

BIOTRANSFORMATIONS OF ALKALOIDS: A CHALLENGE

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Dedicated to Prof. James Peter Kutney on the occasion of his 70th birthday

Abstract - The biotransformation of various alkaloids is reviewed. Special attention is paid to problems in microbial transformations and the different solutions offered by different authors. The biosynthetic potential of the strains selected for biotransformations is compared to the reaction observed in microbial oxidation. An outlook to future developments in other areas, especially from active site modelling, genomics and microbial ecology, are discussed which may contribute to problems in biotransformations.

INTRODUCTION

Alkaloids are nitrogen-containing, secondary metabolites mainly produced by plants. They have been used for thousands of years in various religious, cultural and medical applications. With the advent of modern organic chemistry more and more of the underlying principles for their activities have been elucidated. Nowadays more than 3000 different alkaloids are known, forming a huge diversity of chemical structures. Because of their pharmaceutical activities many alkaloids are the focus of both chemists and pharmaceutical scientists. Due to their often complex structures chemical syntheses are usually difficult and alternatives have been investigated. One alternative is to use the technique of biotransformation with microorganisms for the derivatisation of alkaloids or their precursors. These techniques have successfully been developed for the technical production of steroid precursors¹ and their application in alkaloid biotransformation seemed to be a logic step. However, a simple transfer of techniques from steroids to alkaloids did not produce satisfactory results and the methods had to be adapted.² From this experience more systematic approaches have been developed comprising systematic studies using active site modelling.³ A number of reviews on biotransformations in general⁴ or with special foci have been published⁵ and the reader is forwarded to them for a general overview. Despite our

broad gain in knowledge about the underlying principles of microbial transformations alkaloids are still notorious in their resistance to microbial attacks. The biotransformations of alkaloids remain a challenge.

In this article we want to discuss some results of biotransformations with various alkaloids, the problems encountered in these microbial reactions and the different routes taken by scientists to overcome the obstacles. Finally, we want to summarize the results and give an outlook to possible future developments using novel developments in the field of biotransformations.

Before we can start to discuss the different biotransformations and their results reported in the literature we have to define what we will understand here under the terms alkaloid and biotransformation. Alkaloids are understood here as natural secondary metabolites containing at least one nitrogen atom not including the amino acids found in proteins or peptides. The biotransformations discussed here are transformations carried out with whole microbial cells excluding transformations with plant tissue cultures, genetically engineered microorganisms, or isolated enzymes.

BIOTRANSFORMATIONS OF ALKALOIDS

Morphine alkaloids

To find the best strain for a biotransformation one can either screen the culture collection of the institute or search for the best strain in the environment. The latter has been done for the biotransformation of morphine alkaloids and led to the isolation of a strain of *Pseudomonas putida*, designated strain M10, isolated by selective enrichments with waste liquors from an opiate processing site, which was capable of metabolizing morphine and codeine. Biotransformation of codeine with resting cells of *Pseudomonas putida* M10 led to hydroxylated, hydrogenated and reduced products.⁶ Codeine (**1**) was first oxidized to codeinone (**2**) which could not be detected in the culture broth due to its instability in aqueous solution at neutral pH. Codeinone was then hydroxylated at C-14 (**5**) and further reduced to 14 β -hydroxycodeine (**6**). Another reaction route went from codeinone to hydrocodone (**3**) which was subsequently reduced to give dihydrocodeine (**4**) (Figure 1). Some of the enzymes responsible for the conversions have been purified from this strain and characterized.⁷

The steps initiating the transformation of morphine by M10 proceeded *via* morphine and hydromorphine. The number of unwanted side reactions observed with *P. putida* M10 meant that this organism would be unsuitable for a biotransformation process.⁸ Such a biotransformation can be improved by mutagenesis of the strain either by traditional methods or by genetical engineering. In this study recombinant DNA technology was used to construct a route in *Escherichia coli* that enabled, in high yields, the conversion of morphine to hydromorphine and codeine to hydrocodone. Plasmid curing and Southern hybridization revealed that the structural gene for morphine-dehydrogenase (MDH), *mor A*, was located on a large natural plasmid of about 165 kb in *P. putida* M10. Plasmid pMORAB5, carrying both *morA* and *morB*

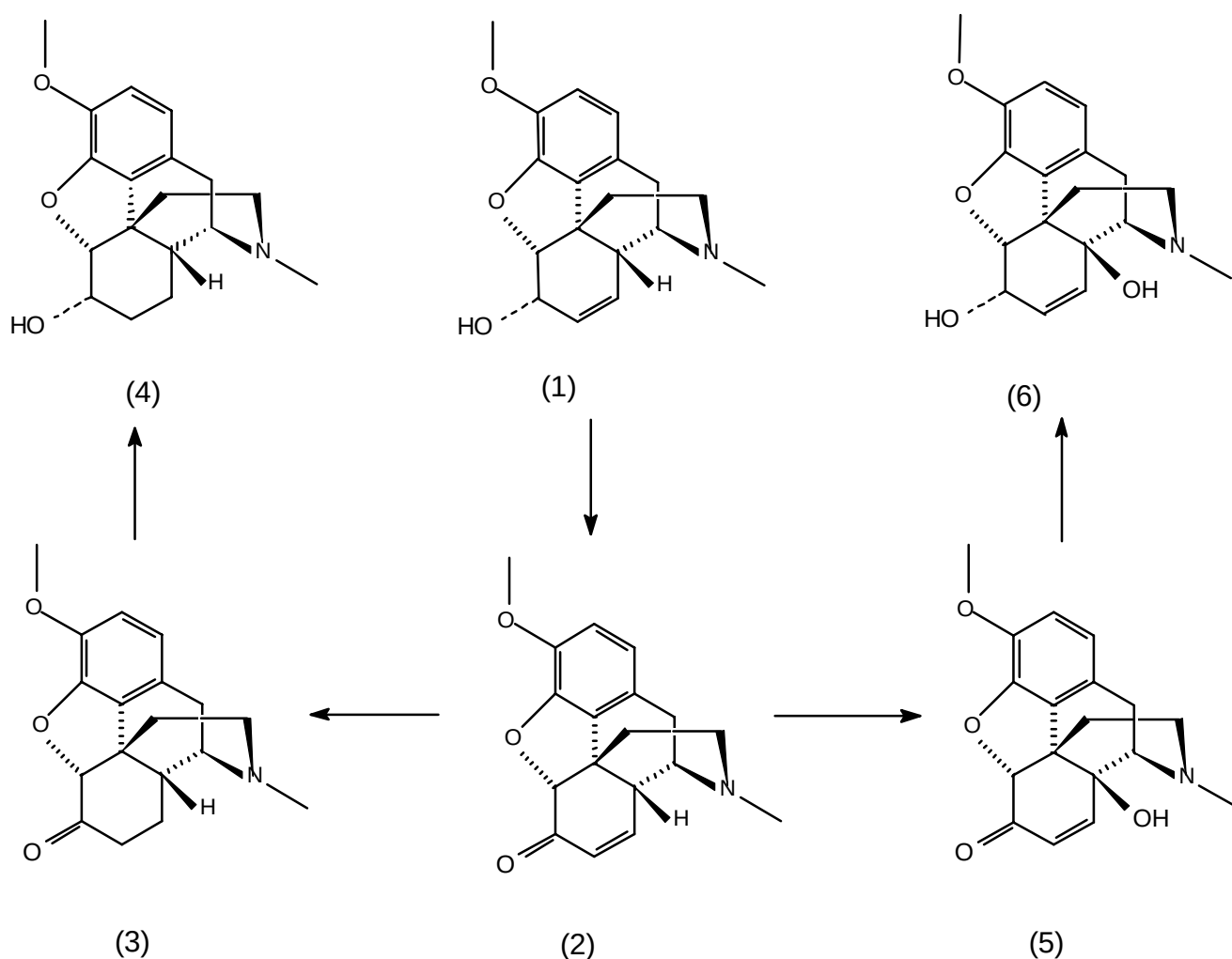


Figure 1: Biotransformations of codeine

was used to transform *E. coli* JM109. The recombinant host exhibited almost 10-fold more MDH than morphine-reductase.⁹

Biotransformation of codeine with *Streptomyces griseus* ATCC 10137 resulted in the formation of 14-hydroxycodeine and norcodeine as the only identifiable products. The ratio of these two compounds was 4:1 after an incubation time of one week.¹⁰ The formation of 14-hydroxycodeine seems to be a rather common microbial reaction with codeine but the formation of the *N*-demethylation products of alkaloids were also often reported. Additionally to the *Streptomyces griseus* strain the fungus *Cunninghamella bainieri* C42 was able to form norcodeine from codeine.¹¹ In an optimization study with this fungus the best results were obtained using 4 h pre-germinated spores, but norcodeine yields were still low. It was necessary to use a stabilized alginate gel for immobilization of the spores and subsequent culture to obtain the desired pelleted growth form. Using perchloric acid, instead of sulfuric acid or hydrochloric acid, for pH control resulted in an increase of the codeine conversions by approximately 99%. It appeared that perchloric acid makes oxygen readily available for demethylation to follow the *N*-oxide route. The purification of the resulted products was optimized at the same biotransformation approach.¹²

A very similar reaction was reported for the biotransformation of thebaine (7) with *Mucor piriformis* where northebaine was the main product. Minor metabolites identified were isomeric thebaine *N*-oxides. Among isomeric thebaine *N*-oxides, the one with the equatorial oxygen attached to the nitrogen was relatively unstable, leading nonenzymatically to rearranged products.¹³ The biotransformation of the same substrate was studied with *Trametes sanguinea*.¹⁴ Here 14 β -hydroxycodeinone (8) and 14 β -hydroxycodeine (9) were characterized as the biotransformation products (Figure 2) similar to the biotransformation of codeine with *Pseudomonas putida* M10, which suggests the *O*-demethylation as the first step resulted in codeinone.

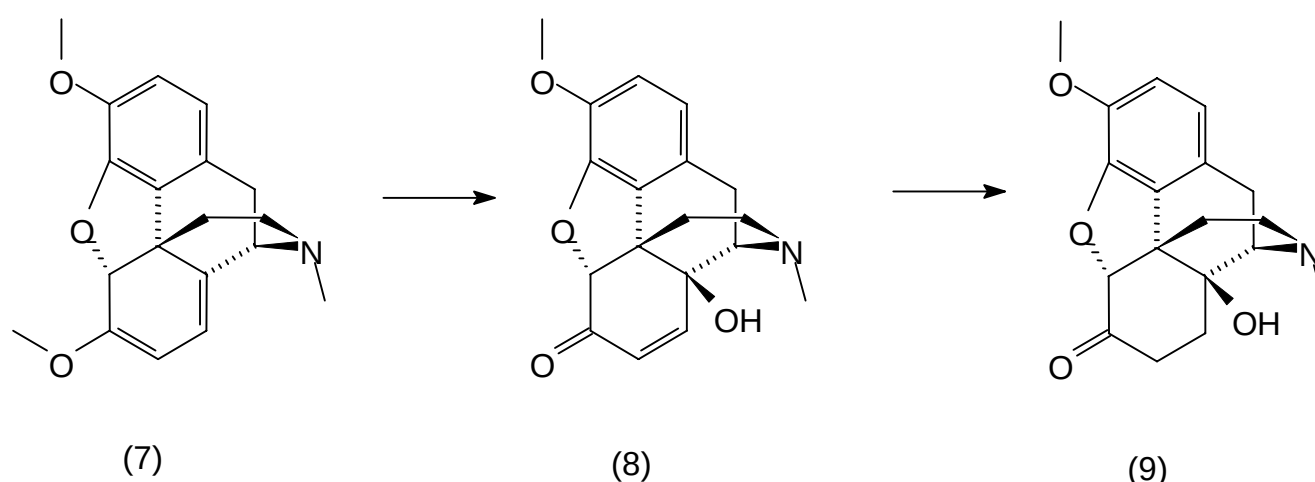


Figure 2: Biotransformation of codeine

3-Desoxydihydromorphinanes are available by simple and well developed chemical syntheses and can be produced in large quantities. To introduce the 3-hydroxy group microbiological transformations have been investigated. Microbial hydroxylation of aromatic rings are rather rare and a few examples are given in this review. Nevertheless, this possibility was tested with different 3-desoxymorphinanes possessing different substituents at the nitrogen atom. About one hundred strains have been tested with the different substrates. The substrate with a hydrogen at the nitrogen was attacked by only 4 of 37 tested strains. The substrate with a methyl group at the nitrogen was attacked by 68 of 190 tested strains. Amides were better substrates and the hydroxyacetate was metabolized by 17 of 23 screened strains. The best substrate was the ethoxyacetate which was used by 209 of the 268 tested strains. It is interesting to compare the capability of the different strains to attack these substrates. A comparison is shown in Table 1.

