **148**

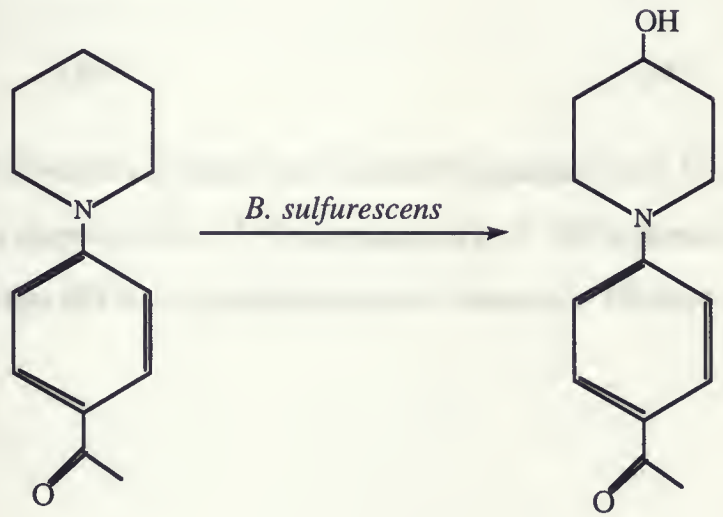



**148**

Only starting material **148** was recovered.

2. THE AMINE SUBSTRATES

Figure 81: 4'-Piperidinoacetophenone **35**



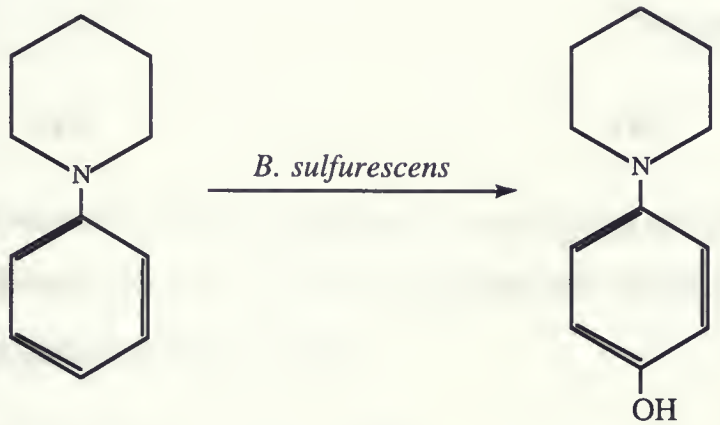

**35** **37**

4'-Piperidinoacetophenone **35** served as a standard for comparison with a literature data.[36, 53]

The 4-hydroxylated product 4-(4'-hydroxypiperidinyl) acetophenone **37** was obtained in 21%

yield (literature yield was 20%[36] and 66%[53]). 4'-Piperidinoacetophenone **35** was also a potential substrate for biotransformation with *Aspergillus niger* ATCC 9142 and *Rhizopus arrhizus* ATCC 11145, both known to perform biohydroxylation. 4'-Piperidino-acetophenone was transformed by *A. niger* ATCC 9142 to 4-(4'-hydroxypiperidinyl) acetophenone **37** (20% yield), whereas only starting material was recovered as a product of biotransformation with *R. arrhizus* ATCC 11145.

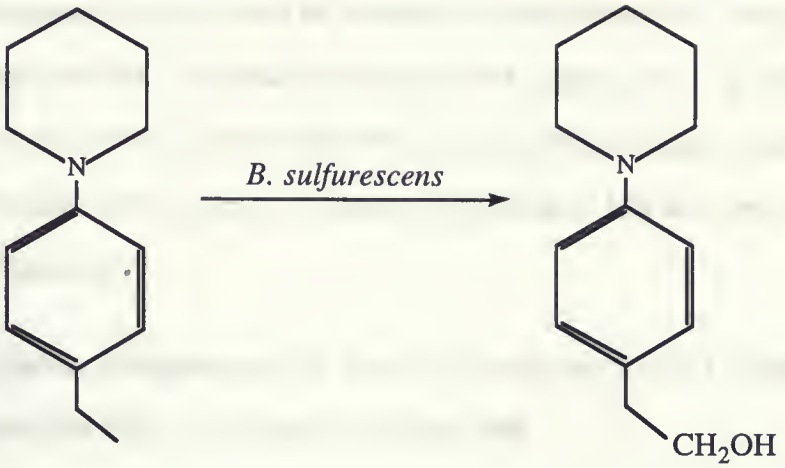
Figure 82: 1-Phenylpiperidine **155**




**155** **185**

Spectral data for **185** revealed a characteristic p-substitution pattern in the $^{1}H$ nmr spectrum as well as characteristic chemical shift in $^{13}C$ nmr spectrum for C-OH in phenols (151.1 ppm). Mass spectrum confirmed that **185** was 4-piperidinyl phenol, obtained in 7% yield.

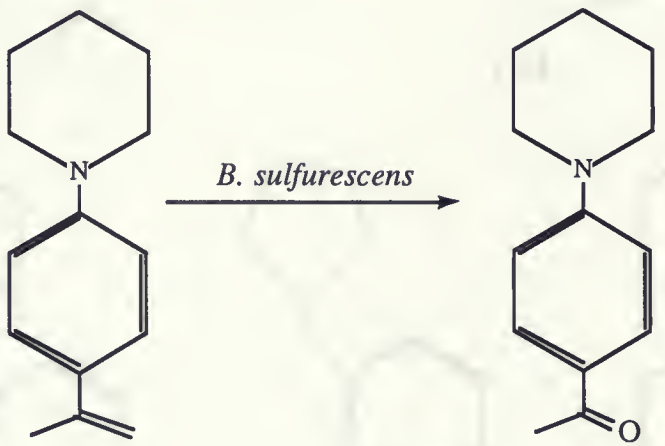
Figure 83: 1-(p-Ethylphenyl) piperidine **160**




**160** **186**

$^1$H nmr spectrum of the product shows disappearance of methyl group signal (3H, t) at 1.3 ppm and a new signal at 3.9 ppm (2H, t) for $CH_2$-OH. The product was identified as 1-(p-2-hydroxyethylphenyl) piperidine **186**, 39% yield.

Figure 84: 1-(p-Isopropenylphenyl) piperidine **165**

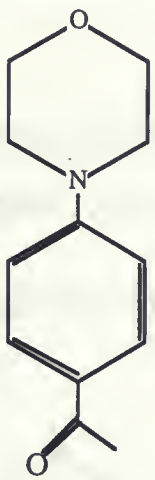



**165** **35**

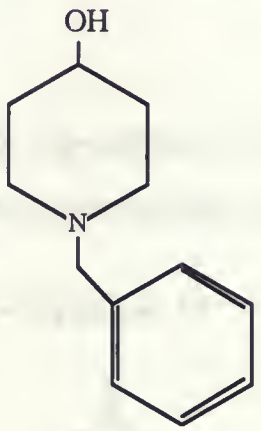
The product was identified as 4'-piperidino acetophenone, since all the spectral data were identical to commercially available **35**.

Only starting materials were recovered from the biotransformation of the following amine substrates: 4-morpholino acetophenone **145**, 1-benzyl-4-piperidinol **154**, 1-benzylpiperidine **158**, 1-benzylpyrrolidine **159**, 1-cyclohexylpiperidine **168** (Figure 85); 1-(p-ethylbenzyl) piperidine **175**, 1-(p-ethylbenzyl) pyrrolidine **176**, 1-(p-acetylbenzyl) piperidine **179** and 1-(p-acetylbenzyl) pyrrolidine **180** (Figure 86); 1-benzyl-4-piperidone **146** and 1-phenethyl-4-piperidone **147**. (Figure 87)

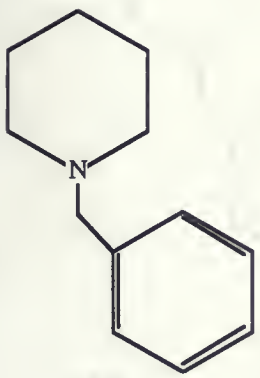
Figure 85: 4-Morpholino acetophenone **145**, 1-benzyl-4-piperidinol **154**, 1-benzylpiperidine **158**, 1-benzylpyrrolidine **159**, 1-cyclohexylpiperidine **168**



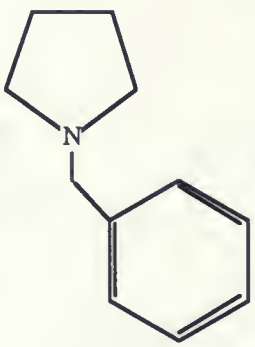

**145**



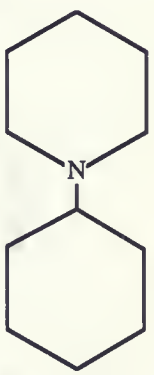

**154**




**158**

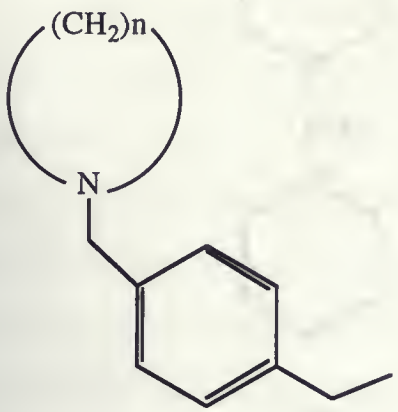


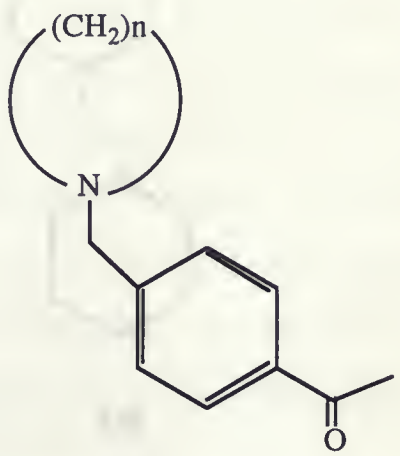

**159**




**168**

Figure 86: 1-(p-Ethylbenzyl) piperidine **175**, 1-(p-ethylbenzyl) pyrrolidine **176**, 1-(p-acetylbenzyl) piperidine **179** and 1-(p-acetylbenzyl) pyrrolidine **180**



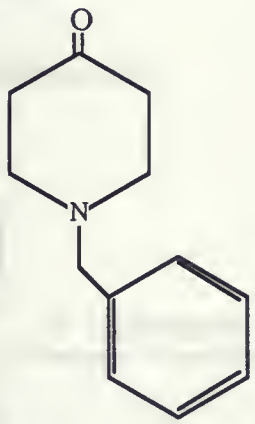

n=5; 1-(p-ethylbenzyl) piperidine **175**

n=4; 1-(p-ethylbenzyl) pyrollidine **176**

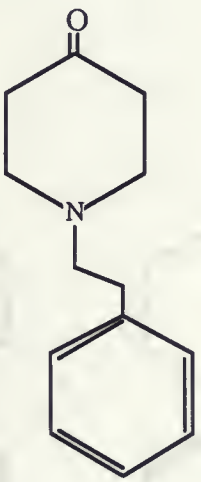
n=5; 1-(p-acetylbenzyl) piperidine **179**

n=4; 1-(p-acetylbenzyl) pyrollidine **180**

Figure 87: 1-Benzyl-4-piperidone **146** and 1-phenethyl-4-piperidone **147**

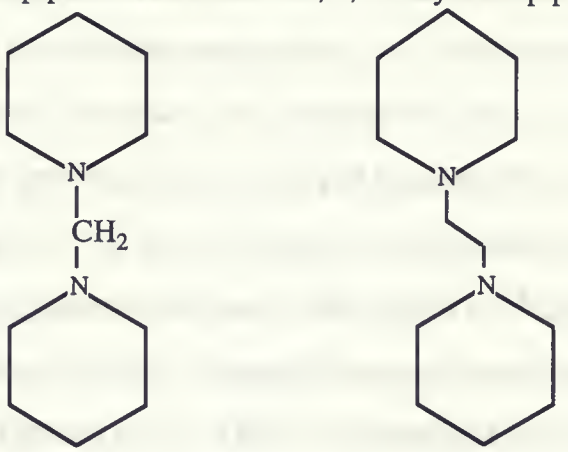



**146**




**147**

Figure 88: Dipiperidinomethane **149**, 1,1'-ethylenedipiperidine **150**

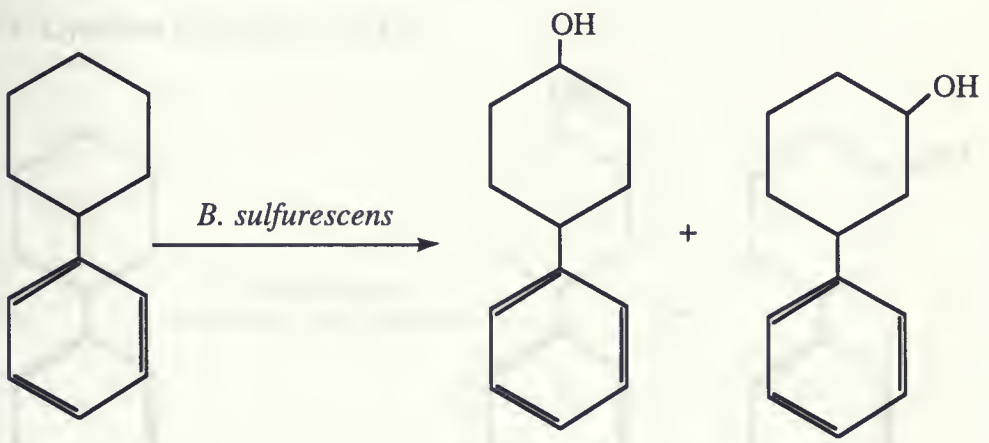



**149** **150**

Dipiperidinomethane **149** and 4,4'-ethylenedipiperidine **150** also did not undergo biotransformation, but the starting materials were not recovered either . Since these are basic compounds and the media is pH 4.85, the filtrate was basified to pH 8 before the extraction with dichloromethane, but again no product or starting material were extracted.