Table 1: Microorganisms capable of biotransformations of 3-desoxymorphinanes having different *N*-substituents

Strains	Substrates			
	N-H	N-CH ₃	N-COCH ₂ OH	N-COCH ₂ OCH ₂ CH ₃
<i>Cercospora beticola</i> DSM 62107				●
<i>Coprinus comatus</i> CBS 150.39		●		
<i>Corticium sasakii</i> DSM 62716		●		●
<i>Laccaria laccata</i> ATCC 24509		●		
<i>Mucor lusitanicus</i> CBS 277.49		●		
<i>Mucor plumbeus</i> DSM 62759		●		
<i>Mucor spinosus</i> DSM 63349				●
<i>Pellicularia filamentosa</i> DSM 843				●
<i>Rhizopus achlamydosporus</i> CBS 385.34		●	●	●
<i>Rhizopus boreas</i> CBS 330.53	●	●		●
<i>Rhizopus circinans</i> CBS 147.22				●
<i>Rhizopus fusiformis</i> CBS 266.30				●
<i>Rhizopus pygnaens</i> CBS 344.29				●
<i>Rhizopus rhizopodiformis</i> CBS 607.33				●
<i>Rhizopus sontii</i> CBS 279.38				●
<i>Rhizopus stolonifer</i> CBS 383.52				●
<i>Rhizopus thermosus</i> CBS 405.51				●
<i>Rhizopus usami</i> CBS 406.61				●
<i>Sporomiella isomera</i> CBS 166.73				●
<i>Stereum rameale</i> CBS 119.16				●
<i>Tanatephorus cucumeris</i> DSM 1907		●		

It is interesting that only fungi and not a single bacterium appear in this list and that most strains accepted only one substrate. Only *Corticium sasakii* DSM 62716 could use two substrates and *Rhizopus achlamydosporus* CBS 385.34 and *R. boreas* CBS 330.53 were able to metabolize three compounds.¹⁵ Both *Rhizopus* strains were the only microorganisms tested which could use the secondary amine and the

hydroxyacetamide derivatives. This versatility of the genus *Rhizopus* was observed in a number of different biotransformations and seems to be a characteristic for this genus.

The reaction performed by these strains is the reduction of the carbonyl moiety to the 6*R*-hydroxy group. This product was not used by any of the strains and no further hydroxylation either at the aromatic ring or at any other position of the molecule was detected. Only *Rhizopus achlamyosporus* CBS 385.34 and *Pellicularia filamentosa* DSM 843 could form, after longer incubation periods with the *N*-ethoxyacetyl derivative (**10**), first the alcohol (**11**) and then another compound (**12**) which was identified as the cleavage product of the ether moiety of the amide (Figure 3).

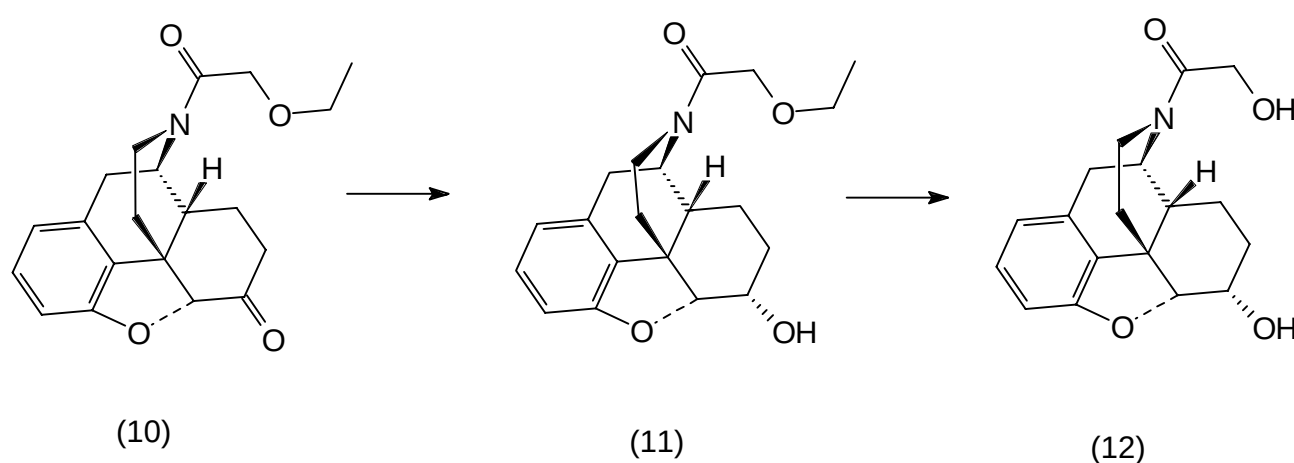


Figure 3: Microbial transformations of desoxyhydromorphinan (**10**)

Ergot alkaloids

Ergot alkaloids are very important pharmaceutical substances with wide medical applications. Their bioproduction by species of the genus *Claviceps* is usually followed by chemical modification. Bioconversions of ergot alkaloids on an industrial scale have become more important in recent years and a number of microbial transformations have been reported. Here only some of these reactions will be listed and the reader is referred to the excellent review on this topic by Kren.¹⁶ Agroclavine is hydroxylated at C-8 by several fungi and the same reaction is observed with elymoclavine. *Corticium sasaki* hydroxylated at C-2 and *Penicillium roqueforti* at C-17. *N*-Demethylation is observed with several *Streptomyces* spp. Elymoclavine is isomerized to lysergol by several fungi. The biotransformation of lysergic acid amides, most notably lysergic acid diethylamide, the famous hallucinogen LSD, by *Streptomyces* spp. was intensively studied. *Streptomyces lavendulae* IFM 1031 demethylated only the *N*-6 position yielding nor-LSD. Conversely, *Streptomyces roseochromogenes* IFM 1081 attacked only the *N*-amide alkyl group to yield lysergic acid ethylamide. The high degree of substrate stereoselectivity in *Streptomyces roseochromogenes* was proved by the fact that this species could not metabolize iso-LSD

while, on the contrary, *S. lavendulae* yielded iso-nor-LSD. Studies of higher alkyl homologs of LSD with *Streptomyces roseochromogenes* revealed that the side chains were hydroxylated by ω -1 hydroxylation, yielding epimeric secondary alcohols.

Indol alkaloids

Ellipticin from *Ochrosia elliptica* is an indolic alkaloid. Its antitumor activity is well known and a number of derivatives have been synthesized and tested. Among them are 9-hydroxy- and 9-methoxyellipticin. They all display significant anticancerous activities. As chemical syntheses of these compounds are often complicated, a number of microorganisms were screened for the biotransformations. Some fungi hydroxylated ellipticin (**13**) at the 8- and 9-positions¹⁷ (**14**) and *O*-demethylation of 9-methoxyellipticine was also observed¹⁸ (Figure 4). The optimal biotransformation conditions for the oxidation of ellipticine with *Aspergillus alliaceus* CBS 536.65 were determined. It was found that a rather short contact time of

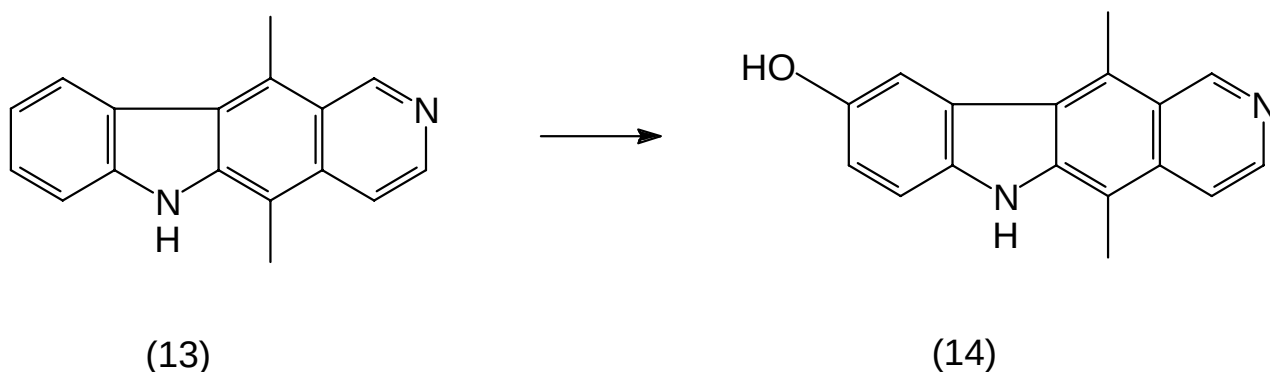


Figure 4: Microbial hydroxylation of ellipticin

24 h and a pH around 7 were the best fermentation conditions. A possible explanation could be that the formed phenolic alkaloid was rather sensitive to further oxidations.¹⁹

Borris and Cordell²⁰ took an interesting approach to form ellipticin or one of its precursors by the biotransformation of uleine (**15**). They speculated that the oxidation of this alkaloid could lead to a compound which could be transferred to ellipticin by this retrobiomimetic approach. From their collection 14 strains showed a potential to transform uleine. While the fungi *Penicillium chrysogenum* ATCC 11709 and *Penicillium adametzi* ATCC 10407 formed both 4*S*- and 4*R*-uleine-*N*-oxide (**17**) only the 4*S*-uleine-*N*-oxide (**18**) was formed by the two bacteria *Streptomyces rimosus* ATCC 10970 and *S. purpureus* NRRL B148. All strains gave 16*R*- (**20**) and 16*S*-hydroxy-16,17-dihydruleines (**19**) (Figure 5). For the formation of this alcohol two possibilities exist. One is the direct addition of water to the double bond as has been observed for the formation of caparrapidiol from *trans*-nerolidol by *Fusarium solani*.²¹ The other one runs via the formation of an epoxide and subsequent reduction to the alcohol as has been found in the biotransformation of caryophyllene by *Diplodia gossypina*²² or

Chaetomium cochlioides.²³ Only the bacteria were able to demethylate the alkaloid to des-*N*-methyluleine (**16**). This is another example where different phylogenetic group showed characteristic reactions which were specific for only one of these groups.

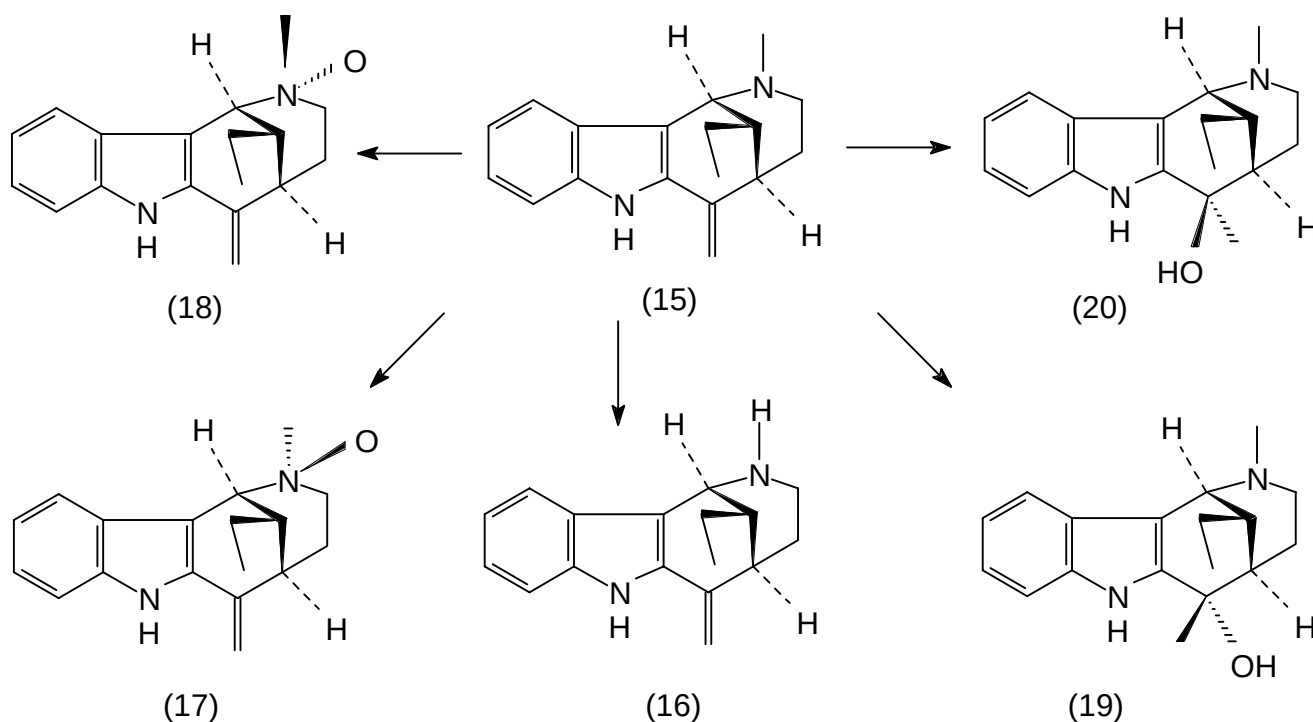


Figure 5: Biotransformations of uleine

Because of its central position in the biosynthesis of many monoterpenoid indole alkaloids 3(*S*)-strictosidine (**21**) is an interesting substrate for biotransformations. It was assumed that microorganisms can oxidize this compound and/or hydrolyze the sugar moiety starting a series of rearrangements which may lead to novel products. All 22 tested bacteria strains, including *Listeria* spp. very seldom used for biotransformations, convert the substrate, all giving the same metabolites which were identified as vallesiachotamine (**22**) and isovallesiachotamine²⁴ (**23**) (Figure 6). It is surprising that all strains were able to do this conversion which is probably only enzymatic in the saponification of the glucose unit. Unfortunately, the authors did not report whether they made sure that this reaction is really related to the presence of the bacteria.

Biotransformations of yohimbine, apoyohimbine, -yohimbine, and ajmalicine by *Cunninghamella blakesleeana*, *C. bertholletiae*, *C. echinulata* and *Calonectria decora* yielded hydroxylations specifically at C-10, C-11 and C-18.²⁵ Obviously, hydroxylations at these compounds occurred preferentially at the allylic and the aromatic carbons and only a few fungi were capable to carry out these reactions.

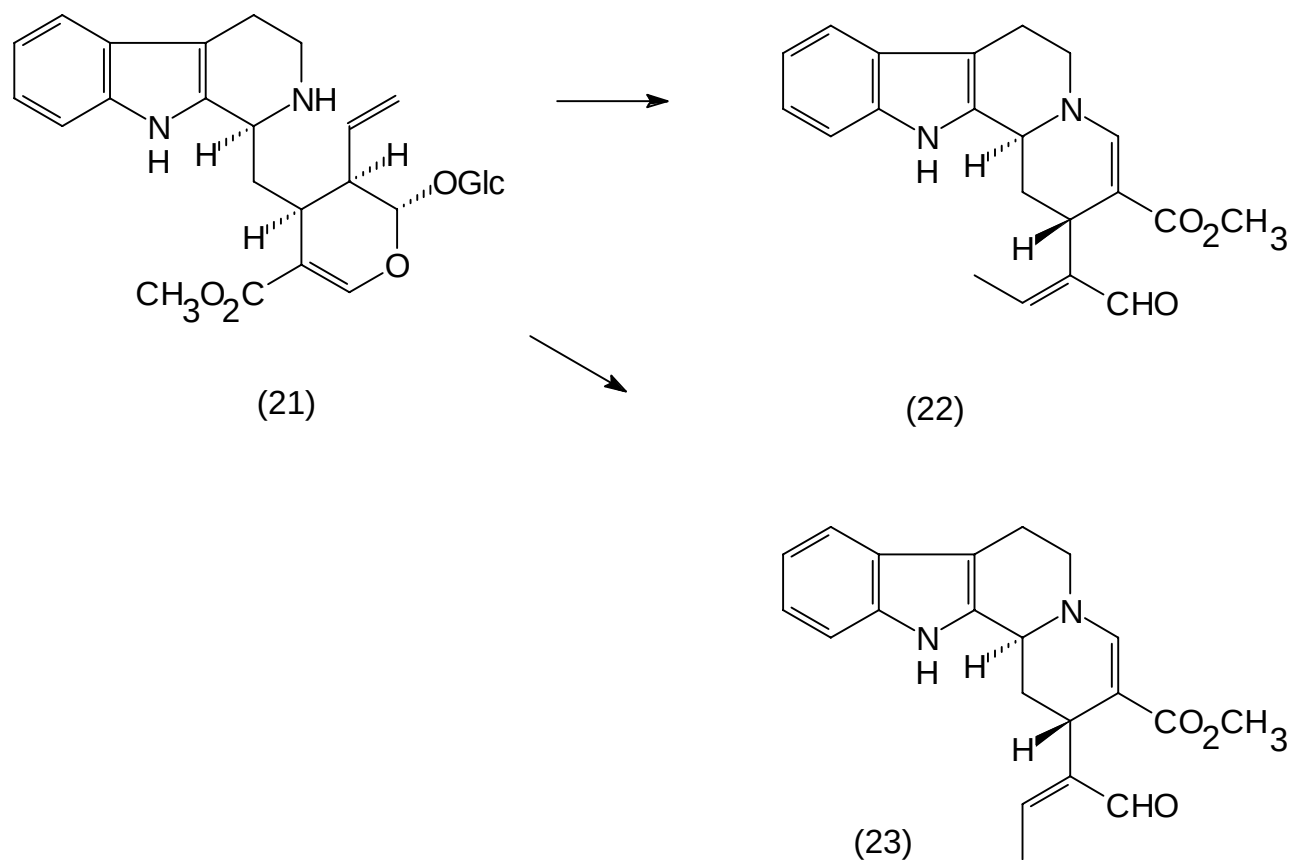


Figure 6: Microbial hydrolysis of 3(S)-strictosidine condensation products

As with other alkaloids synthetic precursors were used in biotransformations to produce intermediates for indol alkaloid syntheses. One such transformation of a *N*-acetylhexamethylene ndoline by *Calonectria decora* has been reported.²⁶ This fungus oxidized *cis*-5-acetyl-5a,6,7,8,9,10,11a-octahydro-5*H*-cyclooct[*b*]indole (24) to the isomeric ketones (25) and (26), introducing a functionality into a chemically inert part of the molecule (Figure 7). This allows the use of these biotransformation products as intermediates in indol alkaloid syntheses.

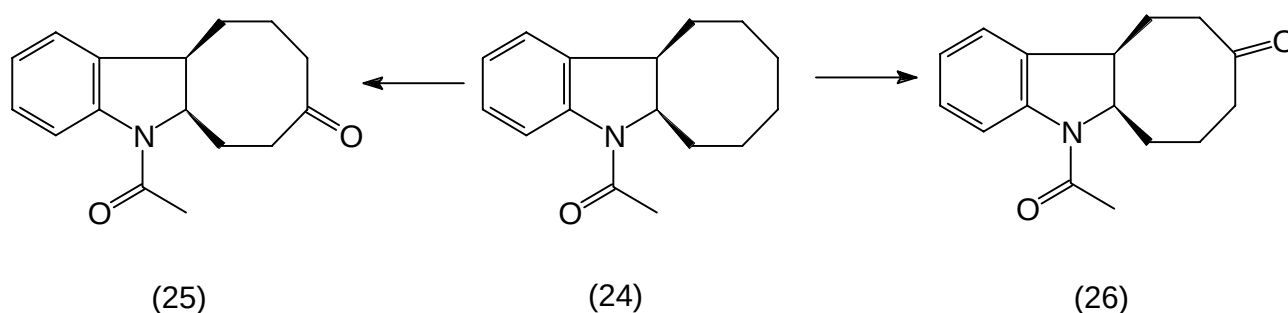


Figure 7: Microbial oxidation of 24 by *Calonectria decora*

The microbiological transformation of *N*-heptylphysostigmine, a semisynthetic physostigmine cholinesterase inhibitor, was investigated using *Verticillium lecanii* ATCC 74148, *Acremonium* sp. ATCC 74164 and *Actinoplanes* sp. ATCC 53771. Nine microbial metabolites were isolated and characterized. The structures of the metabolites were established using spectroscopic techniques including MS and NMR revealing oxidation only within the *n*-heptyl side chain. Here the terminal methyl group was oxidized to the carboxylic acid, the side chain was oxidatively shortened by two carbon atoms and various methylene were oxidized to secondary alcohols. Only the 3'-hydroxy group was further oxidized to yield the carbonyl function. Some of the microbial metabolites were identical to metabolites present in urine of a dog treated with *N*-heptylphysostigmine.²⁷

Steroidal alkaloids

El Sayed reported on the biotransformation of veratramine (27).²⁸ Veratramine is a homosteroidal alkaloid found in *Veratrum* species. It antagonizes the Na⁺ channel-gating mechanism of ceveratrum alkaloids by blocking the channel. Veratramine also displays serotonin agonist activity by acting on presynaptic 5-HT neurons. It was also reported to induce hemolysis of human red blood cells. Because of their interesting

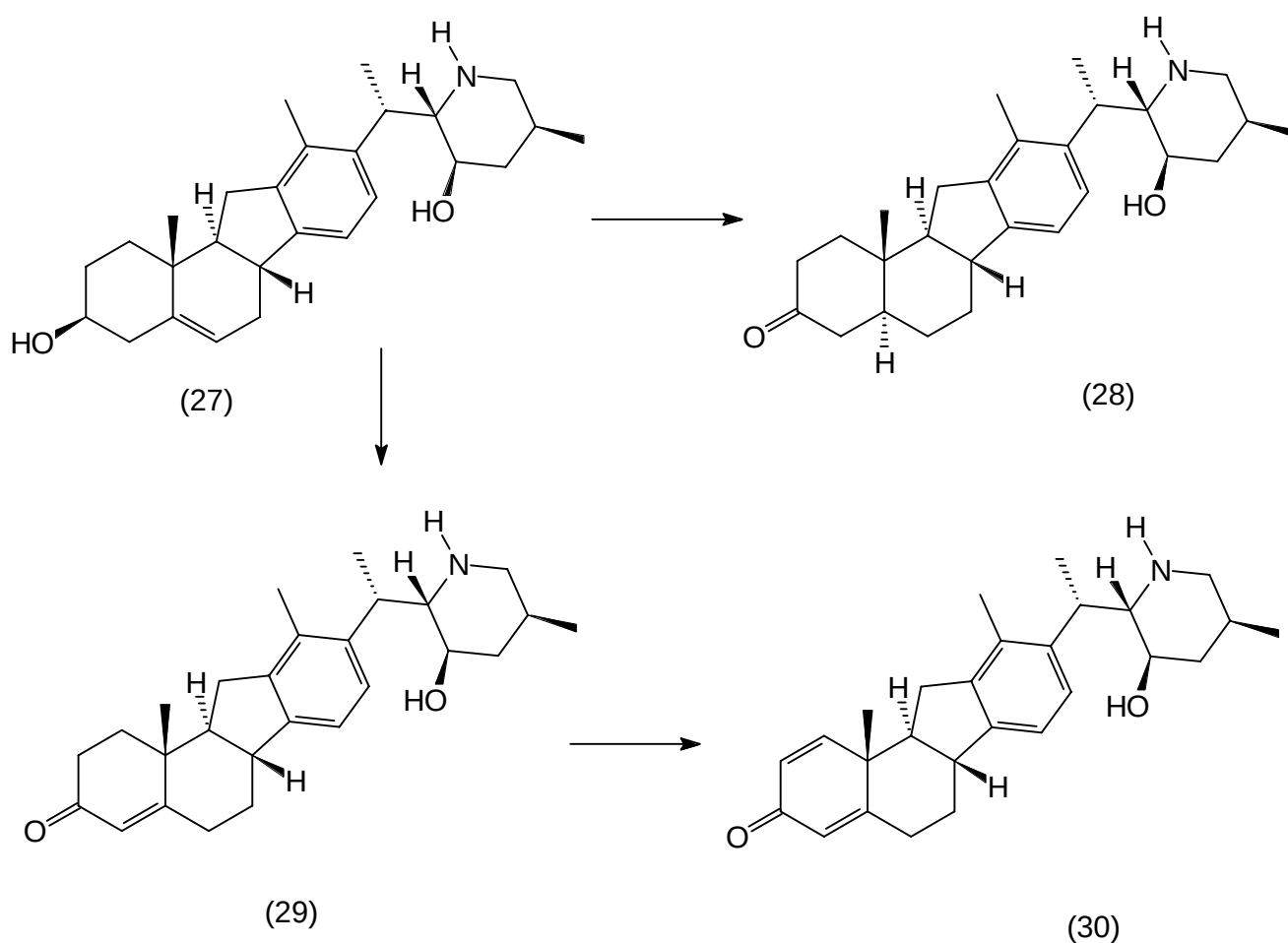


Figure 8: Biotransformations of veratramine (27)