3. NEUTRAL SUBSTRATES

Figure 89: Phenylcyclohexane **152**

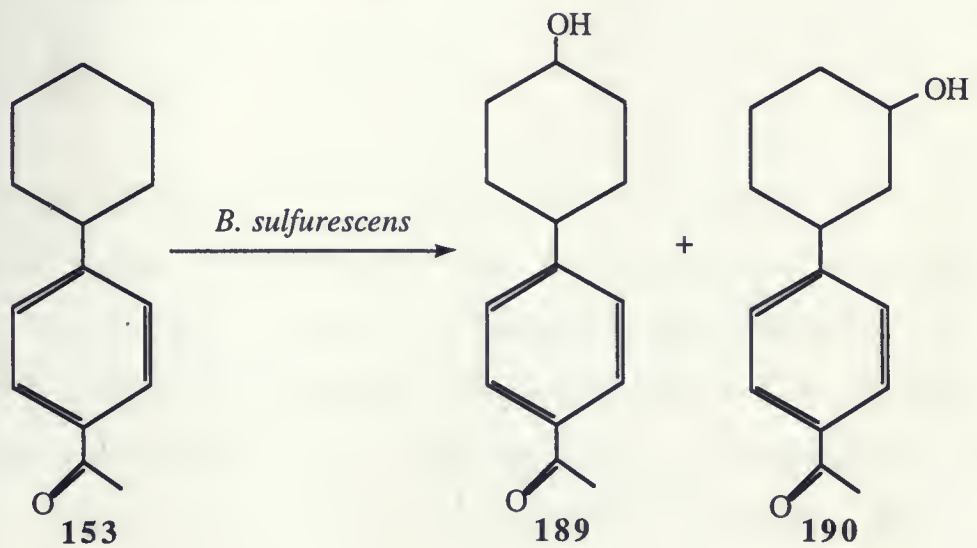


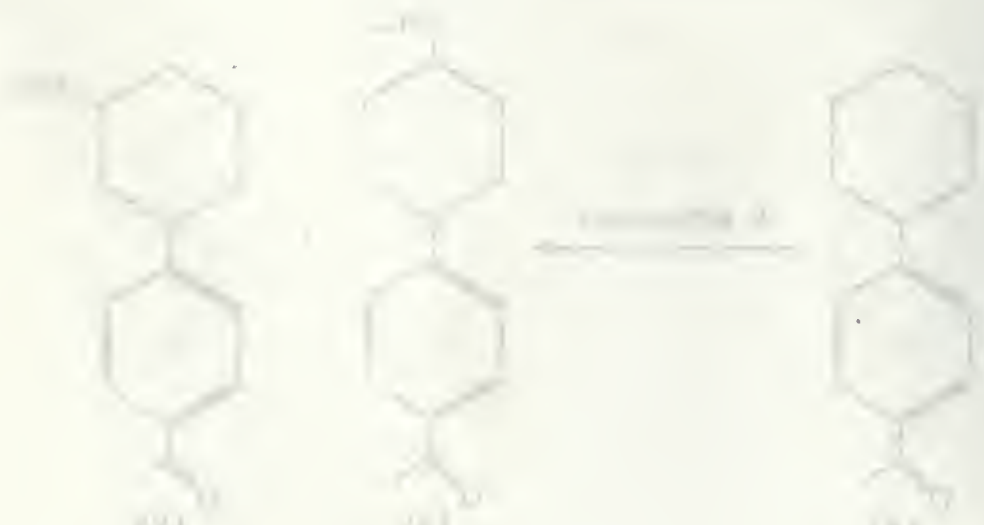

**152** **187** **188**

Products 4-phenylcyclohexanol **187** and 3-phenylcyclohexanol **188** were isolated in the same fraction during purification by column chromatography (yield 14%), and their further separation was not attempted. The site of hydroxylation was determined to be at C-4 in **187** because of the symmetry pattern in carbon spectrum, and at C-3 in **188** because of the lack of symmetry and according to the chemical shifts. The hydroxyl group in both products was found to be in the equatorial position according to the calculations of carbon shifts in $^{13}C$ spectrum. The chemical shifts in $^{13}C$ nmr for cyclohexyl carbons of phenylcyclohexane were corrected for the presence of hydroxyl group at C-4 (in **187**) and C-3 (in **188**). The following table lists ppm values for cyclohexyl carbons in the starting material **152** and in the products **187** and **188** (calculated and found). Numbers for carbons correspond to numbers in **152**.

| | **152** | **187**(calc.) | **187**(found) | **188**(calc.) | **188**(found) |
|---|---|---|---|---|---|
| | | equat. / axial | | equat. / axial | |
| C-1 | 45.3 | 43.3 / 44.3 | 43.8 | 42.3 / 38.3 | 43.2 |
| C-2 | 35.1 | 32.1 / 28.1 | 32.7 | 43.1 / 40.1 | 43.5 |
| C-3 | 27.6 | 35.6 / 32.6 | 36.2 | 70.6 / 66.6 | 71.3 |
| C-4 | 26.8 | 69.9 / 65.8 | 70.9 | 34.8 / 31.8 | 35.6 |

Figure 90: 4'-Cyclohexylacetophenone **153**

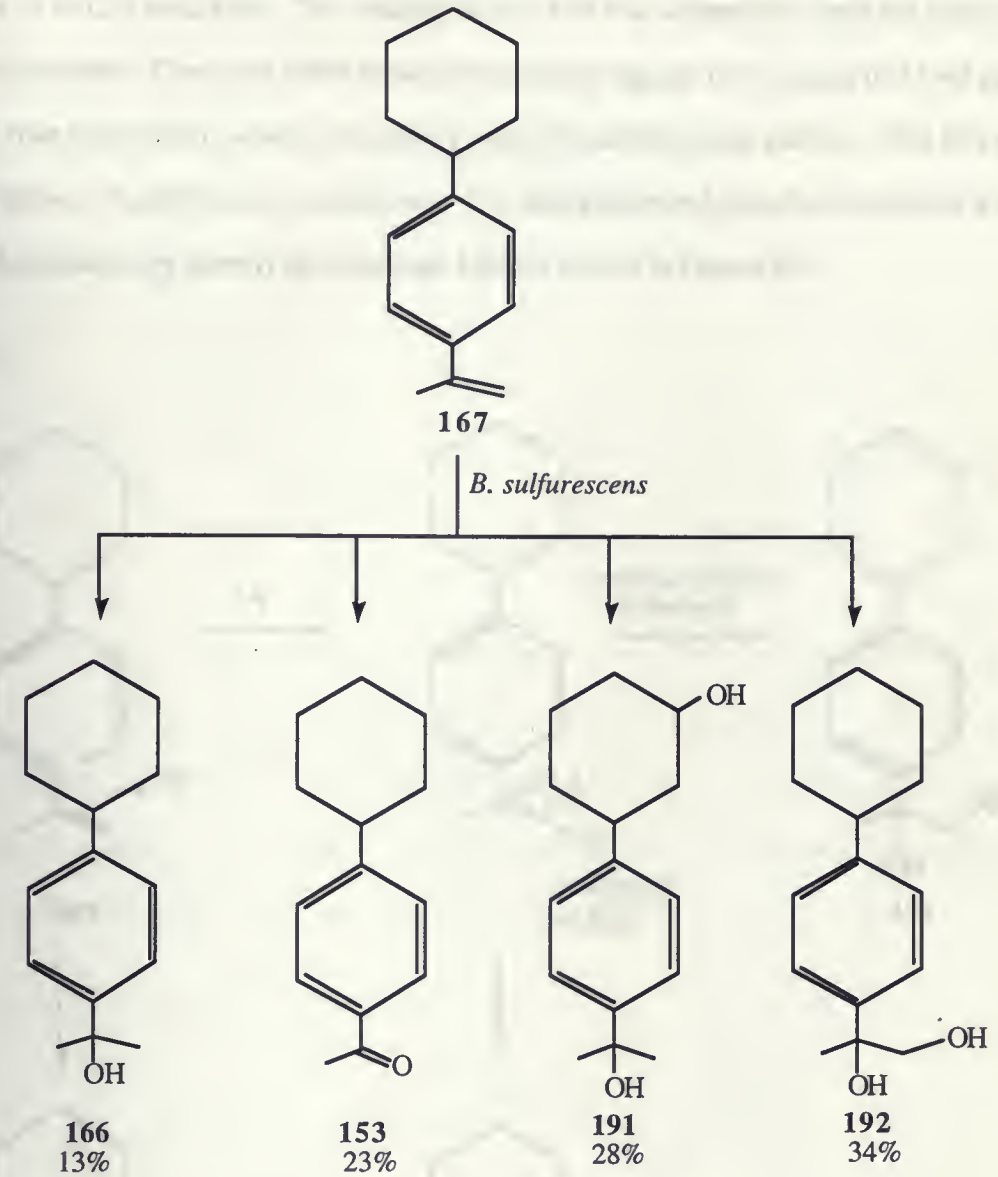




Two products, 4-(4'-hydroxycyclohexyl) acetophenone **189** and 4-(3'-hydroxycyclohexyl) acetophenone **190** were recovered in the same fraction of the column chromatography (combined yield 57%). Their further separation was not attempted. Both products were *trans* isomers, with hydroxyl group in the equatorial position according to the calculations of carbon shifts in $^{13}$Cnmr spectrum (see following table; numbering corresponds to **153**). Chemical shifts in $^{13}$C nmr spectrum were corrected for the presence of hydroxyl group at C-4 (**189**) and C-3 (**190**). Also, as a confirmation of these conclusions, oxidation of the mixture of isomers to ketones was performed using Jones' reagent. If the products were mixture of *cis* and *trans*, the $^{13}$Cnmr spectrum of the oxidised mixture would be simpler (showing one compound), but in this case two signals corresponding to carbonyl carbon replaced the signals of two carbinol carbons on oxidation.

| | **153** | **189**(calc.) | **189**(found) | **190**(calc.) | **190**(found) |
|---|---|---|---|---|---|
| | | equat. / axial | | equat. / axial | |
| C-1 | 45.4 | 43.6 / 44.5 | 43.9 | 42.6 / 37.9 | 43.1 |
| C-2 | 34.5 | 31.7 / 27.0 | 32.5 | 43.0 / 40.1 | 43.2 |
| C-3 | 26.8 | 35.3 / 32.4 | 36.1 | 70.5 / 65.7 | 71.1 |
| C-4 | 26.4 | 70.1 / 65.3 | 70.7 | 34.9 / 32.0 | 35.6 |

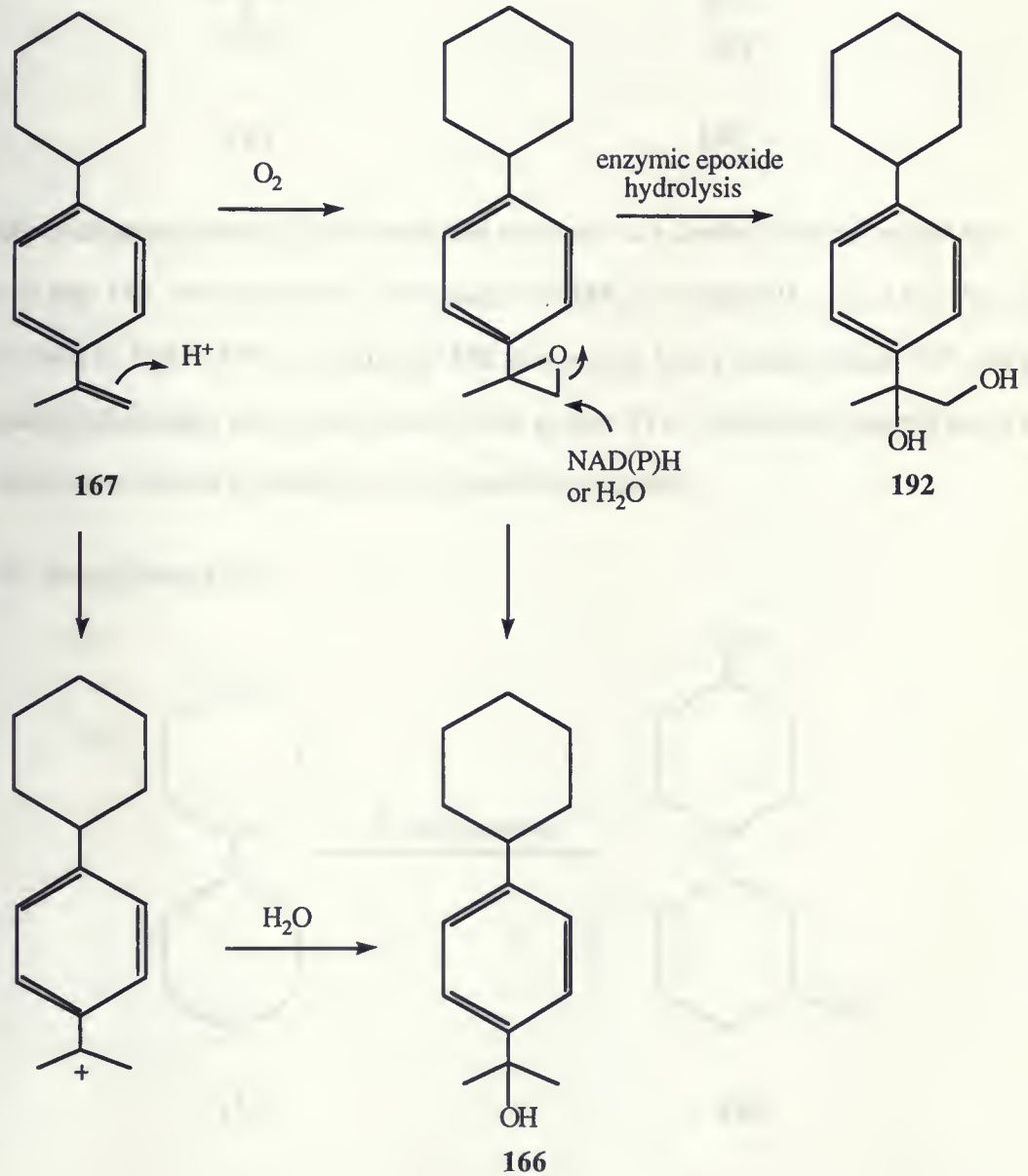
Figure 91: p-Isopropenylphenyl cyclohexane **167**