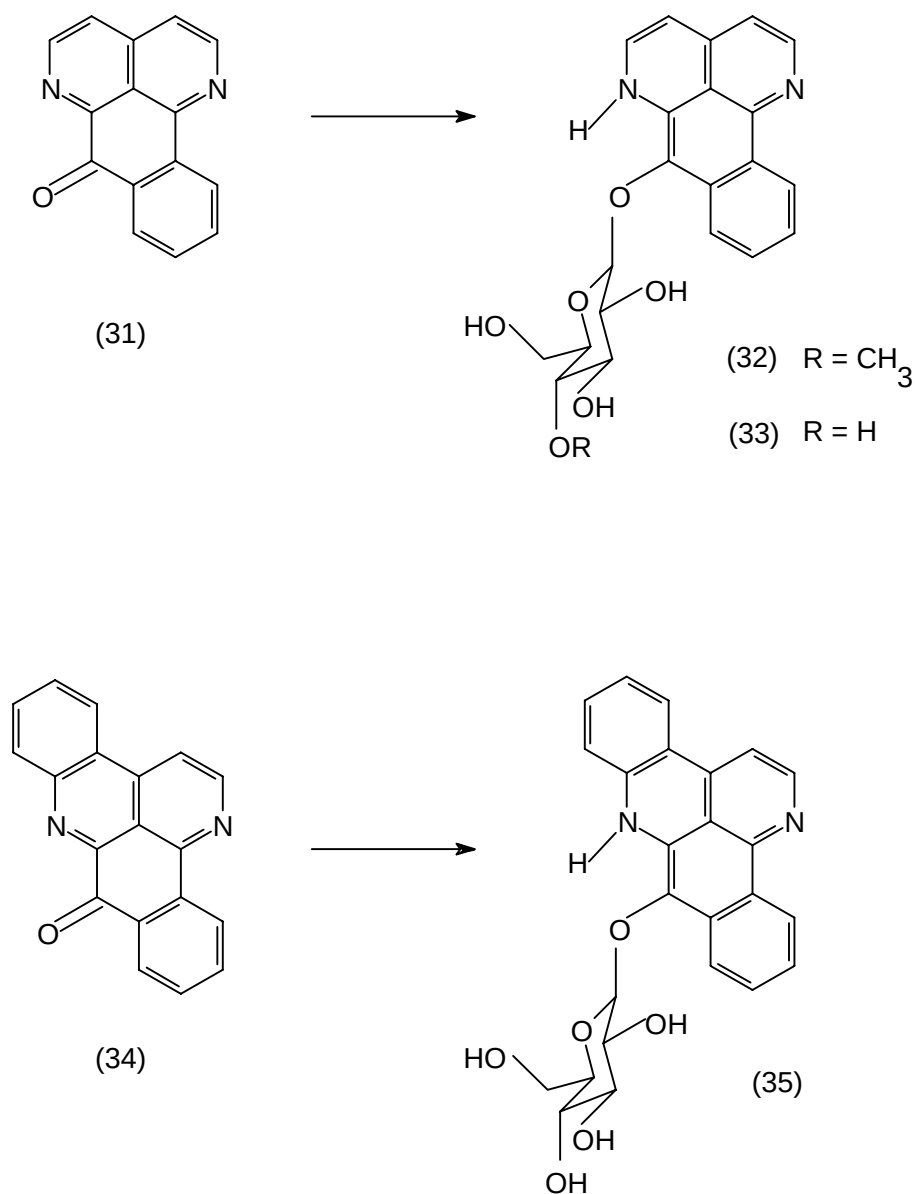
biological effects steroidal alkaloids have been investigated in biotransformation studies in attempts to prepare less toxic and more bioactive analogues. Incubation of the spirosolane-type *Solanum* alkaloid tomatidine with *Nocardia restrictus*, *Mycobacterium phlei*, or *Gymnoascus reesii* resulted in the conversion of this compound to 1,4-tomatidien-3-one, tomatidone, tomatanin-3-ol, 1-tomatiden-3-one, and 4-tomatiden-3-one.²⁹ Only few strains out of 25 tested were able to convert veratramine (**27**). Among them was *Nocardia* sp. ATCC 21145, which transformed veratramine into its 3-dehydro-5,6-dihydro- (**28**), 3-dehydro- (**29**), and 1,2,4,5-didehydro-3-oxo-derivative (**30**). No attack at the nitrogen bearing ring was observed (Figure 8).

Conessine (3 β -dimethylamino-con-5-enine) is the principal compound of the kurchi alkaloids from the Indian shrub *Holarrhena antidysenterica* showing antiamoebic activity. Microbial transformation of conessine was investigated in order to improve its pharmacological properties. From the biotransformations of conessine with *Aspergillus niger* A2 and F5, *Aspergillus oryzae* A1, *Aspergillus* sp. F9 and three bacteria 7 -, 7 β -, 9 -, 11 -, and 12 -hydroxyconessines, con-4-enine-3-one, 11-hydroxycon-4-enin- 3-one have been isolated from the fermentation broths.³⁰ Beside the more or less typical hydroxylations of the sterine skeleton the deamination to con-4-enin-3-one is worth mentioning.

Sampangine and the relatives

To cure fungal infections is often more difficult than to deal with bacterial infections because fungi are eucaryotes and, therefore, in their metabolism more similar to humans than bacteria. From this difficulty results a large demand for effective antifungal drugs with little side effects. Especially fungal opportunistic infections like cryptococcosis and candidiasis in AIDS patients which have the burden of the characteristic breakdown of their immune system have intensified the search for new, more effective, and less toxic antifungal agents. One of these promising drugs is sampangine (**31**), isolated from the West African tree *Cleistophathis patens* (*Annonaceae*). To establish the metabolic fate of the new drug biotransformations with fungi were investigated because they can serve as convenient, reliable, and predictive models for mammalian drug metabolism. This method produces significant quantities of metabolites that would be otherwise difficult to obtain from either animals or chemical syntheses. Only seven out of 70 microbial strains tested showed a definite biotransformation.³¹ *Absidia glauca* ATCC 22752, *Cunninghamella elegans* ATCC 9245, *Cunninghamella* sp. NRRL 5695, and *Rhizopus arrhizus* ATCC 11145 produced one compound which was elucidated using spectroscopic methods as the β -glucopyranose conjugate (**33**). However, *Beauveria bassiana* ATCC 7159, *Doratomyces microsporus* ATCC 16225, and *Filobasidiella neoformans* ATCC 10226 produced a somewhat less polar metabolite which was identified as the 4'-O-methyl- β -glucopyranose conjugate (**32**) (Figure 9). Both products were active against *Cryptococcus neoformans* but were inactive against *Candida albicans*. The free glucopyranose conjugate (**33**) was inactive *in vivo* in a mouse model of cryptococcosis, demonstrating once again that promising *in vitro* activities may be useless in an animal model.

Another promising antifungal agent is benzosampangine (**34**), a synthetic analogue of sampangine (**31**).



This compound was also screened for biotransformation products with 54 different strains. It is often observed in biotransformations that a small change in the molecule of the starting material results in new active strains which were previously inactive but also making some of the good converters inactive. In the case of the biotransformation of benzosampangine only some of the strains active in the biotransformation of sampangine could be used. *Absidia glauca* ATCC 22752, *Cunninghamella blakesleeana* ATCC 8688a, *Cunninghamella* sp. NRRL 5695, *Fusarium solani* f. sp. *cucurbitae* CSH #C-5, and *Rhizopogon* sp. ATCC 36060 produced a more polar compound which was identified as the β -glucopyranose conjugate (**35**) analogous to the one

Figure 9: Microbial glycosidation of sampangine (**31**) and benzosampangine (**34**)

found with sampangine.³² In marked contrast to the biotransformation of sampangine the methoxy derivative was not observed.

Cinchona alkaloids and camptothecine

Cinchona alkaloids, especially quinine, are often used as schizonticides against the erythrocytic stage of the malaria parasites and as beverage bitter. Quinidine (**40**) is used as a cardiac depressant or

antiarrhythmic agent. Other major alkaloids are cinchonidine (**44**), the nonmethoxylated form of quinine (**36**), cinchonine, the nonmethoxylated form of quinidine, and dihydroquinidine. A bioconversion of quinone to quinidine by resting cells of *Hansenula anomala* var. *schneggii* has been reported.³³ In the human body quinidine is mainly metabolized to (3*S*)-3-hydroxyquinidine, *O*-desmethylquinidine, and the *N*-oxide. Although microbial transformations are very useful to model the human metabolism only few studies have been reported with these alkaloids. Ten different fungi and two *Streptomyces* spp. formed exclusively the (3*S*)-3-hydroxy compounds from quinine and quinidine.³⁴ In a broad study 96 fungi and 52 bacteria were selected for the screening of biotransformations with quinine, quinidine, dihydroquinidine, cinchonine, and cinchonidine. Quinine (**36**) was oxidized by *Microsporium gypseum* ATCC 11395 at both nitrogen atoms and both mono-*N*-oxides (**37**) and (**39**) were formed (Figure 10).

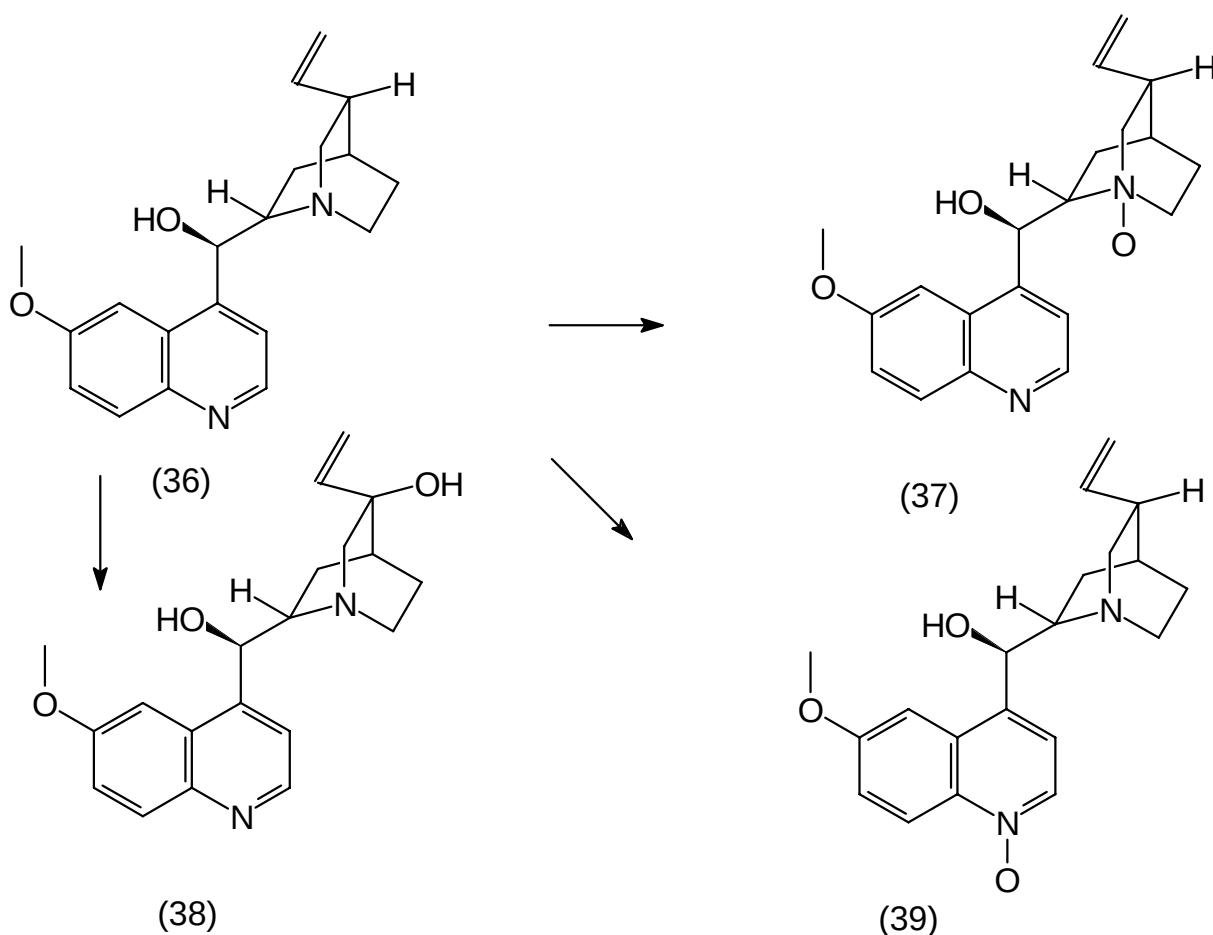


Figure 10: Microbial oxidations of quinine (**36**)

Pellicularia filamentosa DSM 843 oxidized only the tertiary amine to the *N*-oxide and did not attack the nitrogen in the quinoline ring. *Cunninghamella echinulata* ATCC 9244 gave exclusively the 3-hydroxyquinine (**38**), but in low yield. As the genus *Xylaria* is known to produce a variety of different secondary metabolites including alkaloids³⁵ *Xylaria* spp. were included in the screening in an attempt to use some of these biosynthetic enzymes. Quinidine (**40**) was transformed by *Xylaria digitata* ATCC 14962 and *Mycobacterium smegmatis* DSM 43061 to the aliphatic *N*-oxide (**41**). The same reaction occurred with

the dihydro derivative (**42**) yielding (**43**) but here *Microsporium gypseum* ATCC 11395 was the most active strain (Figure 11). No strain could be found which could transform cinchonine.

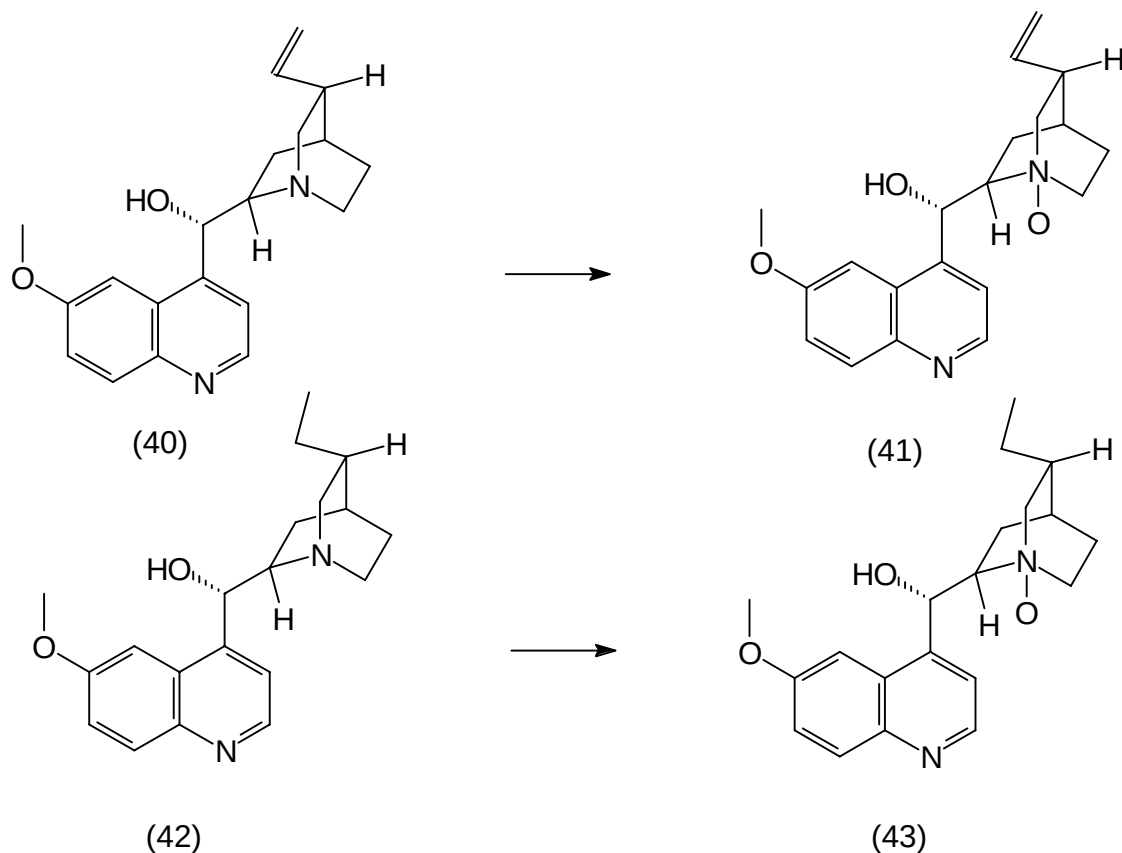


Figure 11: Biotransformations of quinidine (**40**) and dihydroquinidine (**42**)

Cinchonidine (**44**) was oxidized at the tertiary amine to the *N*-oxide (**45**) by *Pellicularia filamentosa* DSM 843 which showed the same reaction with quinine. *Rhizopus arrhizus* strains ATCC 10260 and ATCC 11145 hydroxylated cinchonidine and formed 3-hydroxycinchonidine (**46**) (Figure 12).³⁶ Interestingly, only fungi showed the hydroxylation reaction with these alkaloids and only *Microsporium gypseum* formed the aromatic *N*-oxide. Most yields were between 1-9% which is quite good for biotransformations of alkaloids. Although a number of different media and cultivation procedures were tested no strain could be found which attacked all five alkaloids.

Tremorgenic and neurotrophic intoxication has been reported in humans as a result of the ingestion of *Aspergillus fumigatus*, growing on rice and home-made “miso”. A number of indol alkaloids, called fumitremorgins, have been described from this fungus. 12,13-Dihydroxyfumitremorgin C is a higher oxidized member of this family and was first isolated from *Aspergillus fumigatus* DSM 790.³⁷ In an attempt to exploit the capability of this strain to produce alkaloids it was included in a screen for the microbial oxidation of reserpine derivatives. Chemical synthesis starting from reserpine acid lactone gave the pivalate (**47**) in good yield. This pivalate served as starting material for a chemical synthesis of camptothecin, an antitumor agent, but the functionalization of the D-ring to lead to the unsaturated system was difficult. Therefore, the intermediate was subjected to screening with some strains selected for their

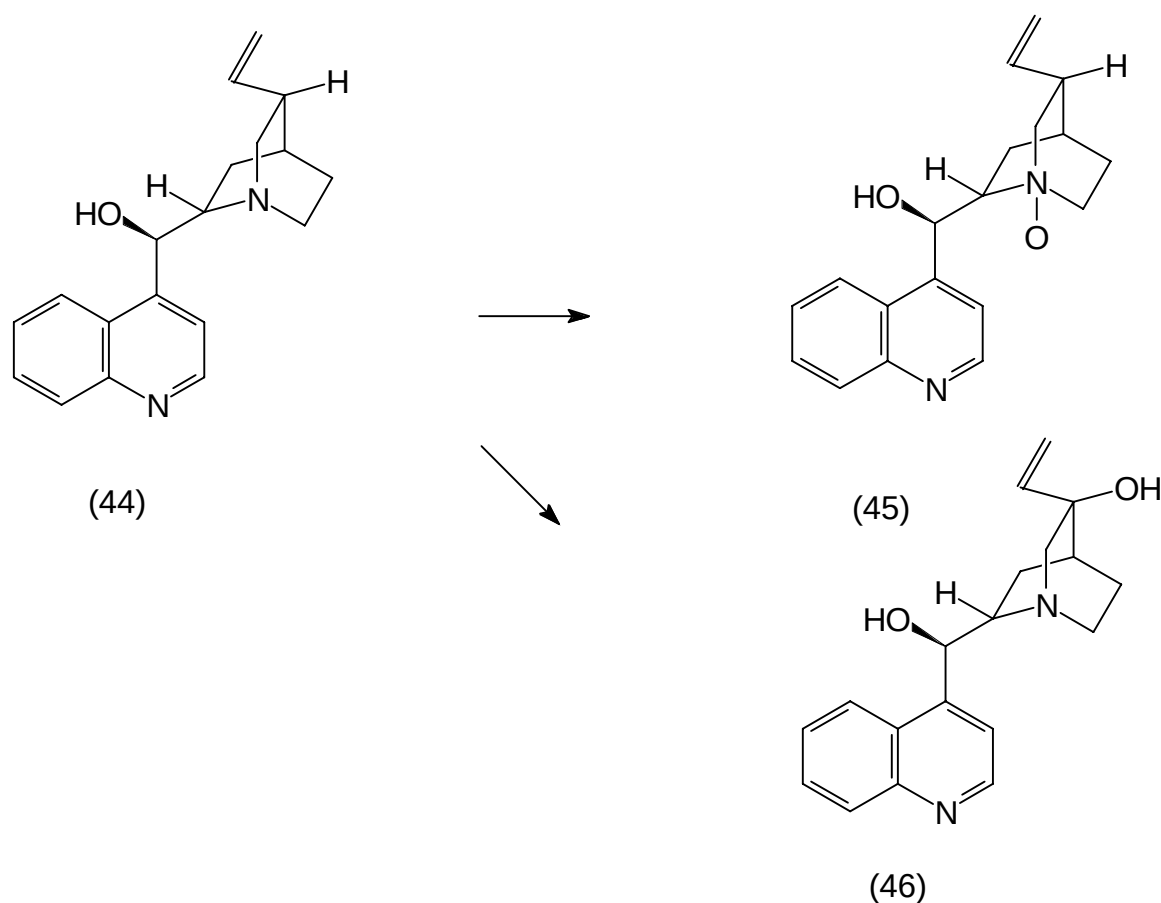


Figure 12: Microbial oxidation of cinchonidine (**44**)

high activity in microbial hydroxylation. The most active strains were *Streptomyces californicus* DSM 40801 and *Mucor circinelloides* CBS 394.68 but they only saponified the pivalate (**47**) forming the chinolone derivative (**48**).³⁸ To prevent the cleavage of the ester moiety more stable derivatives were synthesized. The methoxy- and the chloro derivative (**49**) were again screened with various strains. *Aspergillus fumigatus* DSM 790 and *Pseudomonas aceris* DSM 50257 isomerized some of the substrate at C-3 to (**50**) and formed the C-ring lactam (**51**) with a hydroxylation at the tertiary carbon at the same ring. This metabolite fulfilled the requirement for the functionalization of the D-ring but the yields were below 1%. A better yield with essential the same reaction gave *Aspergillus ochraceus* NRRL 405, *Aspergillus sclerotiorum* DSM 63357, and again *Pseudomonas aceris* DSM 50257 with the chloro-derivative ((**49**), R = Cl) (Figure 13). The yield of the hydroxylated metabolite was almost 10% with the *Pseudomonas aceris* strain.³⁹ In this example the alkaloid producing *Aspergillus fumigatus* strain was active in the biotransformation of the substrate and it was possible to support chemical synthesis with functionalized synthons.

Catharanthus alkaloids

Alkaloids from *Catharanthus roseus* (Apocynaceae) are well known for their antitumor activities and

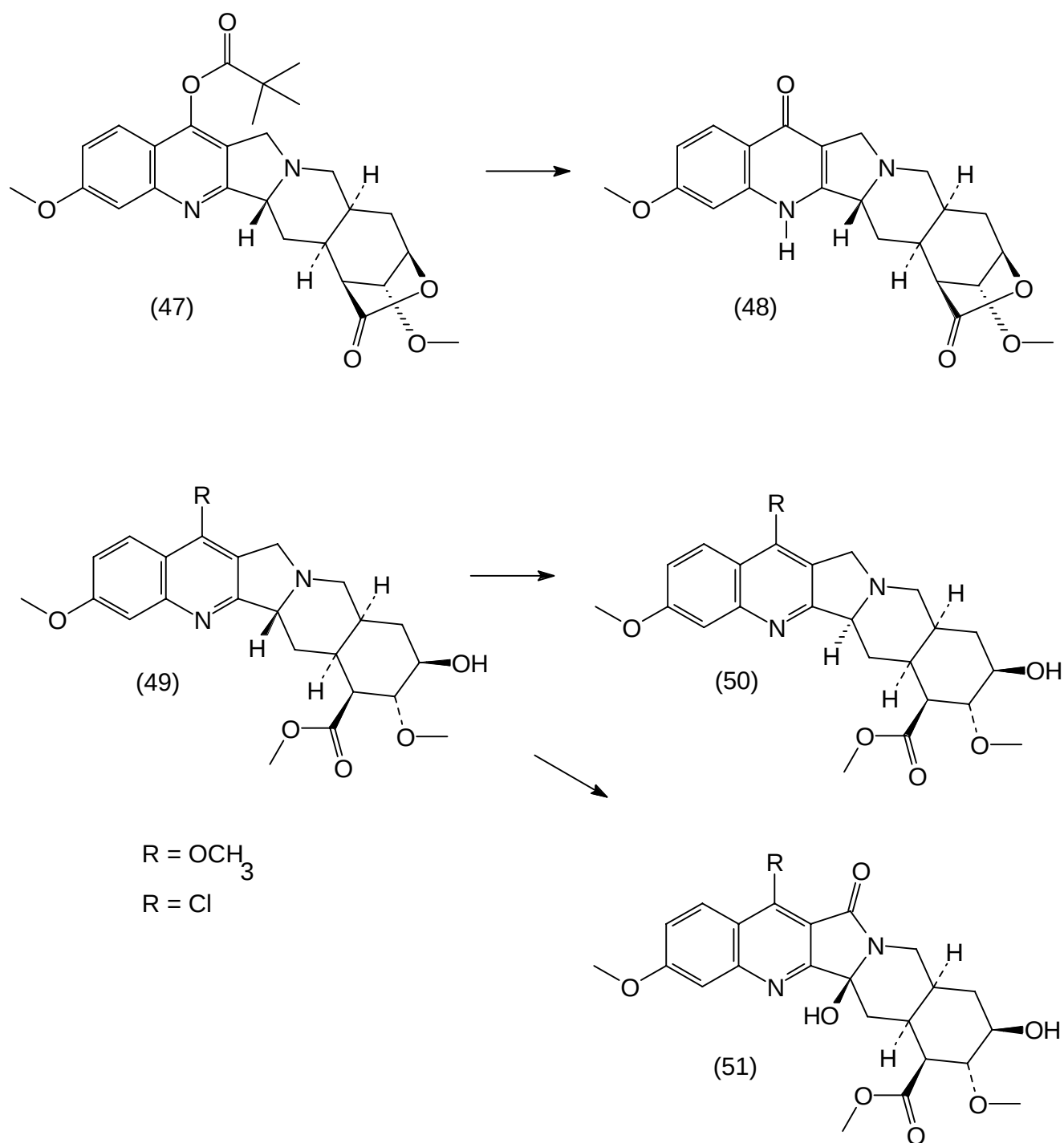


Figure 13: Biotransformations of reserpine derivatives

vincristine and vinblastine are used clinically as antineoplastic agents. However, little is known about their mode of action and their structure-activity relationships. Again, biotransformations were tried to shed more light on these questions. The most abundant dimeric antitumor alkaloid of this plant is leurosine (52) and it was used in a study to elucidate the biotransformation products probably occurring in living systems. A number of strains were able to metabolize the alkaloid. One of these metabolites formed

by *Streptomyces* was identified as 10'-hydroxyleurosine (**54**).⁴⁰ Biotransformation of leurosine with *Aspergillus terricola* UI-AT or *Aspergillus ochraceus* NRRL 398 resulted in the formation of a slightly less polar product which was identified as an epimeric mixture of 5'-hydroxyleurosines (**53**) (Figure 14). The same product was obtained from reactions with copper oxidase enzyme systems, benzoquinone and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone indicating that this position at the leurosine molecule is susceptible to oxidations.⁴¹