Bioconversion of **167** gave four different products: p-1-hydroxyisopropylphenyl cyclohexane **166**, 4'-cyclohexylacetophenone **153**, 1-hydroxy-3-(p-1-hydroxyisopropylphenyl) cyclohexane **191** and p-1,2-dihydroxyisopropylphenyl cyclohexane **192**. Identification of the structures for **166** and **153** was confirmed by comparison of their spectral data with the data of commercially available and synthesized **153** and **166**, respectively. A second site of hydroxylation in **191** was determined to be at C-3 because of the lack of symmetry in $^{13}C$ nmr spectrum and because of the

fact that **191** was optically active (C-4 hydroxylated product would not be optically active, whereas C-2 or C-3 would be). The identity of diol **192** was determined from the chemical shifts in carbon spectrum. Chemical shifts showed five carbon signals in the range of 23-43 ppm, which represent four cyclohexyl carbons (symmetry) and one methyl group carbon. Diol **192** was also optically active. Possible routes leading to p-1-hydroxyisopropylphenyl cyclohexane **166** and p-1,2-dihydroxyisopropylphenyl cyclohexane **192** are shown in Figure 92.

Figure 92:



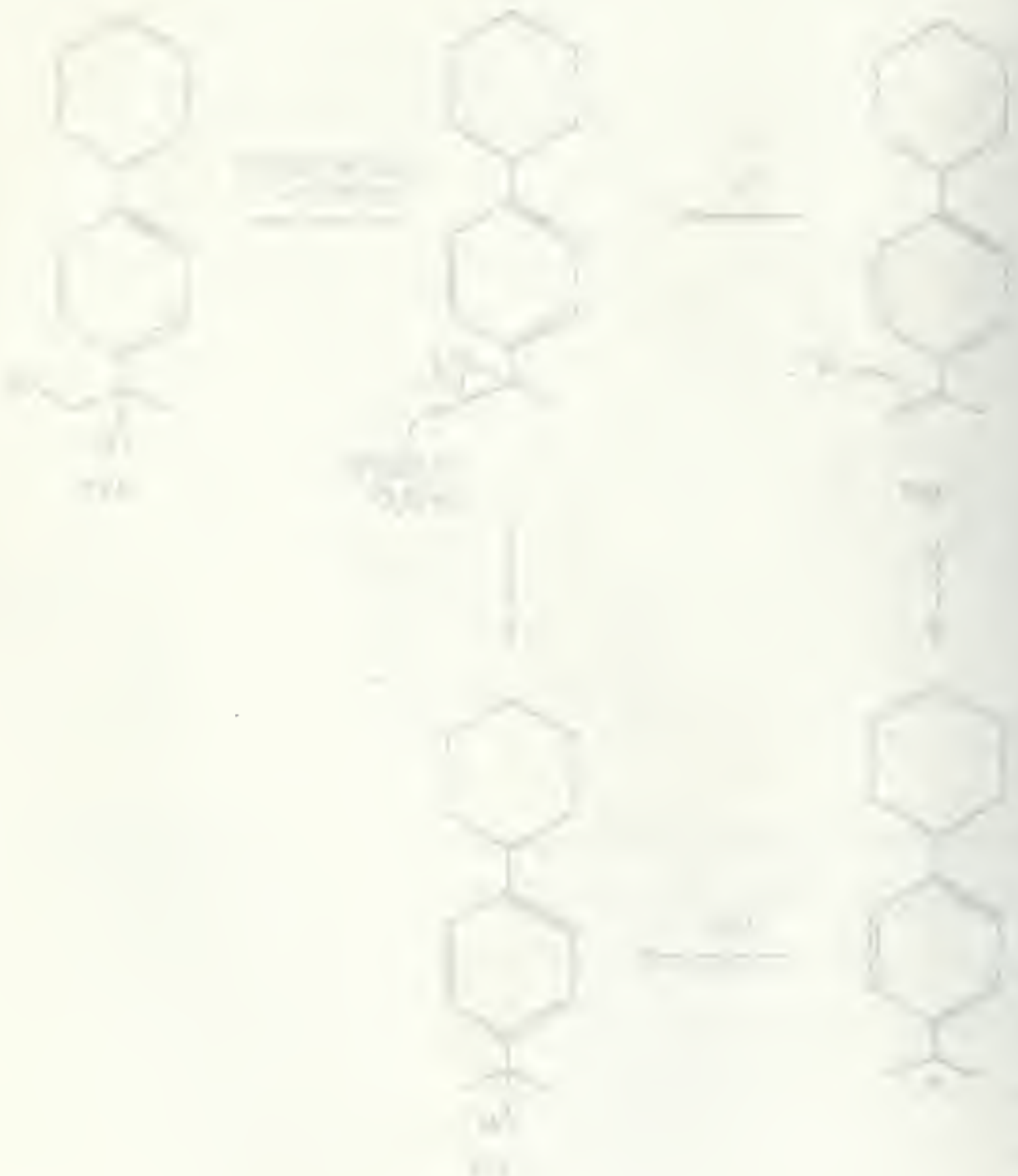
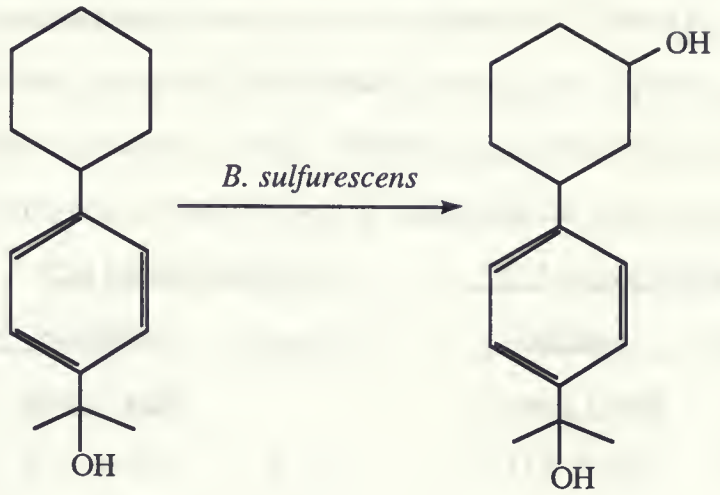


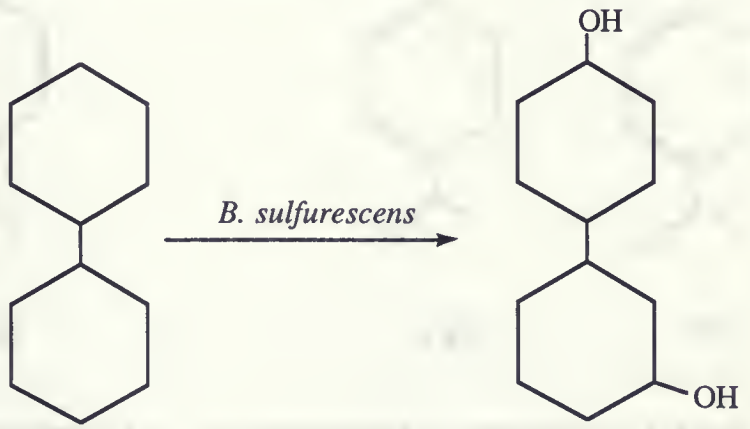
Figure 93: p-1-Hydroxyisopropylphenyl cyclohexane **166**




**166** **191**

p-1-Hydroxyisopropylphenyl cyclohexane **166** was used as a control in order to test the hypothesis that **191** was a result of hydroxylation of **166**, see Figure 91. From the results of the bioconversion of **166** to **191**, it seems that **166** was indeed first produced from **167** and then underwent hydroxylation at an unactivated carbon to give **191**. This would suggest that a terminal alkene would be oxidized in preference to an unactivated carbon.

Figure 94: Bicyclohexyl **151**

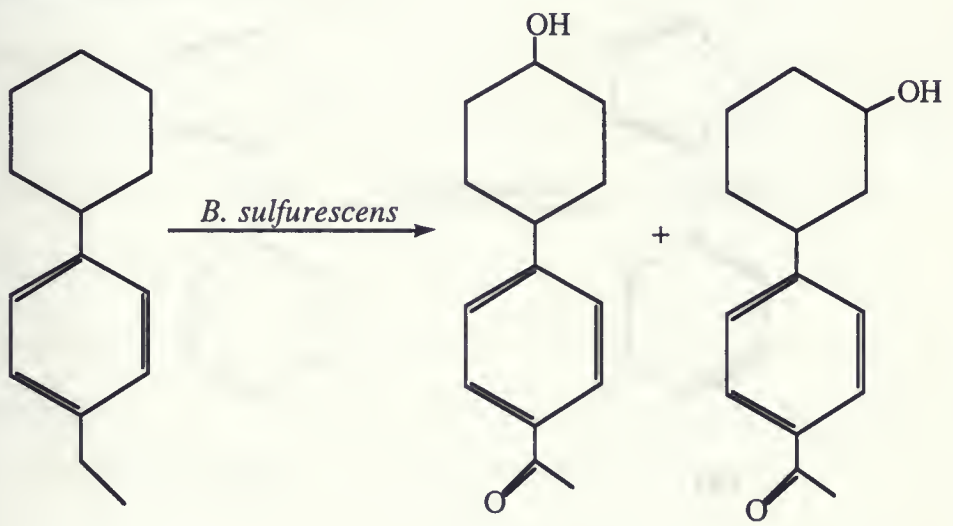



**151** **193**

Product was 3,4'-dihydroxydicyclohexyl **193** (13% yield). The calculation of the carbon shifts in $^{13}$Cnmr spectrum, compared with equatorial/axial calculation of $^{13}$C shifts for cyclohexanol suggests that both hydroxyl groups are predominantly in the equatorial position (see following table, where numbering corresponds to **151**). $^{13}$C nmr analysis also indicates the presence of about 15-20% of axial material (either both axial or axial/equatorial hydroxyl groups).

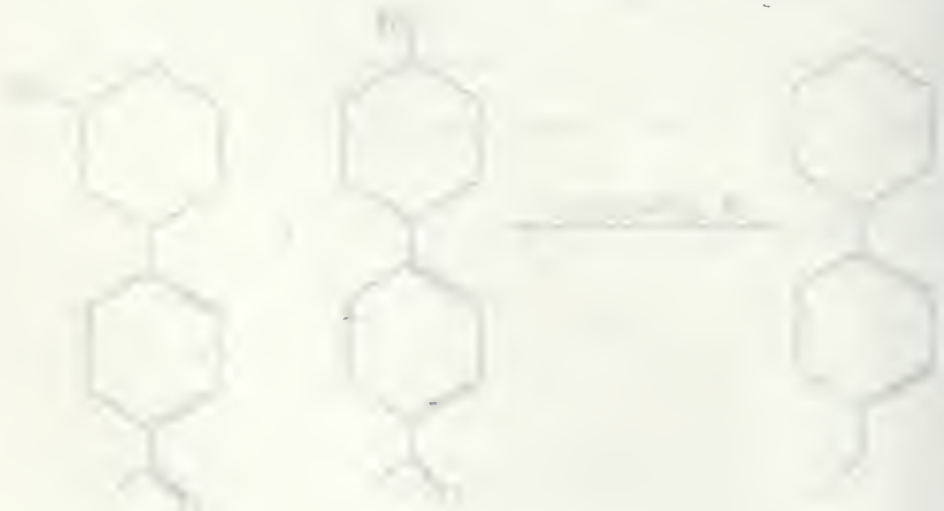
| | | C-4 hydroxylated ring | | C-3 hydroxylated ring | |
|---|---|---|---|---|---|
| | **151** | calculated | found | calculated | found |
| | | equat. / axial | | equat. / axial | |
| C-1 | 43.8 | 42.1 / 42.9 | 42.5 | 41.0 / 36.3 | 39.9 |
| C-2 | 30.5 | 27.7 / 23.0 | 27.7 | 39.0 / 36.1 | 38.3 |
| C-3 | 27.3 | 35.7 / 32.9 | 35.7 | 70.9 / 66.2 | 69.9 |
| C-4 | 27.3 | 70.9 / 66.2 | 70.9 | 35.8 / 32.9 | 34.5 |

Figure 95: p-Ethylphenyl cyclohexane **161**




**161** **189** **190**

Two hydroxylated products: 4-(4'-hydroxycyclohexyl) acetophenone **189** and 4-(3'-hydroxycylohexyl) acetophenone **190** were recovered in the same fraction of column chromatography (12% yield) and their further separation was not attempted. The hydroxyl groups

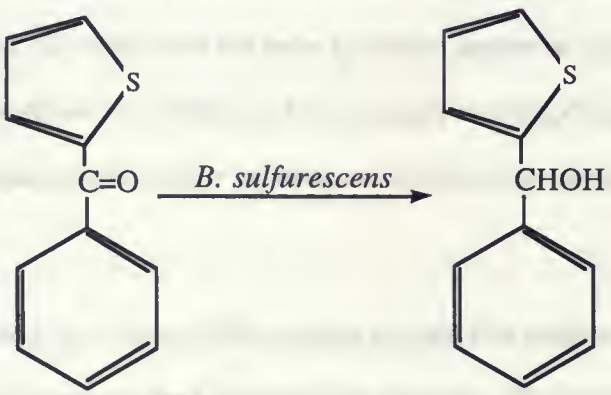


in both products are in the equatorial position. The calculation of the chemical shifts in the $^{13}C$ spectrum revealed the same products as for biotransformation of 4'-cyclohexylacetophenone **153** (see following table). The initially hydroxylated benzylic position was further oxidized to a ketone, a process known to occur with *Beauveria sulfurescens*.

| | **153** | **189**(calc.) | **189**(found) | **190**(calc.) | **190**(found) |
|---|---|---|---|---|---|
| | | equat. / axial | | equat. / axial | |
| C-1 | 45.4 | 43.6 / 44.5 | 43.9 | 42.6 / 37.9 | 43.0 |
| C-2 | 34.5 | 31.7 / 27.0 | 32.5 | 43.0 / 40.1 | 43.2 |
| C-3 | 26.8 | 35.3 / 32.4 | 36.0 | 70.5 / 65.7 | 71.0 |
| C-4 | 26.4 | 70.1 / 65.3 | 70.7 | 34.9 / 32.0 | 35.5 |

4. OTHER SUBSTRATES

Figure 96: 2-Benzoylthiophene **157**




**157** **194**

Product was 2-(1-hydroxybenzyl) thiophene **194**, single enantiomer. Control chiral shift reagent work was done with racemic **194**, where the separation of the enantiomers was clearly seen using the same chiral shift reagent.

DISCUSSION:

The hydroxylation reaction performed by fungus *Beauveria sulfurescens* ATCC 7159 has been studied for three decades and a lot of data have been generated. Although the first model describing the active site of the hydroxylating enzyme was proposed 30 years ago (Figure 17, p.17)[29], the ability to predict the regio- and stereochemistry of the reaction did not improve over the years. In fact, it seems that there is a lot of misconception created by focusing in detail on just one type of substrate (i.e. amides), completely overlooking the fact that other compounds could also be hydroxylated, although they did not have any of the requirements proposed earlier for amide hydroxylation.

The first model was developed after hydroxylation of cyclododecanol[29] (Figure 17), but then it was mainly used to study hydroxylation of the amides (see introduction). In the amide examples studied later, it seems that hydroxylation did occur mainly 5.5 Å away from the electron-rich binding site.[31-34] But this model did not account for hydroxylation of other substrates such as dialkylbenzenes[40] (Figure 32), which did not have an amide, amine or hydroxyl functionality. Nevertheless, this was somehow overlooked and later papers continued proposing electron-rich group requirement and measuring distances between an electron-rich center and the site of hydroxylation.

The examples studied during the course of this project suggest that neither amide, amine, carbonyl group or aromatic ring are necessary for hydroxylation to occur. The best example of this is bioconversion of bicyclohexyl **151** to diol **193**. (Figure 94).

The octant model proposed by Johnson et al.[35] was used for defining the orientation of the substrate-product molecules in space. (Figure 25, p.24) Several examples studied suggested that the bulk of the molecule was placed in the upper right (UR) rear part of the octant system.[35]

The hydroxylated products obtained from biotransformation of the substrates used in this study are presented in Figure 97 (see Appendix). In addition to a structural formula, each product is also

shown in the octant system. After drawing each product using the Spartan molecular modeling program, energy minimization was done and the molecule then positioned in such a way that C-O bond of the hydroxylated carbon is projected towards the viewer.

The following observations apply:

-The assumption was that the substrate will take minimum energy conformation once in the enzyme-substrate complex

-Hydroxyl groups are in the equatorial position where applicable

-Because of the conformational mobility of some products (i.e. amide conformations, free rotation of the alkyl group), they do not necessarily have to be positioned as drawn in Figure 97 . The postulate was to position them in a way that the bulk of the molecule is in the upper right octant in order to see which of them cannot be positioned in this way.

-For some of the bioconversion products, the absolute configuration is not known. In these cases both *(R)* and *(S)* configurations are presented in Figure 97.

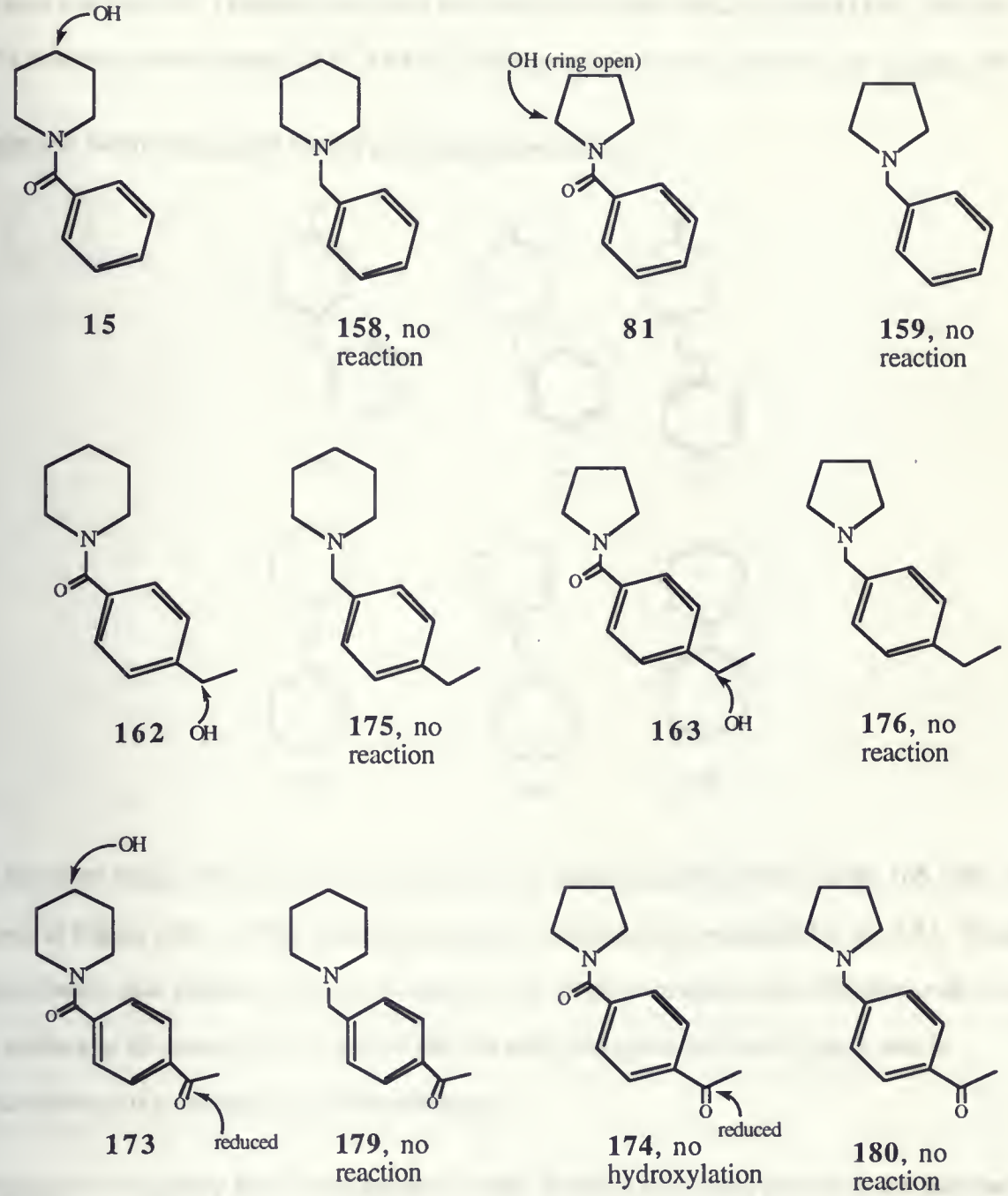
The following observations were made:

Products **181**, **182**, **183**, **186**, **188**, **190**, **191**,**192** and **193** can be positioned in a way that the bulk of the molecule occupies UR octant. **181** and **182** produced as a result of hydroxylation reaction were found to be almost racemic mixture of *(R)* and *(S)* enantiomers. This means that the bulk of the molecule can be placed in either UR and UL octant in order for the reaction to occur. The same observation applies to **188**, **190**, **191** and **193** of unknown configuration: generally the *(S)* enantiomer has the bulk of the molecule in UR octant, whereas the *(R)* enantiomer in UL octant. When **181** and **182** were produced as a result of the reduction reaction, they were found to be single enantiomer of *(S)* configuration, but they are not considered here, being a result of the action of an oxidoreductase. **184** and **194** are also result of the reduction reaction and they too are not considered here.

Near planar molecules such as **35**, **37**, **185**, **187**, **189**, **166** and **153** have bulk of the molecule placed along the x, y or z-axis in space, (also **189** and **190**, view from carbonyl oxygen).

Using this model still does not explain why hydroxylation did not occur in some of the substrates, which can still be positioned in the UR, UL or LR octant. For example, all the amide substrates were hydroxylated (with the exception of **174** substrate which was just reduced), but none of their amine counterparts was hydroxylated. (Figure 98, where literature data for hydroxylation of **15** and **81** are also included) In the case of the amide substrates, it was observed that the benzylic position was preferentially hydroxylated if it was available (**162** and **163**).

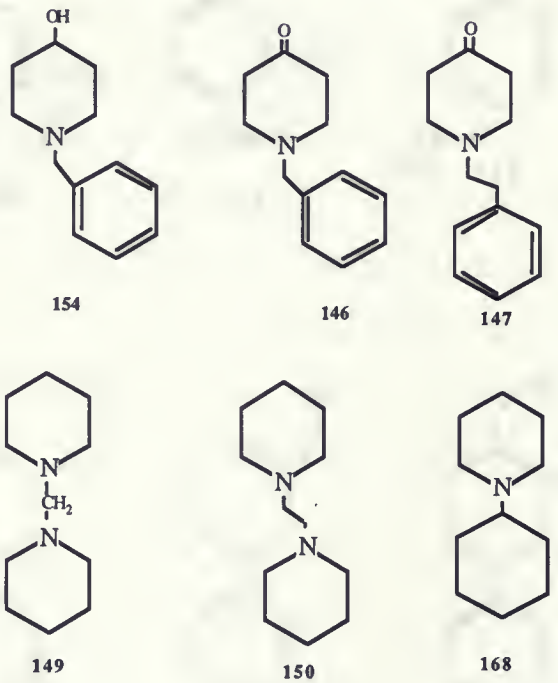
Figure 98: Amide and amine substrates




This would suggest that the amide function can serve as a binding point in the enzyme's active site, but that does not explain hydroxylation of the hydrocarbon substrates. A complicating factor is the differences in solubility properties of amides and amines. Amines would be protonated at pH 4.85

(pH of the media) and probably too water soluble to cross the cell membrane and reach the enzyme's active site. Hydroxylation was not observed in other benzyl amines (**154**, **146** and **147**) or hydrocarbon amines (**149**, **150** and **168**), probably for the same reason. (Figure 99)