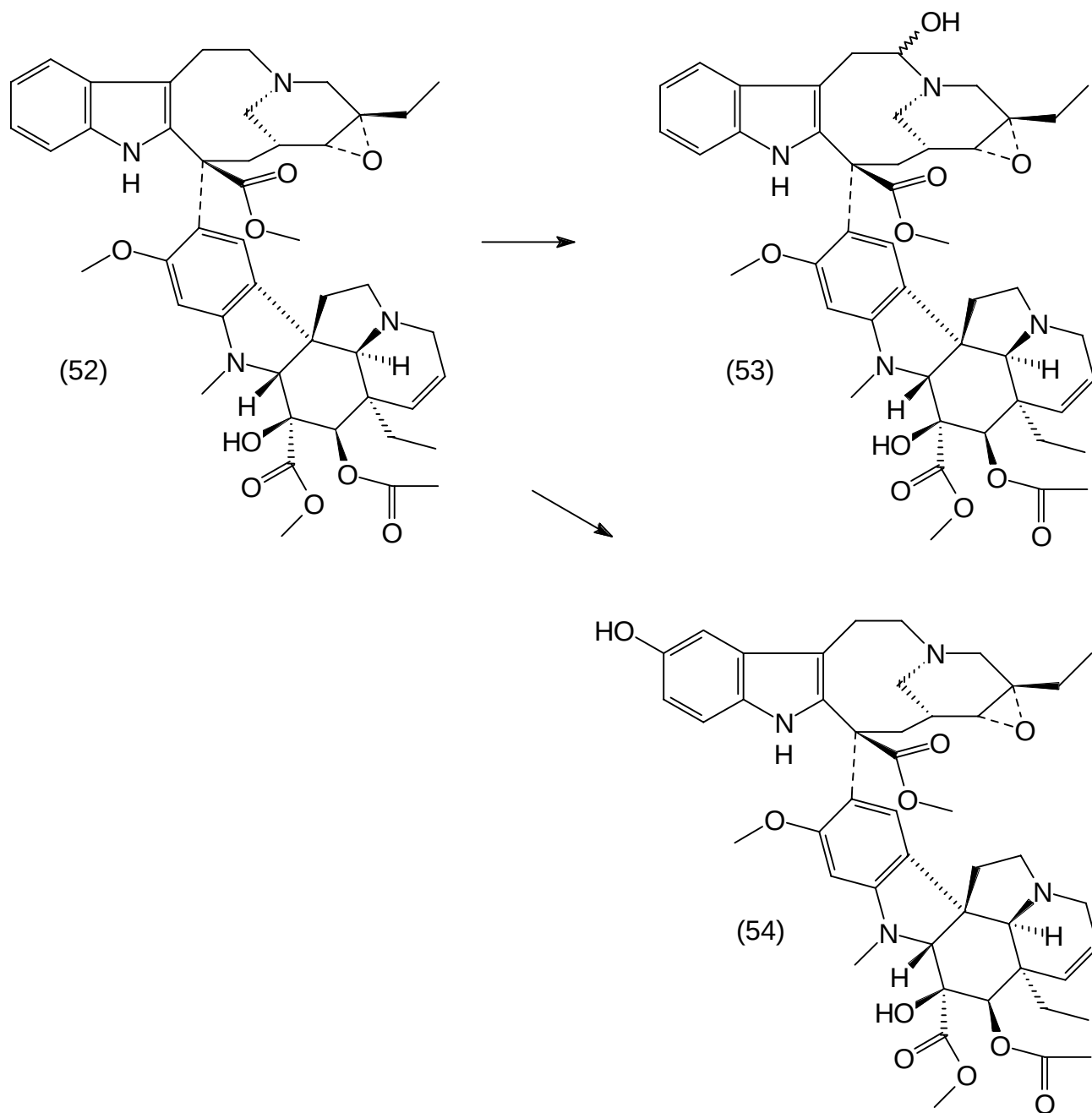


Figure 14: Microbial oxidation of leurosine

Studies with *Streptomyces griseus* revealed a novel *Vinca* (*Catharanthus*) alkaloid metabolic pathway

involving enamine and iminium intermediates. The use of other microbial enzymes from fungi, the laccases, enabled the identification of free radical pathways of oxidation for this group of compounds. The existence of chemically reactive intermediates, imines, carbinolamines and enamines, has been demonstrated. The compounds may have a role in the cytostatic activity of *Catharanthus* alkaloids.⁴² The dimeric *Catharanthus* alkaloids are recalcitrant to oxidations catalyzed by monoamine oxidases and to both normal and induced P-450 rat microsomal preparations. However, the *Catharanthus* alkaloids appear to be selective reversible inhibitors of monoamine oxidase B.⁴³ Thus, biotransformations of *Catharanthus* alkaloids together with enzymatic studies form another example where biotransformations contributed to an improved understanding of alkaloid interactions in the human body.

Benzylisoquinoline and related alkaloids

Laudanosine (55) is a minor opium alkaloid possessing the benzylisoquinoline skeleton. Its biotransformation with a strain of *Pseudomonas putida*, isolated from a biological waste water treatment plant by an enrichment in the presence of laudanosine as the sole carbon and energy source gave four different compounds. Only the structure of two of these compounds could be elucidated and *O*-methylcoripalline (56) and the new metabolite 9-keto-11-hydroxy-13-demethylaudanosine (57) was identified⁴⁴ (Figure 15). This represents one of the rare examples where a hydroxylation of the aromatic ring together

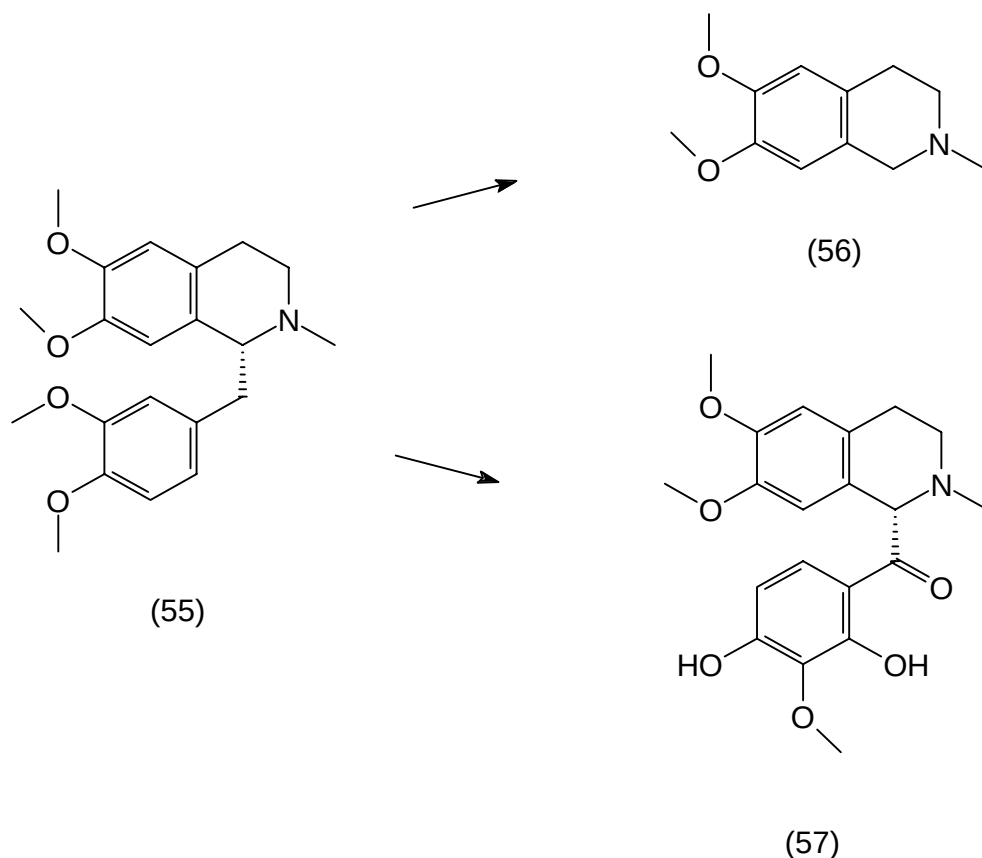
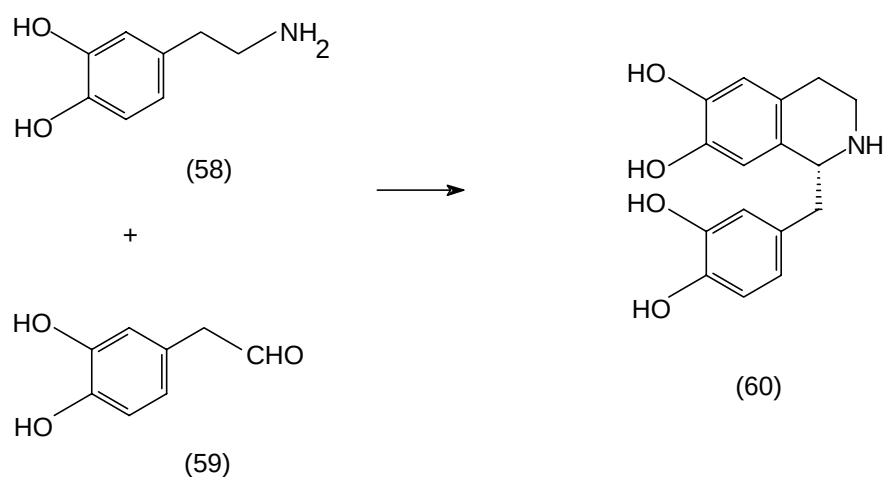


Figure 15: Biotransformation of laudanosine

with the oxidation of the benzylic methylene group occurred.



In an interesting approach to close the gap between biotransformation and biosynthesis Hoover *et al.* used *Aspergillus niger* ATCC 10864 to synthesize (R,S)-norlaudanosoline (60) from dopamine (58). In this reaction dopamine is oxidized by monoamine oxidase to 3,4-dihydroxyphenylacetaldehyde (59) which reacts spontaneously in a Pictet-Spengler reaction to

Figure 16: Biomimetic biotransformation using *Aspergillus niger*

(R,S)-norlaudanosoline⁴⁵ (Figure 16).

Papaveraldine (61) is one of the minor benzyloquinoline alkaloids of *Papaver somniferum* and shows antispasmodic and protective activity against histamine-induced bronchospasm but no analgesic activity.

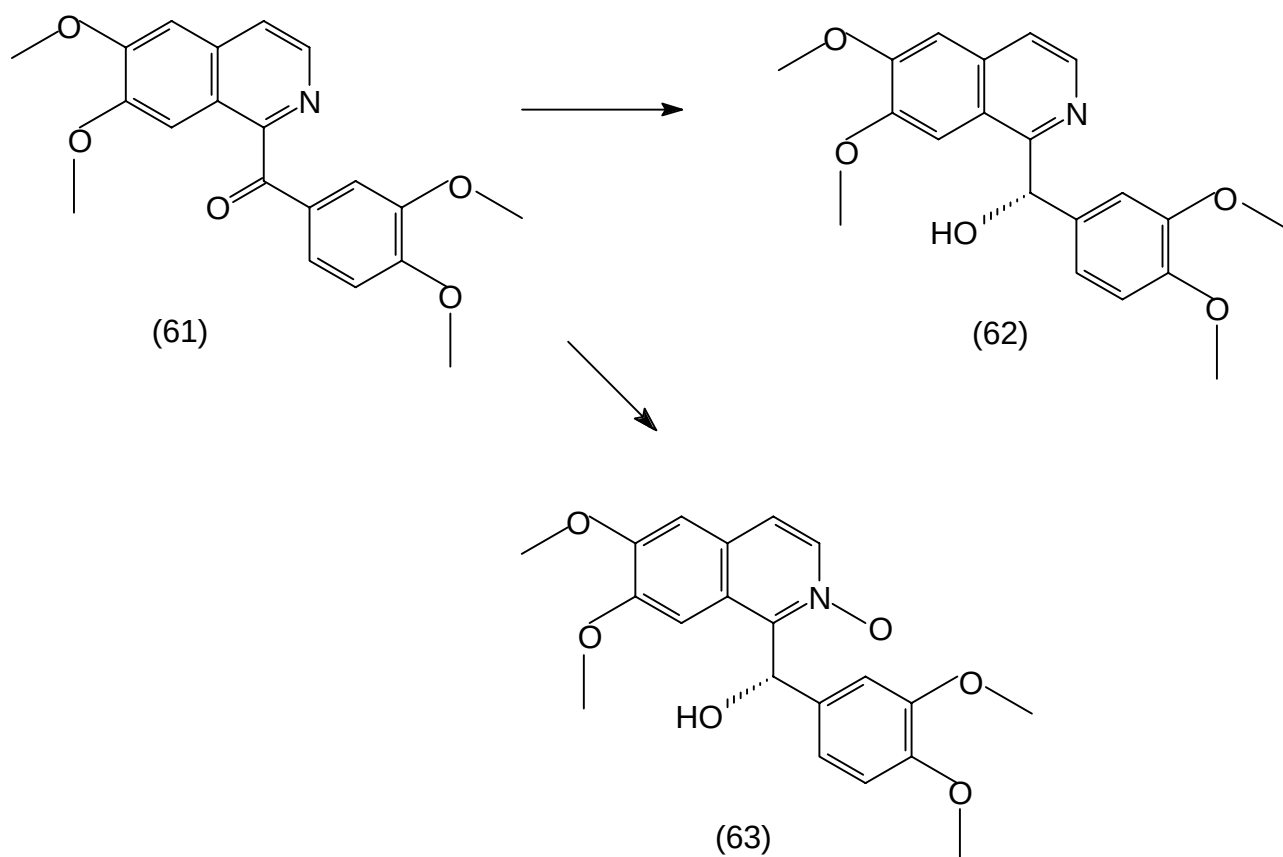


Figure 17: Microbial oxidations of papaveraldine

It is one of the active ingredients of a hair tonic preparation which promotes melanin formation for grey hair. Twenty-eight growing cultures were screened for their ability to oxidize papaveraldine but only few showed transformation products. From the biotransformation with *Mucor ramannianus* 1839 two products were isolated and their structures elucidated (Figure 17). The fungus reduced papaveraldine stereoselectively to (S)-papaverinol (**62**) which was further oxidized to the N-oxide (**63**).⁴⁶ (S)-Papaverinol showed no antimicrobial, antiviral, or antimalarial activity. The biotransformation products observed were similar to those found for papaverine with plant tissue cultures consistent with the general observation that fungi have a metabolism related to higher organisms.