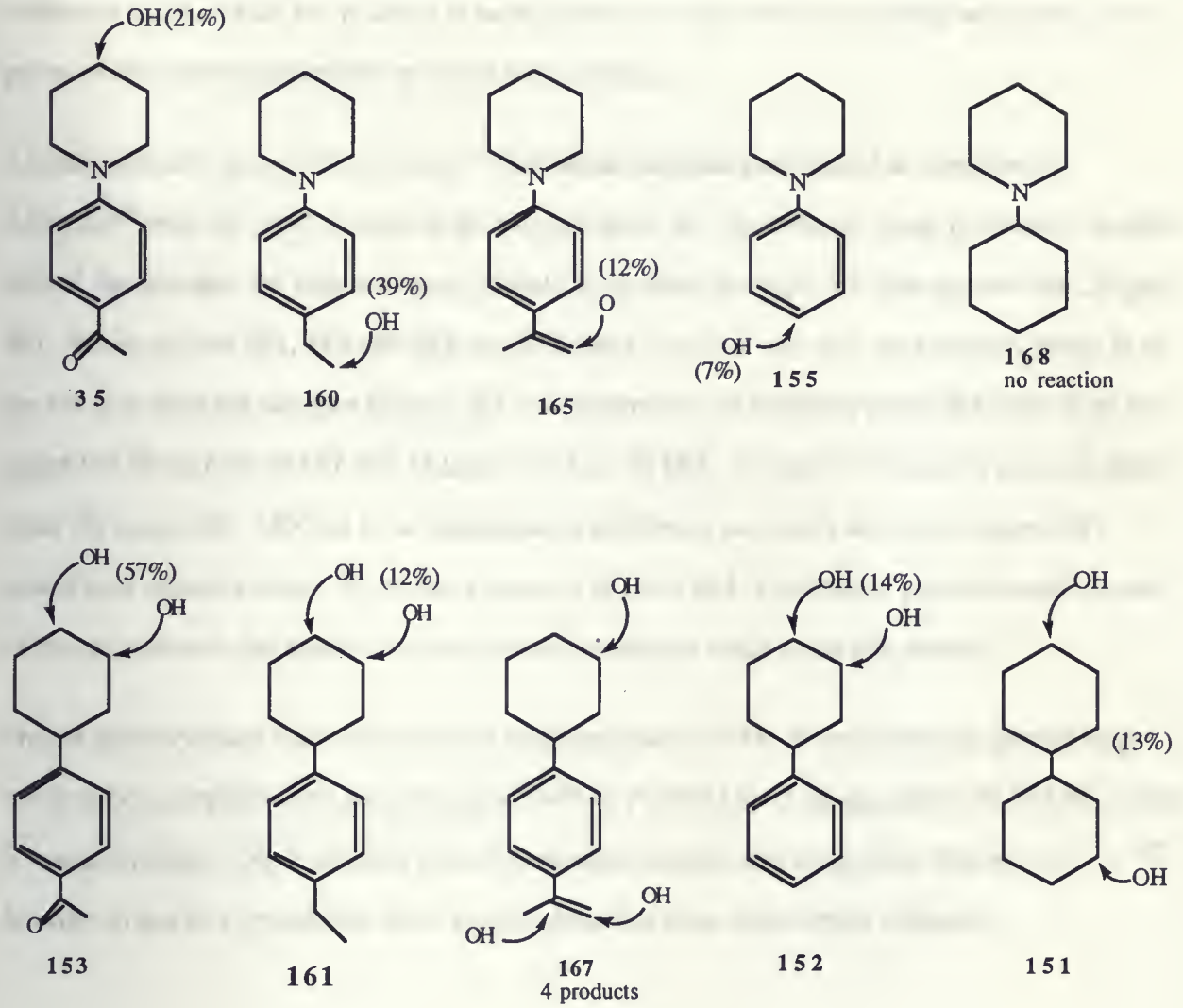
Figure 99: Nonhydroxylated benzyl and hydrocarbon amines



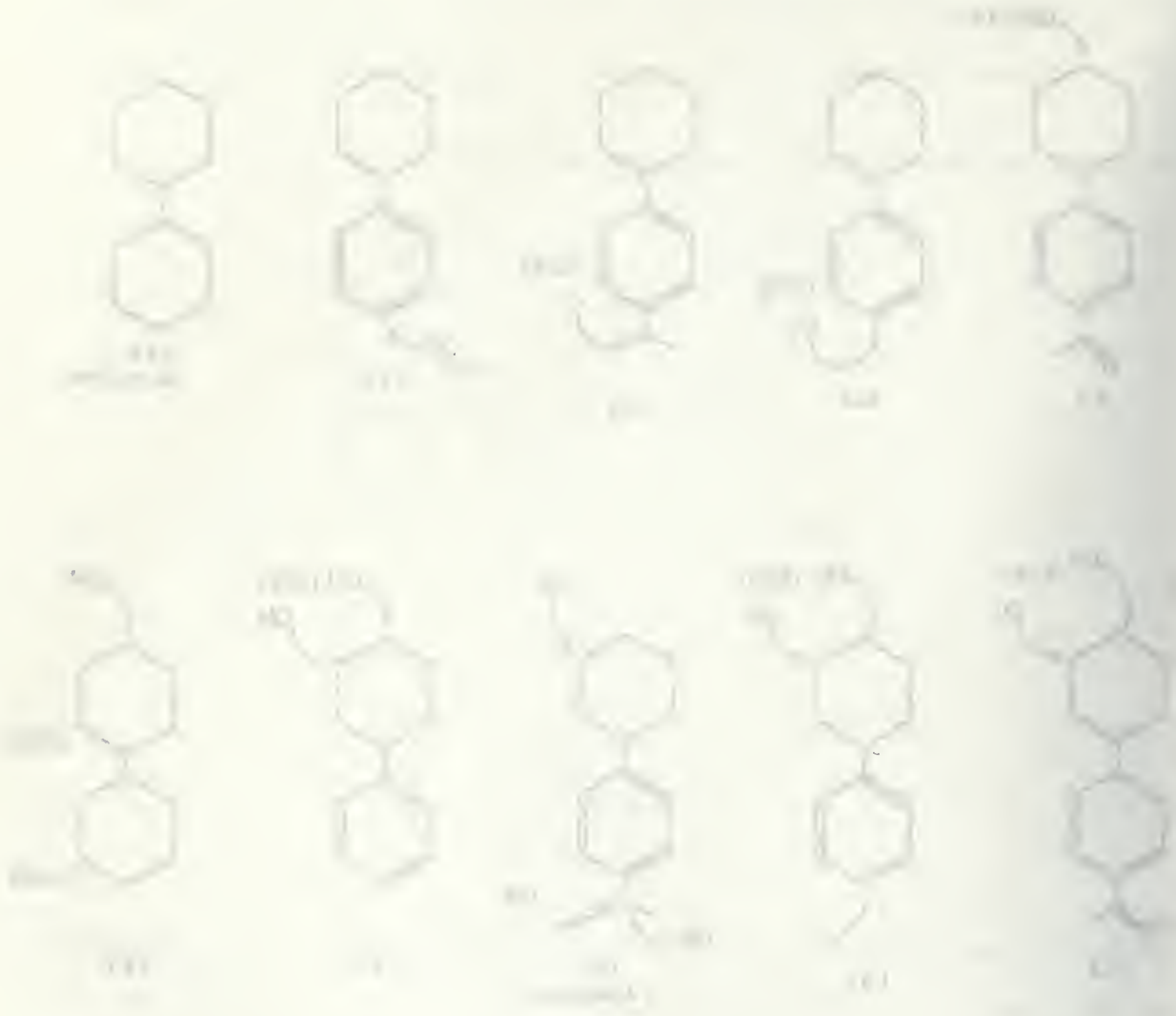

On the other hand, hydroxylation did occur in all phenylpiperidine amines (**160**, **165**, **155**, **35** shown in Figure 100). 1-Phenylpiperidine amines would also be protonated at pH 4.85. Being weaker bases than amines in Figures 98 and 99, they might coordinate with different acids from the media and the counter ion properties (its size and hydrophobicity) could play a role in bioavailability (i.e. crossing the cell membrane).

Although not necessary for hydroxylation to occur, different functional groups on the substrate seem to direct different regioselectivity of the hydroxylation and yields. This is illustrated in Figure 100, where the site of hydroxylation and yield within the same type of the compounds (i.e. amines and neutral compounds) can be compared, in addition to a comparison of amines and neutral substrates with the same functional groups.

Figure 100: Functionality and regioselectivity relationships




The regioselectivity of hydroxylation changes depending on the presence/absence of nitrogen; i.e. compare **160** and **161**, **155** and **152**. (Figure 100) Generally, neutral compounds were always hydroxylated at an unactivated ring carbon, whereas amines were not (with the exception of **35**, which is a vinylogous amide). The regioselectivity did not change with the functionality within the group of neutral substrates, but it varied depending on functionality within the group of amine substrates (i.e. **35**, **160** and **155**, Figure 100).

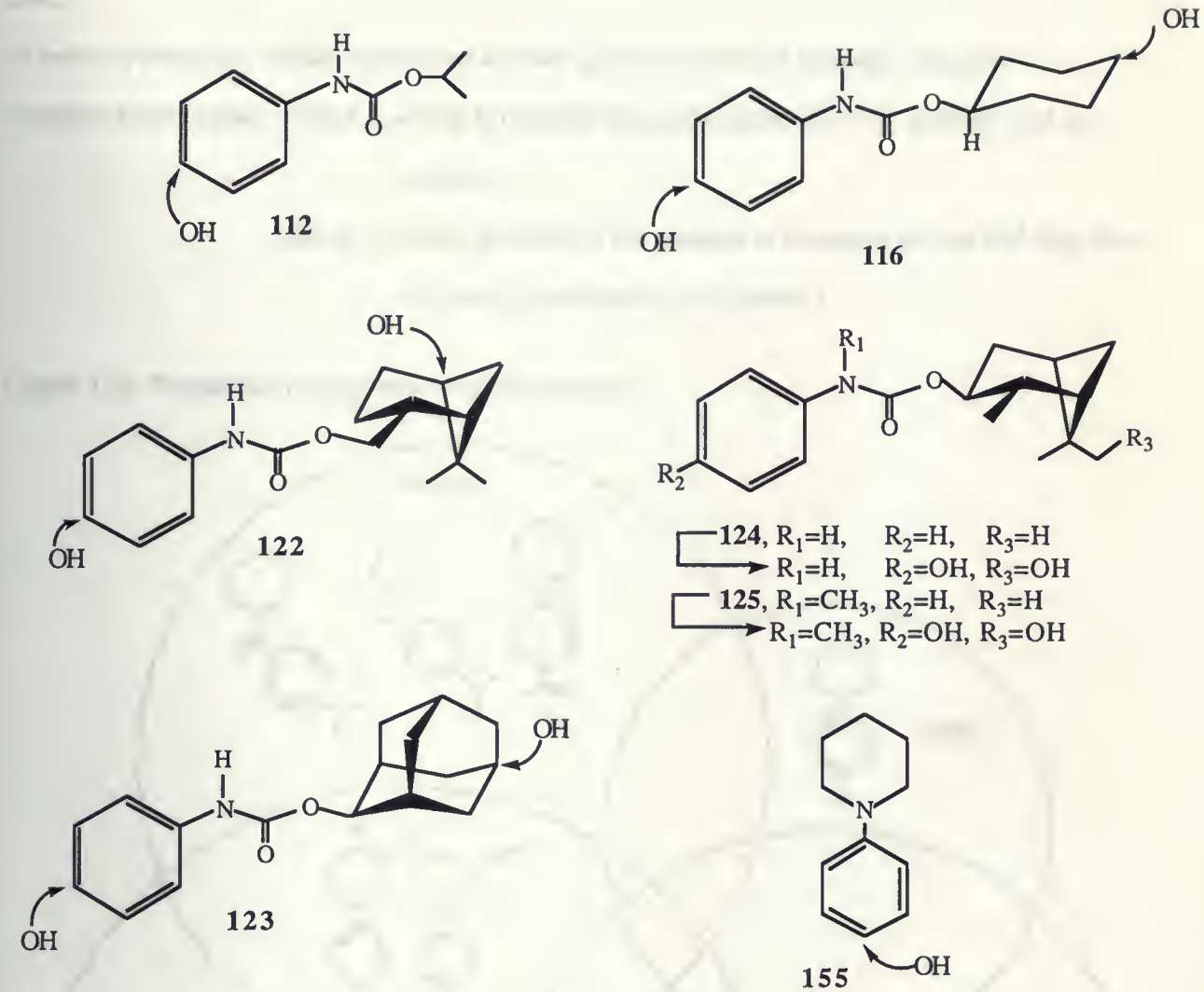


The hydroxylation reaction may not be stereoselective, since both enantiomers were produced from substrates **162** and **163**, but it seems to be regioselective since the functionality within the same group of compounds did influence site of hydroxylation.

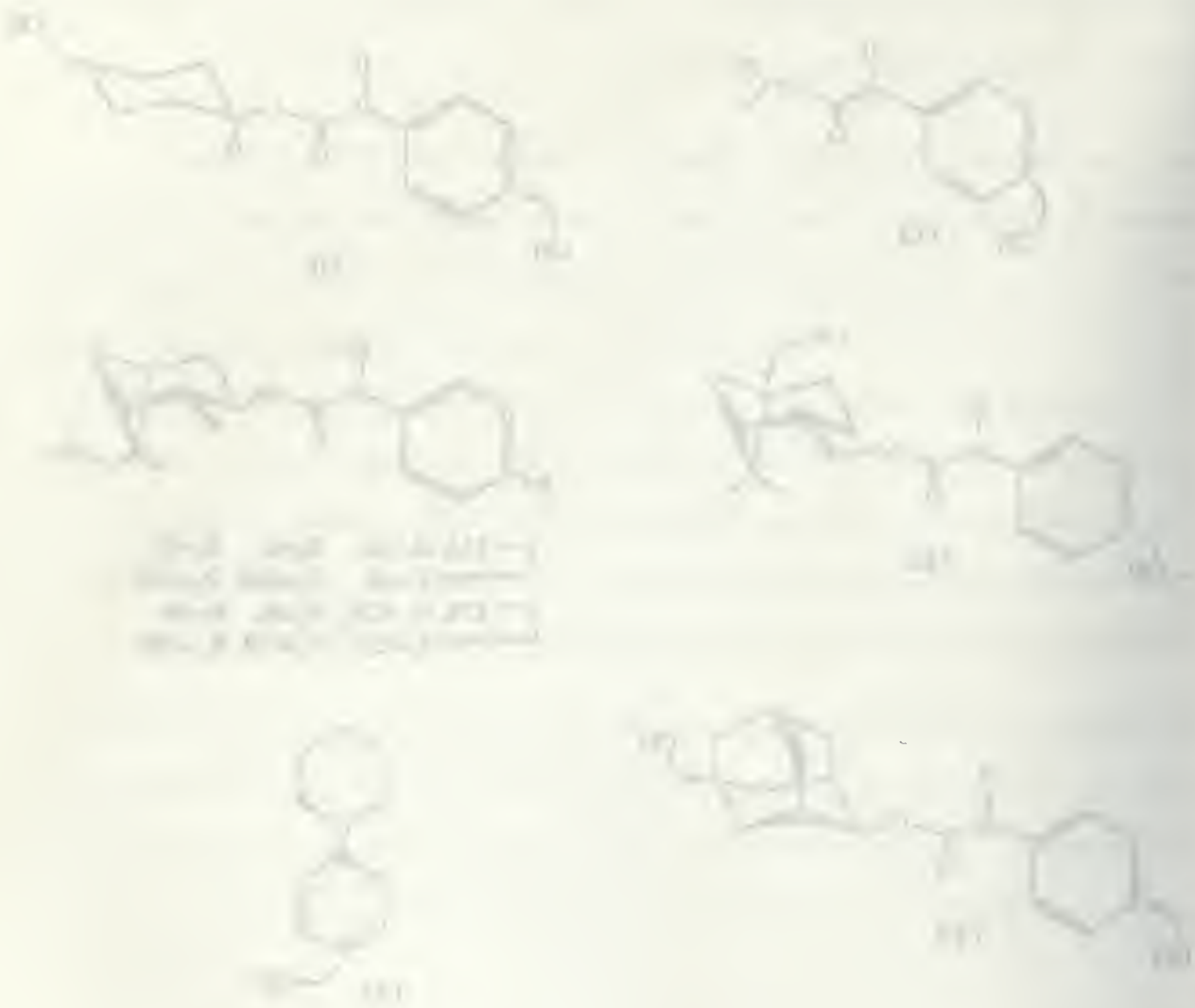
Amides generally gave the best yields. When amide substrates are placed as suggested by Archelas[43] with the fixed position of the nitrogen atom and the carbonyl group positioned on either side of the nitrogen, the hydroxyl group should come from the upper left (see introduction, Figure 38). When amides **181**, **182** and **183** are positioned in such a way that the carbonyl group is on the left side from the nitrogen (Figure 101, see Appendix), the hydroxyl group did come from the upper left in the case of **181** and **182**, but it did not in **183**. In order for hydroxyl group to come from the upper left , **183** had to be positioned in a different way (also shown in Figure 101), which then cannot account for hydroxylation of **181** and **182**. Obviously, predictions of the site of hydroxylation in the amide substrates cannot be reliably made using this model.

Phenyl hydroxylation was observed only with the substrate **155**. It seems that the phenyl ring needs to be activated with a *para* nitrogen (N-Ph or Ph-NH-CO-O, as opposed to N-CO-Ph, which is less activating). Other reported phenol formations support this suggestion (Figure 102).[51, 52] In order to test this hypothesis, more studies should be done with similar substrates.

Figure 102: Phenyl hydroxylation[51, 52]




The conclusion of our studies is that earlier proposed model[29] predicted on the necessity of the electron- rich site is not correct. The octant model[35] also does not apply to all substrates. Because there is no unique explanation (model) which fits all the substrates found to undergo hydroxylation reaction with *B. sulfurescens*, it is very reasonable to suggest that there is more than one enzyme responsible for this reaction. It could be speculated at this point that *B. sulfurescens* contains at least three different hydroxylase enzyme activities (Figure 103):



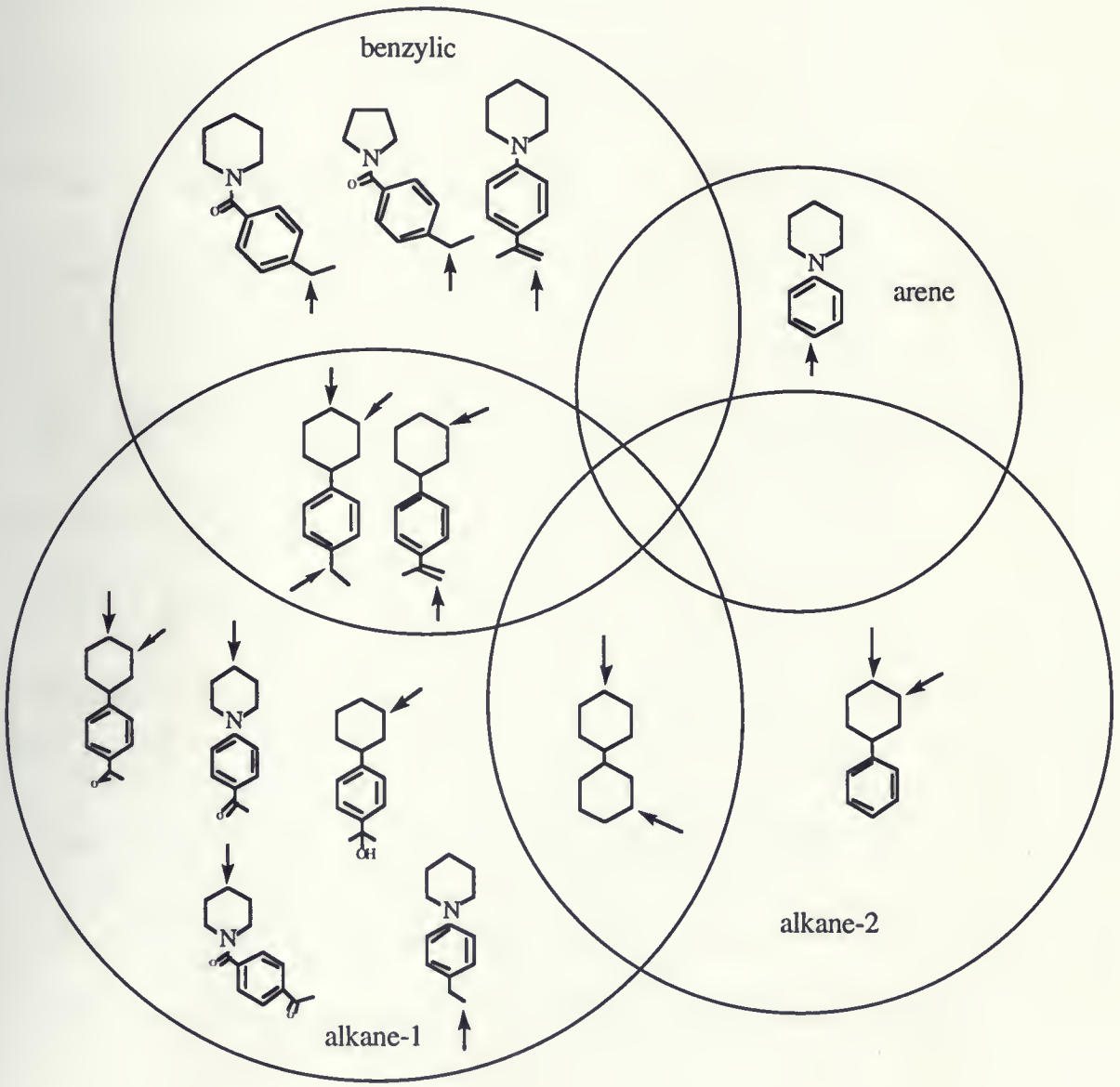
a) benzylic hydroxylase, which requires a free secondary or methyl benzylic position, or a benzylic C=C

b) arene hydroxylase, which requires an activating *para* substituent (usually nitrogen)

c) alkane hydroxylase: alkane-1, which is directed by amide, carbonyl C=O, alcohol -OH or nitrogen

alkane -2, which is active in the absence of directing groups and may also act directly on substrates of alkane-1

Figure 103: Postulated hydroxylase enzyme activities



The overlaping benzylic and alkane-1 area shown in Figure 103 is made on the assumption that hydroxylation occurs first at the benzylic position, and then alcohol group acts as a directing group for alkane-1 hydroxylase. The same applies for overlaping alkane-1 and alkane-2 area, where dicyclohexyl was presumably first hydroxylated at one position, and the hydroxyl group then acts as a directing group for alkane-1 hydroxylase.

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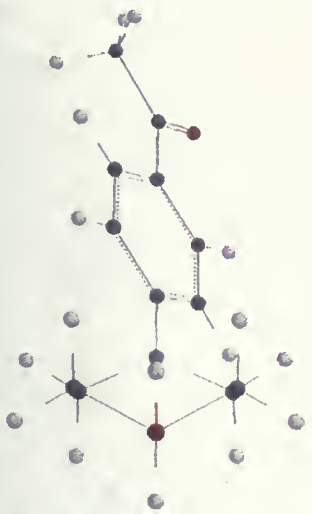
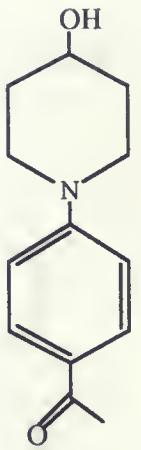
APPENDIX

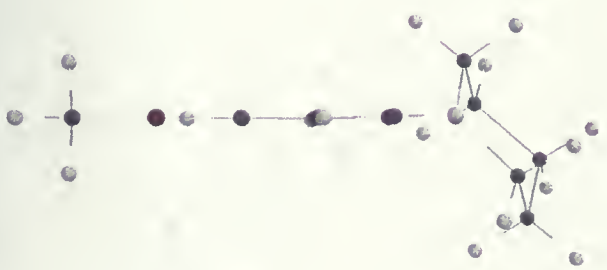
Figure 97: Orientation of the products in the octant system