A stereoselective oxidation of glaucine (**64**)/(**66**) and 7-methylglaucine (**67**)/(**69**) by fungi had been observed and details of the stereochemical course of the reaction had been reported.⁴⁷ The naturally occurring (S)-(+)-glaucine (**64**) is converted by *Fusarium solani* ATCC 12823 to dehydroglaucine (**65**) while the unnatural enantiomer (R)-(-)-glaucine (**66**) is converted to the same product by *Aspergillus flavipes* ATCC 1030.⁴⁸ The stereochemical course of this reaction was elucidated using the conversion of the 7-methyl derivative (Figure 18). Here the 6aS,7S stereoisomer (**67**) is stereoselectively oxidized by *F.*

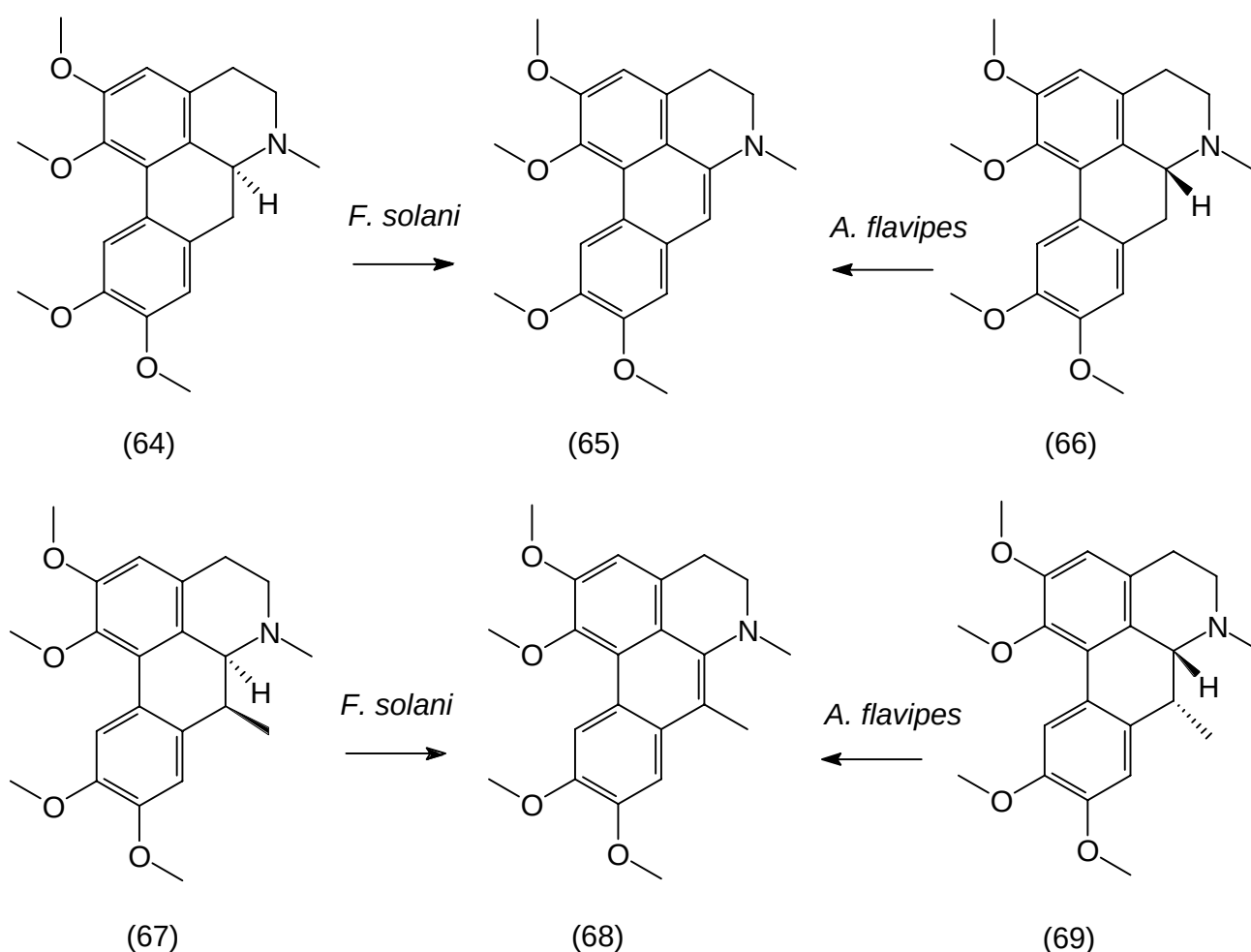


Figure 18: Stereoselectivity of microbial dehydrogenations

solani yielding the dehydro derivative (**68**) while the 6a*R*,7*R* stereoisomer (**69**) is attacked by *A. flavipes* resulting in the same product as that of *F. solani*. This mechanism allows a resolution of a racemic mixture of this alkaloid by a microbial *cis*-elimination of hydrogen because the 6a*S*,7*R*- and the 6a*R*,7*S*-isomers are not attacked by both fungi.

Galanthamine and precursors

Galanthamin is an alkaloid which is used for the posttreatment of polyomyelitis. Its production from the plant *Galanthus nivalis* is limited by climatic factors and by very low yields. Therefore, chemical synthesis and biotransformations of suitable precursors were undertaken. The ring closure of the norbelladine precursor to narwedine is achieved in the biosynthesis via phenol oxidation. In search of a similar microbial reaction a large number of strains were screened with *para,meta'*-*O*-dibenzyl-*N-para'*-dimethyl-norbelladine (**70**) as substrate. 377 strains, mainly fungi and *Streptomyces* spp. were screened, 108 showed only low yields, and 16 were selected for the structure elucidation of the metabolites. The norbelladine derivative (**70**) was oxidized to the *N*-oxide (**71**) by a number of fungi. The main reaction of the biotransformations was the cleavage of the benzyl group at the *para*-substituted aromatic ring to the phenol (**72**) which was formed by *Glomerella cingulata* DSM 1166 in more than 10% yield. Most of the other metabolites were derived from this product. One reaction was the cleavage of the methyl group from the methoxy ether yielding **73** or the cleavage of the second benzyl group to give **74**. The second *N*-oxide found was the norbelladine derivative (**75**) lacking the *para*-benzyl group. Only *Septomyxa affinis* ATCC 6737 gave more than 10% yield of this product which was optically active. The same strain was the only one which attacked *ortho-bromo-para,meta'*-*O*-dibenzyl-*N-para'*-dimethylnorbelladine, forming again the *para*-phenol derivative. From **72** the *N*-methyl was cleaved to yield **76**. Products (**77**) and (**78**) gave the remaining metabolites so that all possible *N*-cleavage products were formed (Figure 19).

Because no sign for microbial phenoloxidation was found further substrates were synthesized and tested. The benzamide (**79**) was transformed by *Nocardia lurida* DSM 43134 leading again to the cleavage of the benzyl moiety at the *para*-substituted aromatic ring in **83**. Two *N*-demethylations were detected, one was **81** derived from **83** and the other one was the isomer (**80**). The most interesting product found was the oxygenation product (**82**) where the benzyl moiety at the *para*-substituted aromatic ring was hydroxylated in *para*-position (Figure 20).⁴⁹ With the strains tested it was possible to cleave one benzyl moiety selectively leaving the other one intact. *Septomyxa affinis* formed the chiral *N*-oxide (**75**) which may be suitable for electrochemical phenoloxidation and hopefully its chirality can direct the ring closure preferentially or exclusively to the desired stereoisomer of narwedine.

In an alternative approach a narwedine type enone (**84**) was subject of an extensive screen for microbial transformations. *Septomyxa affinis* DSM 6737 gave in the course of the microbial reduction the (-)-epigalanthamine derivative (**86**) in 50 % yield. The X-Ray analysis revealed its absolute configuration as

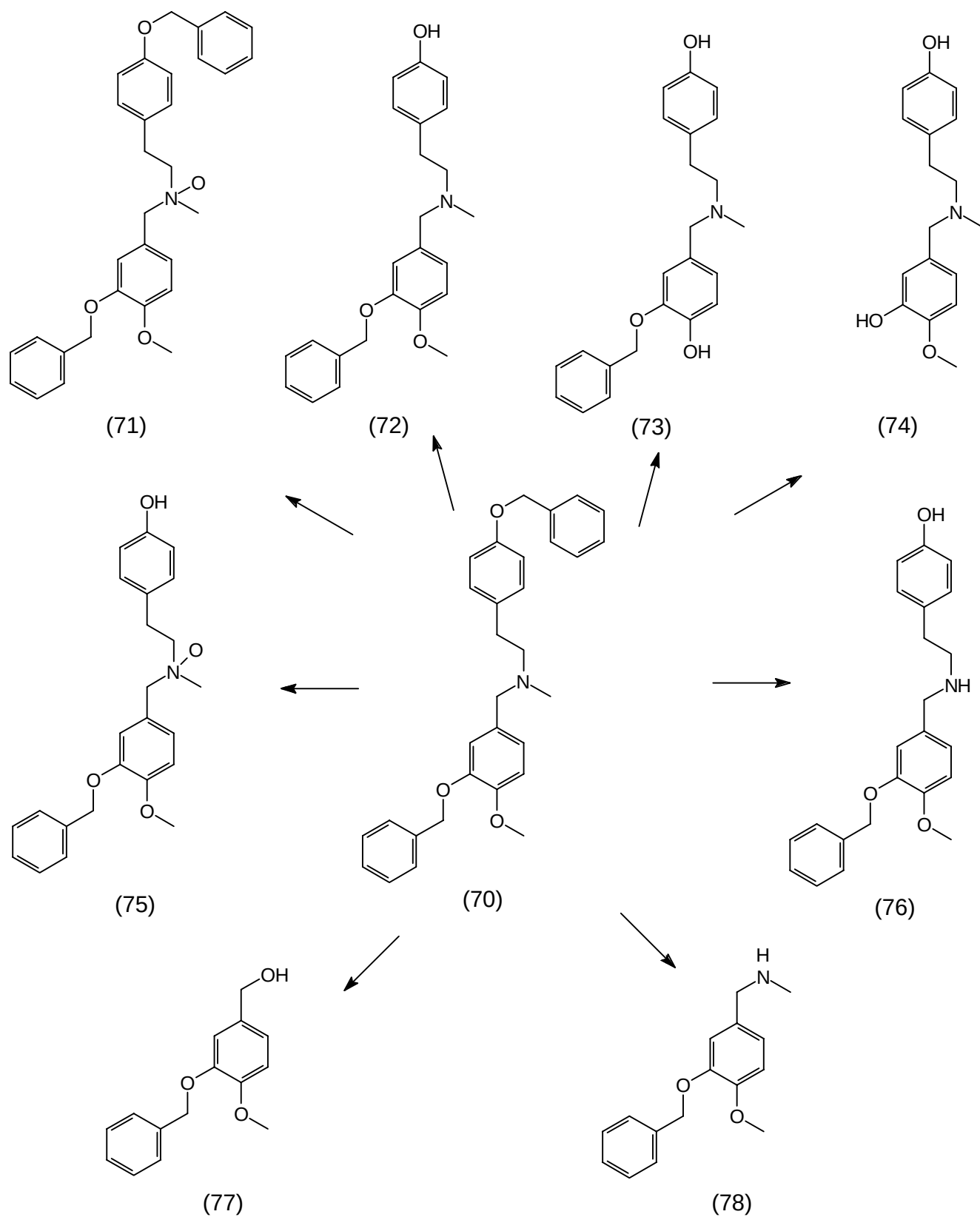


Figure 19: Various biotransformation products of the norbelladine derivative (70)