AMINES

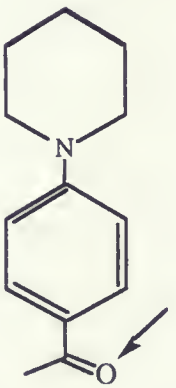


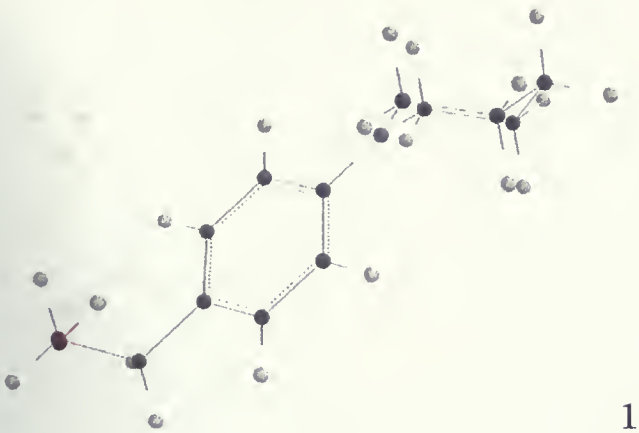
37

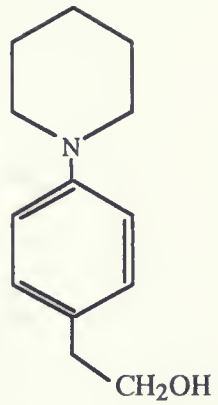


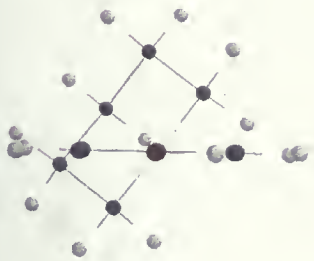
35

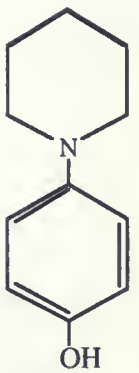






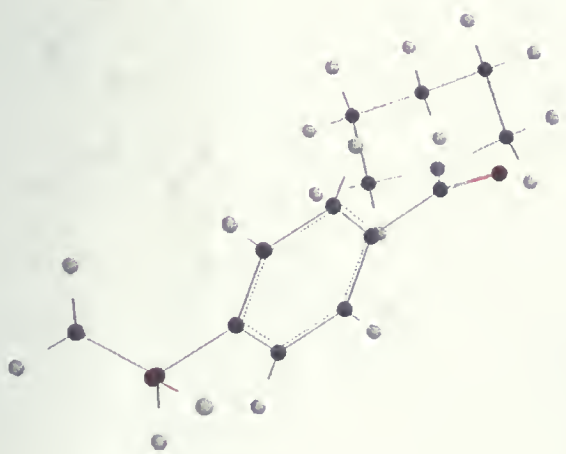

186

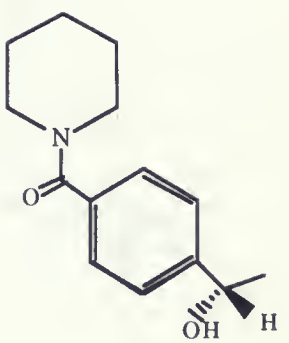




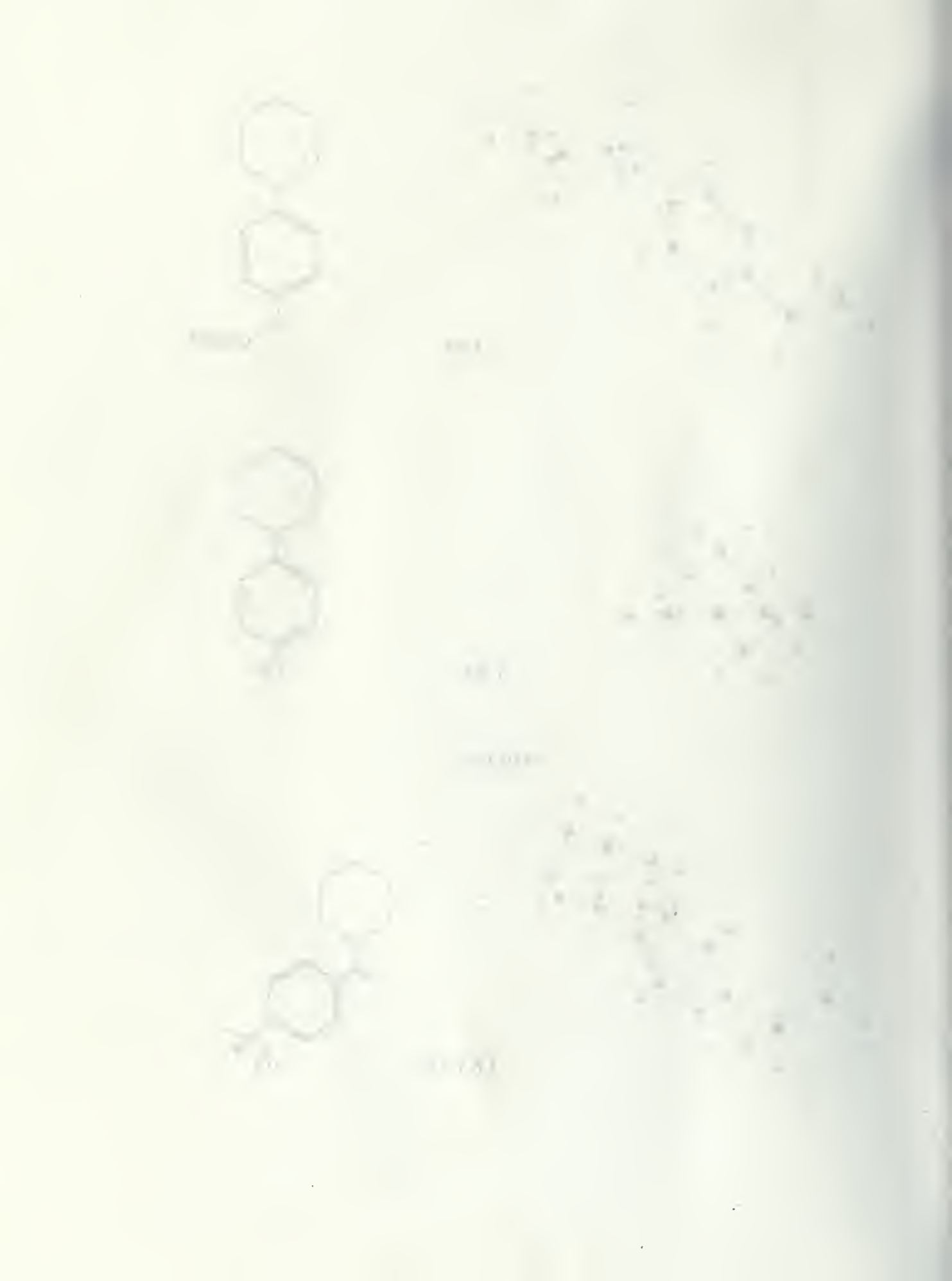

185

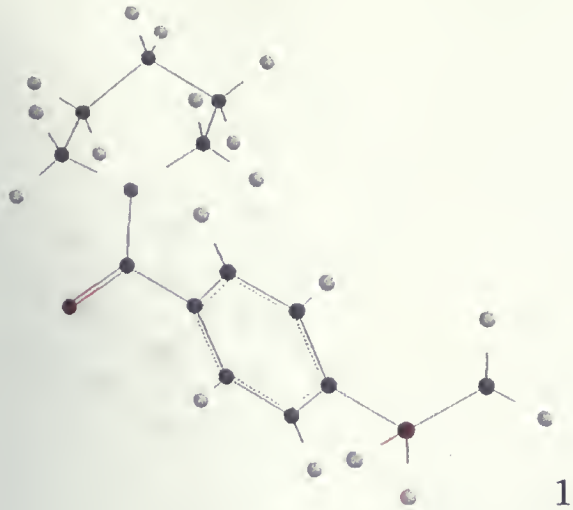
AMIDES

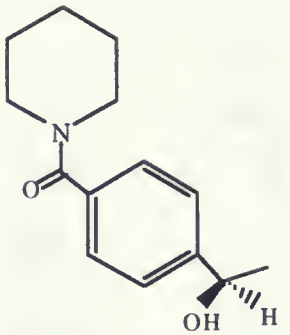




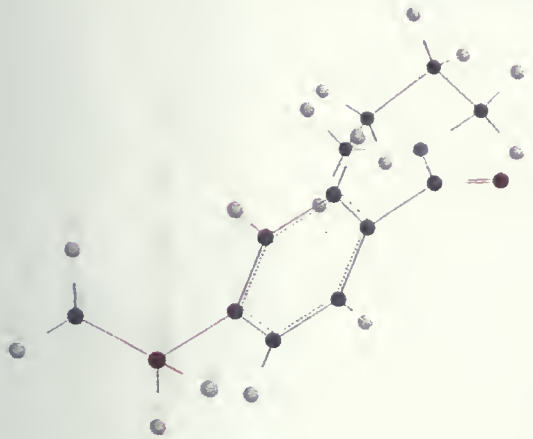

181 (S)

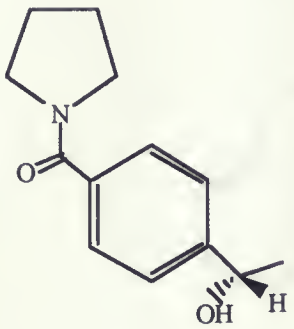




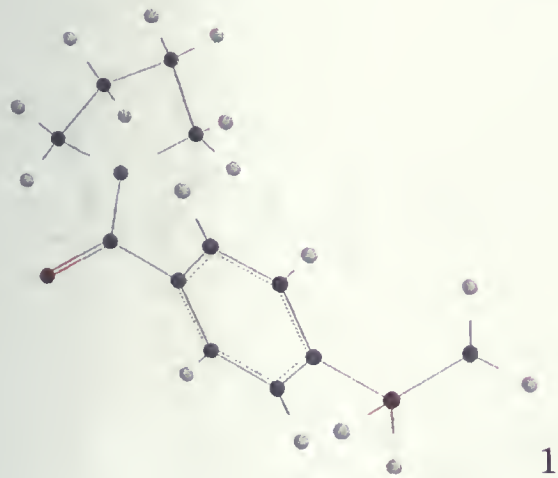


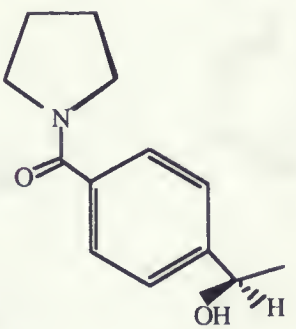

181 (R)



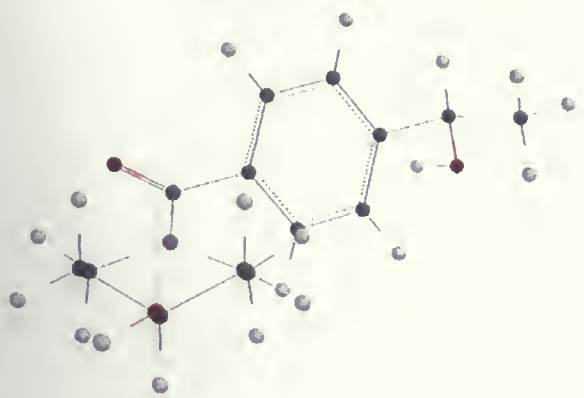


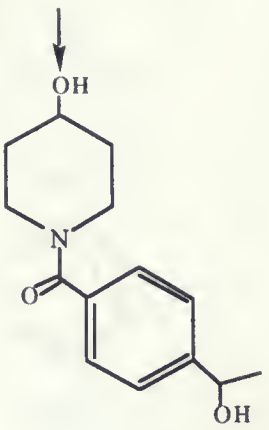

182 (S)





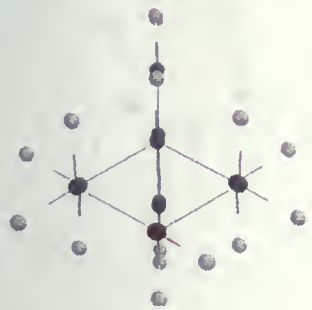

182 (R)



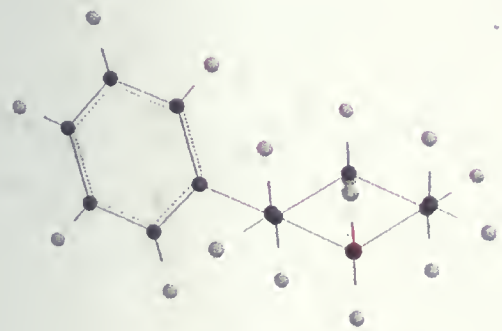


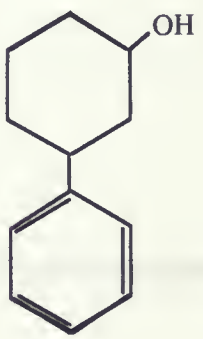

183

NEUTRAL SUBSTRATES

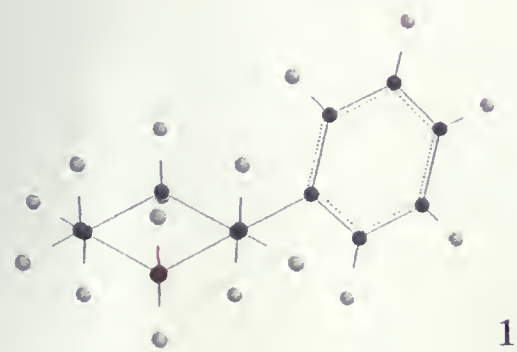



187

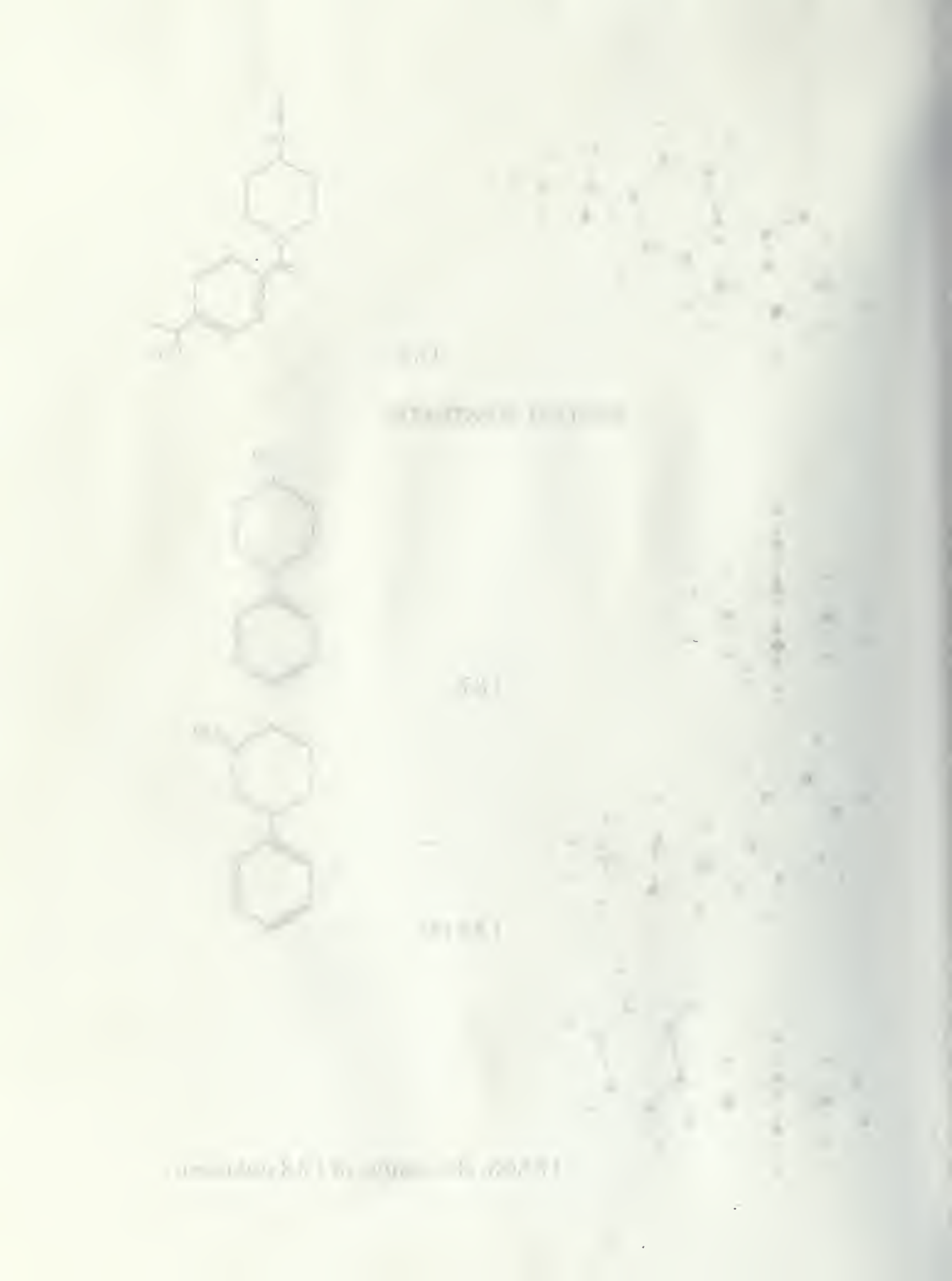


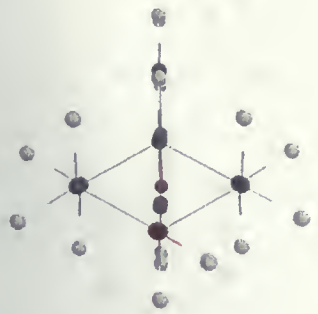


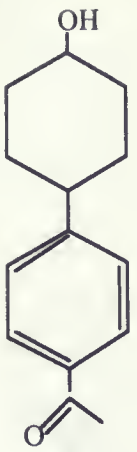

188 (R)



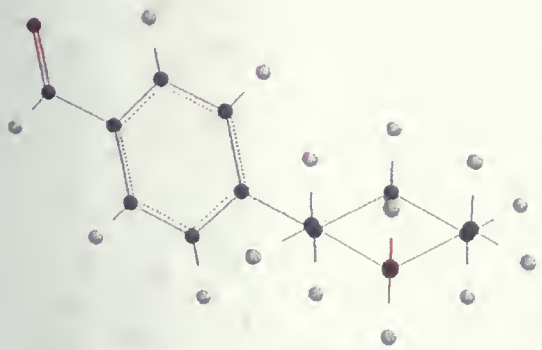
188(S), abs. config. of 188 unknown

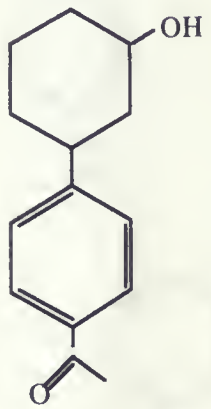




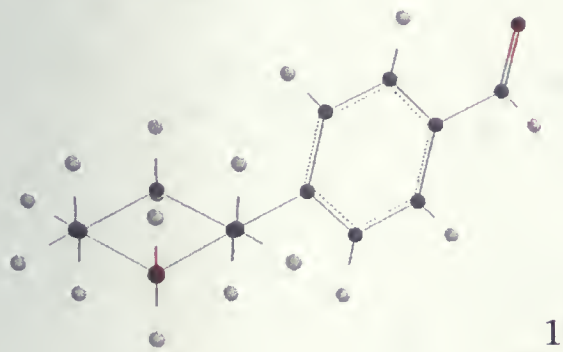



189

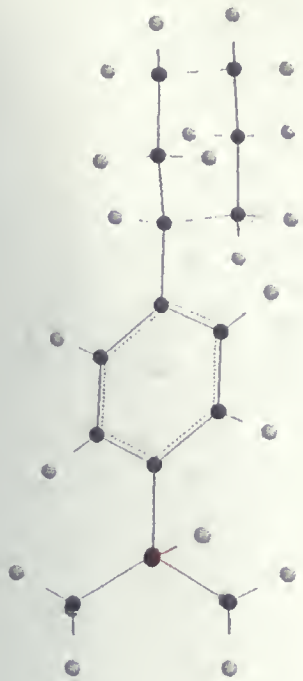


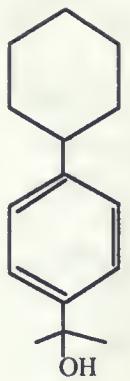



190 (R)

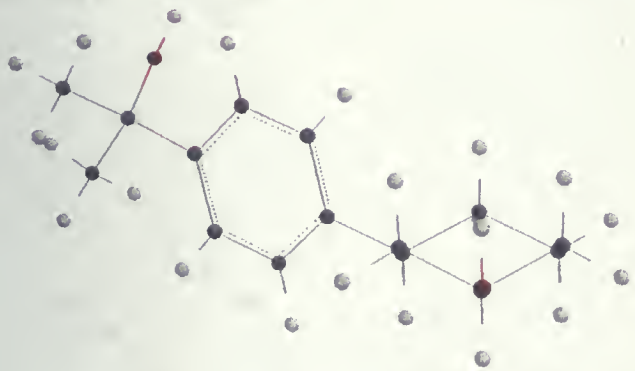


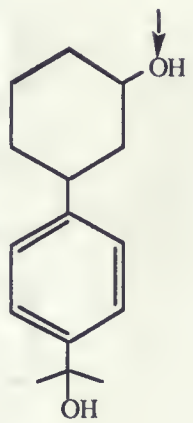
190 (S), abs. conf. of 190 unknown



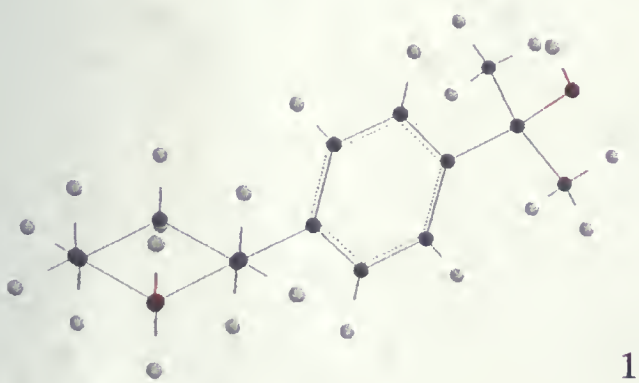



166

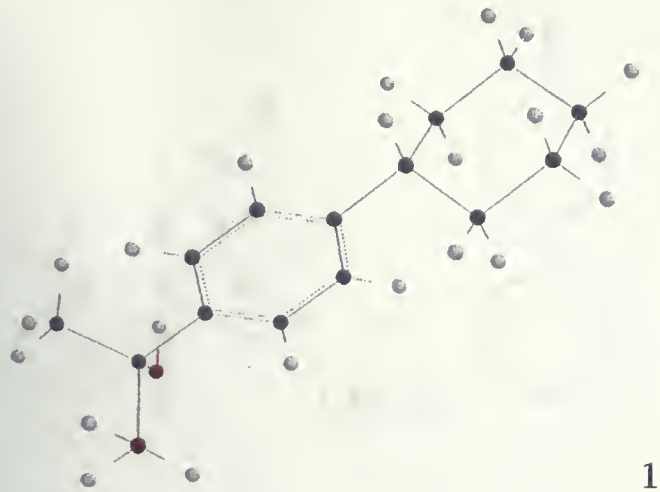


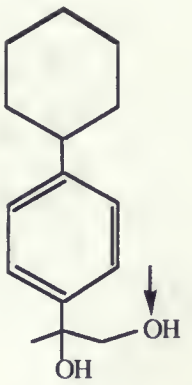



191 (R)

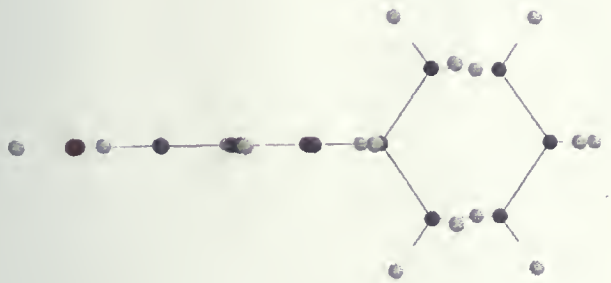


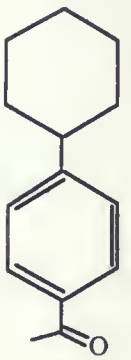
191 (S) (abs. conf. unknown)



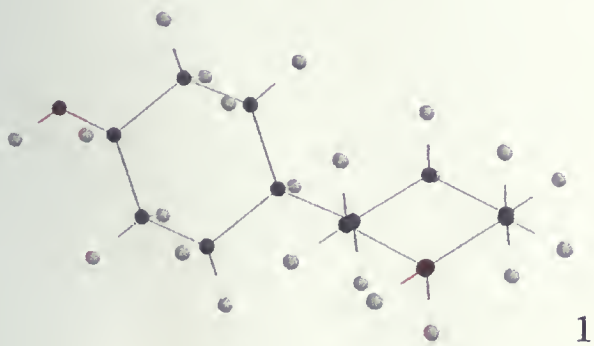


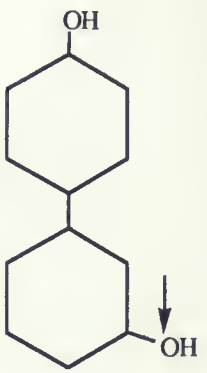

192 (S)



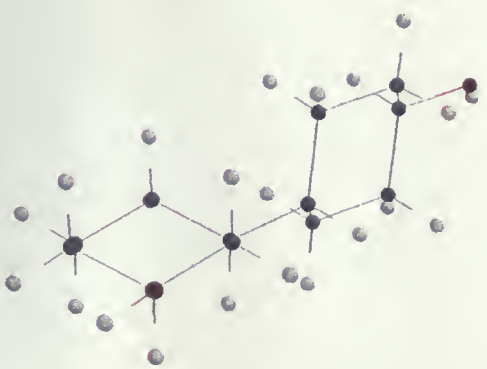



153

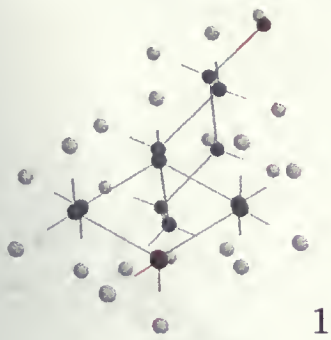




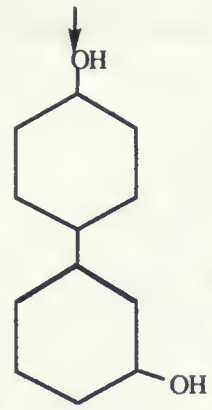

193 (R)

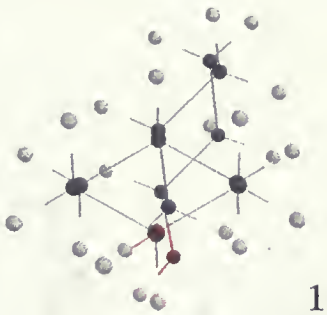


193 (S) abs. conf. unknown



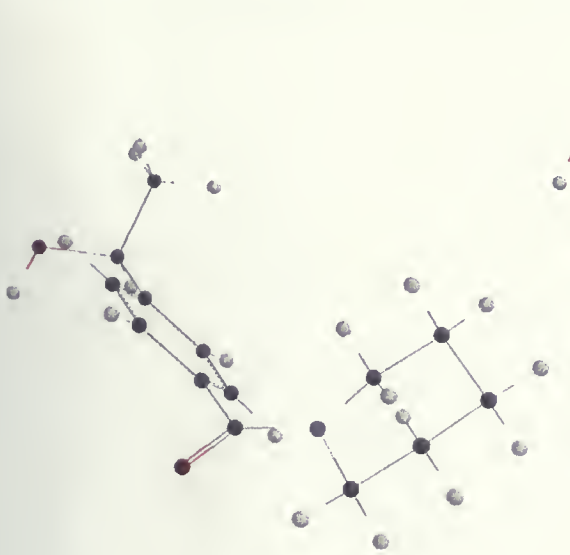
193 (S)



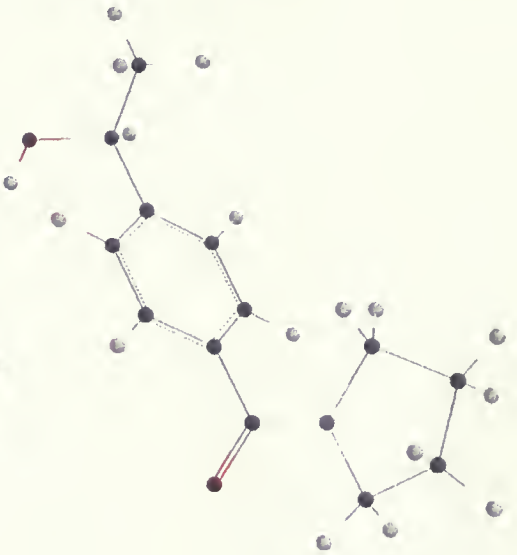



193 (R)

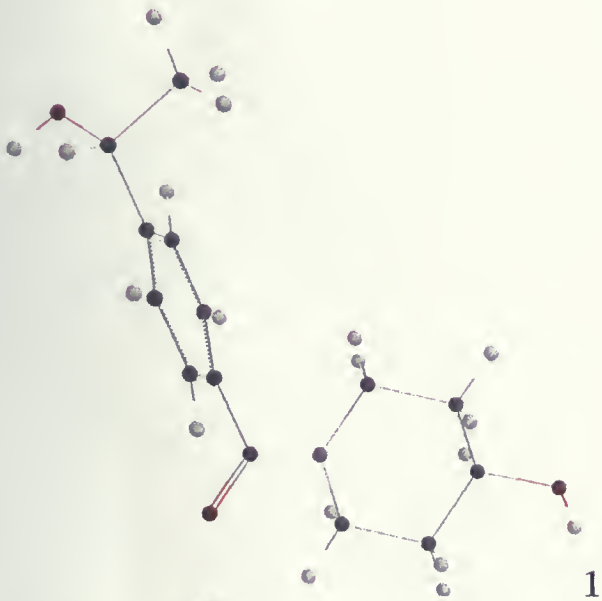
Figure 101: Amides with a fixed nitrogen atom



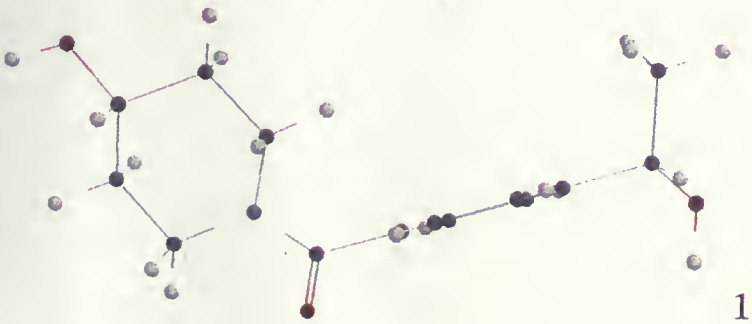
181 (S)



182 (S)



183



183