1'R,2'S,4'S-epigalanthamine. *Nematospora corylii* CBS 26.08 formed the racemic galanthamine derivative

(85). *Ashbya gossypii* IFO 1355 yielded optical pure epigalanthamine and racemic galanthamine derivatives in a 1:2 ratio. Finally, *Nocardia alba* DSM 43130 and *Bacillus cereus* DSM 508 showed a

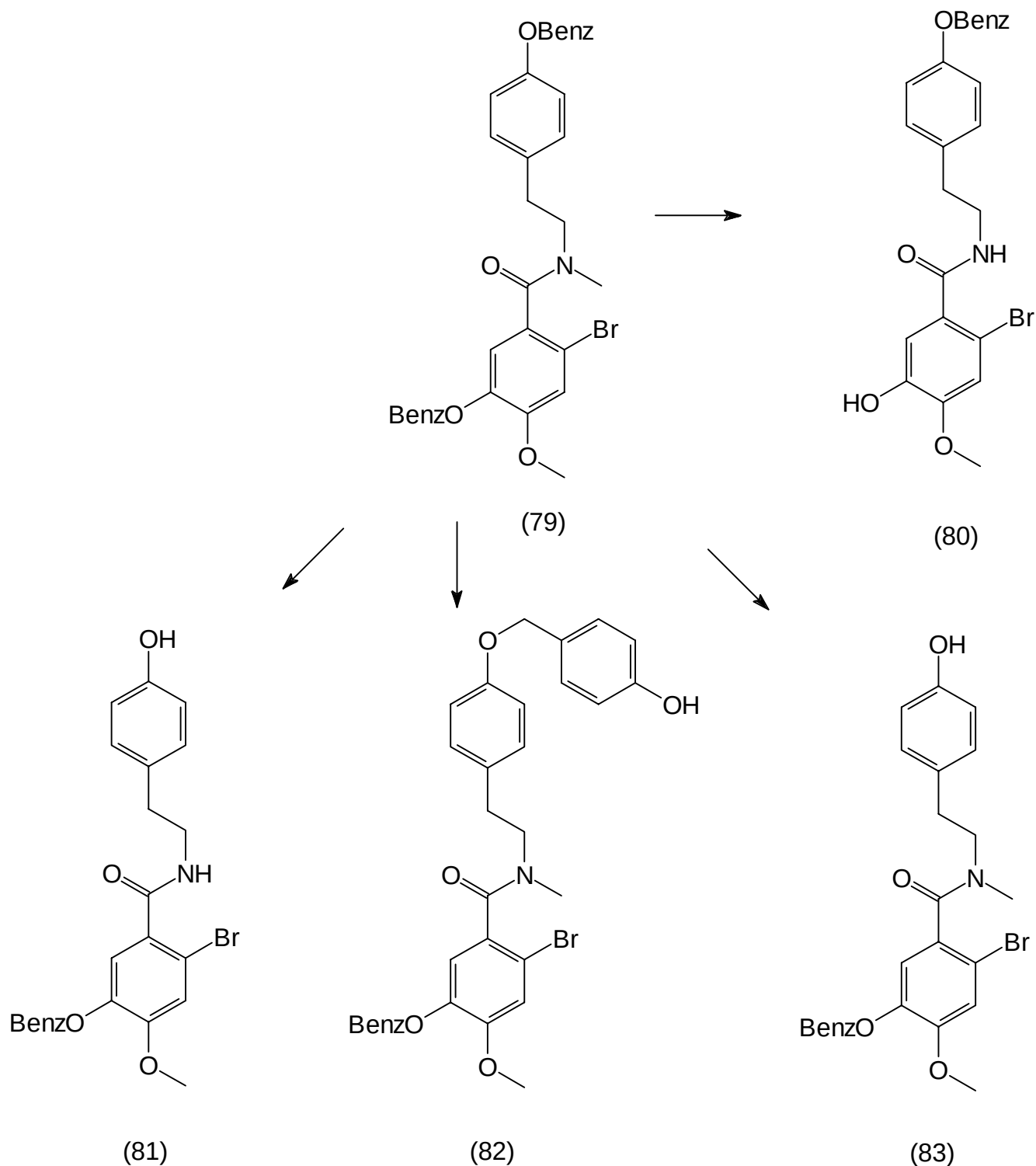
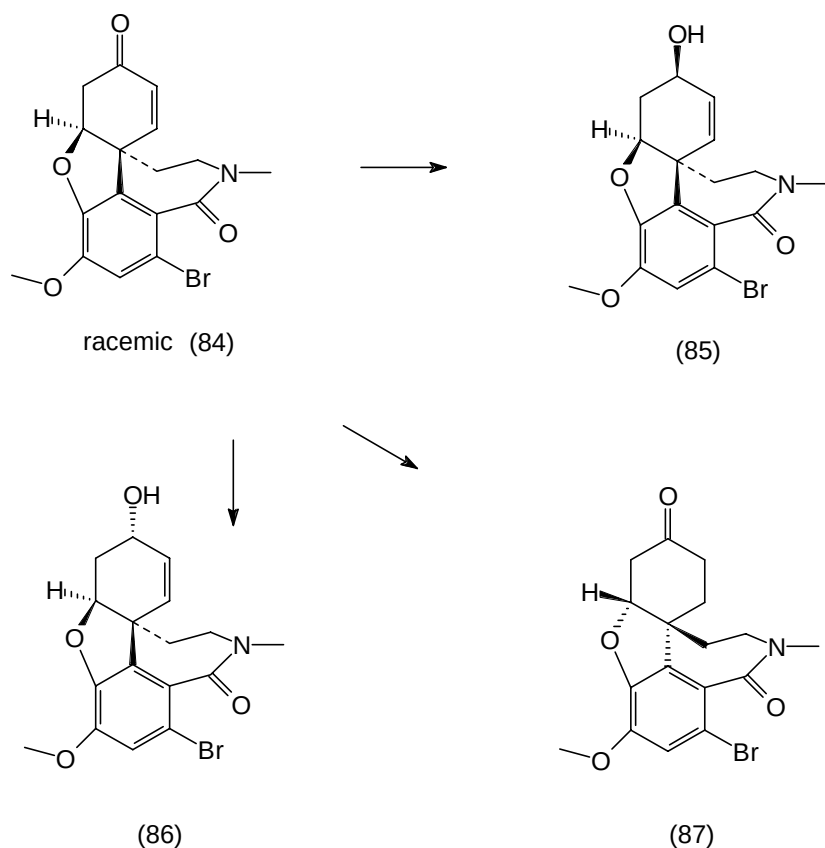


Figure 20: Biotransformation products of the bromonorbelladine derivative (79)

different reaction type forming optically pure (+)-lycoramine bromide (87) by hydrogenating the double bond of the unsaturated carbonyl group⁵⁰ (Figure 21). The remaining starting material was still racemic in

the biotransformations with the two bacteria. The formation of either optical or racemic products can be explained by strict stereospecificity of the enzyme present in *Septomyxa affinis*, *Nocardia alba*, and *Bacillus cereus*, while that of *Nematospora corylii* did only possess such a specificity with respect to the carbonyl group. The formation of optically pure products and the presence of racemic starting material at

the same time were due to the fact that the starting material displayed an equilibrium by opening to the achiral dienone and closing again to the narwedine type enone under racemization. This equilibrium prevented the enrichment of one enantiomer of the starting material.



Biotransformation of miscellaneous alkaloids

Caffeine (88) is a mildly stimulating drug from coffee and tea and is available in large quantities from decaffeinated coffee. For many people consumption of caffeine leads to a supersensitive reactions. Therefore, in some remedies caffeine is replaced by milder acting substances, like theobromine and theophylline. Monomethylxanthines

are interesting alternatives as well. They are not natural products and the chemical synthesis is difficult.

The biotransformation of caffeine is an alternative for the production of monomethylxanthines. *Pseudomonas putida* WS was able to grow on caffeine as the sole source of carbon and nitrogen. Mutants

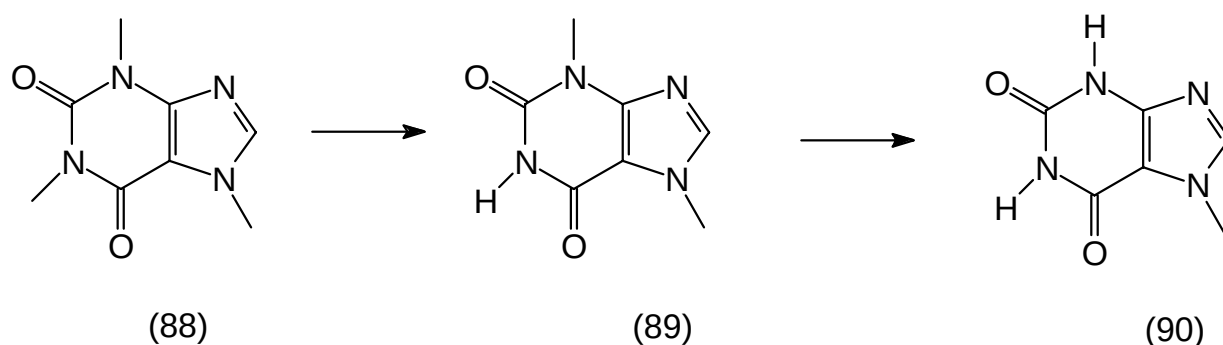


Figure 22: Microbial demethylations of caffeine

of this strain converted caffeine (**88**) to theobromine (**89**) and heteroxanthine (**90**) in resting cell experiments (Figure 22). Growing the mutant H8 on diluted complex media led to formation of heteroxanthine in a yield of 50% based on the amount of caffeine.⁵¹

From soil *Agrobacterium* sp. 19-1 was isolated which was able to oxidize pseudo- β -D-glucose at the 2-hydroxy group and further

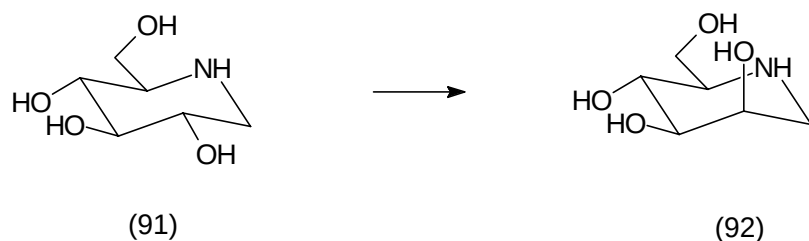


Figure 23: Microbial isomerization of 1-desoxynojirimycin

transformed this substrate. 1-Deoxynojirimycin (**91**) is transformed by this strain to 1-deoxymannojirimycin (**92**),⁵² a potent mannosidase inhibitor (Figure 23). The course of this epimerization probably went *via* the formation of the 2-carbonyl compound which is then reduced to the product.

For pharmaceutical studies it is often essential to produce some of the metabolites of a drug. As mentioned above biotransformations have been used to produce such metabolites and a study has been performed on the biotransformation of *N*-(4-chlorophenyl)benzothiazolone (**93**). Like numerous derivatives of benzothiazolone this compound shows a moderate tuberculostatic effect and in addition bacteriostatic and fungistatic properties. In mammalian systems this compound is metabolized to 2-thiomethyl-*N*-(4-chlorophenyl)benzamide (**97**) which is further enzymatically hydroxylized at the phenyl ring. To produce more of these metabolites and to compare the mammalian and the microbial metabolism of *N*-(4-chlorophenyl)benzothiazolone a screen with more than 100 strains was started. No fungi showed a metabolism of the substrate. Out of the active strains the bacteria *Arthrobacter oxydans* DSM 20119, *Bacillus lentus* ATCC 13805, *Corynebacterium fascians* DSM 20131, *Streptomyces alboflavus* DSM 40761, and *S. griseus* ATCC 21897 were selected for preparative fermentation and elucidation of the transformation products. Only *Streptomyces griseus* formed the sulfhydryl metabolite by reductive opening of the heterocyclic ring. All strains formed the 2-thiomethyl derivative (**97**) probably formed by the methylation of the primarily formed sulfhydryl product (**95**). This product, also observed in the mammalian metabolism of the drug, was the only metabolite found in the biotransformation with *Corynebacterium fascians* DSM 20131 *Bacillus lentus* ATCC 13805, *Streptomyces alboflavus* DSM 40761, and *S. griseus* ATCC 21897 oxidized this product subsequently forming the 2-sulfoxymethyl- (**94**) and the 2-sulfonylmethyl-*N*-(4-chlorophenyl)benzamides (**96**)⁵³ (Figure 24). Phenols resulting from the oxidation of one of the aromatic rings were not found. This underlines the difficulties if bacteria phylogenetically quite different from mammals were used for biotransformations. The sulfoxide was optically inactive, i. e. racemic, therefore, from this observation it could not be excluded that these oxidative reactions were based on autoxidations. Such a process, however, could be excluded by the observation that only some of the tested strains were able to oxidize the thiomethylmetabolite. This

confirms that the oxidation of the thioether is catalyzed by enzymes otherwise it would be found in all culture broths containing the 2-thiomethyl metabolite.

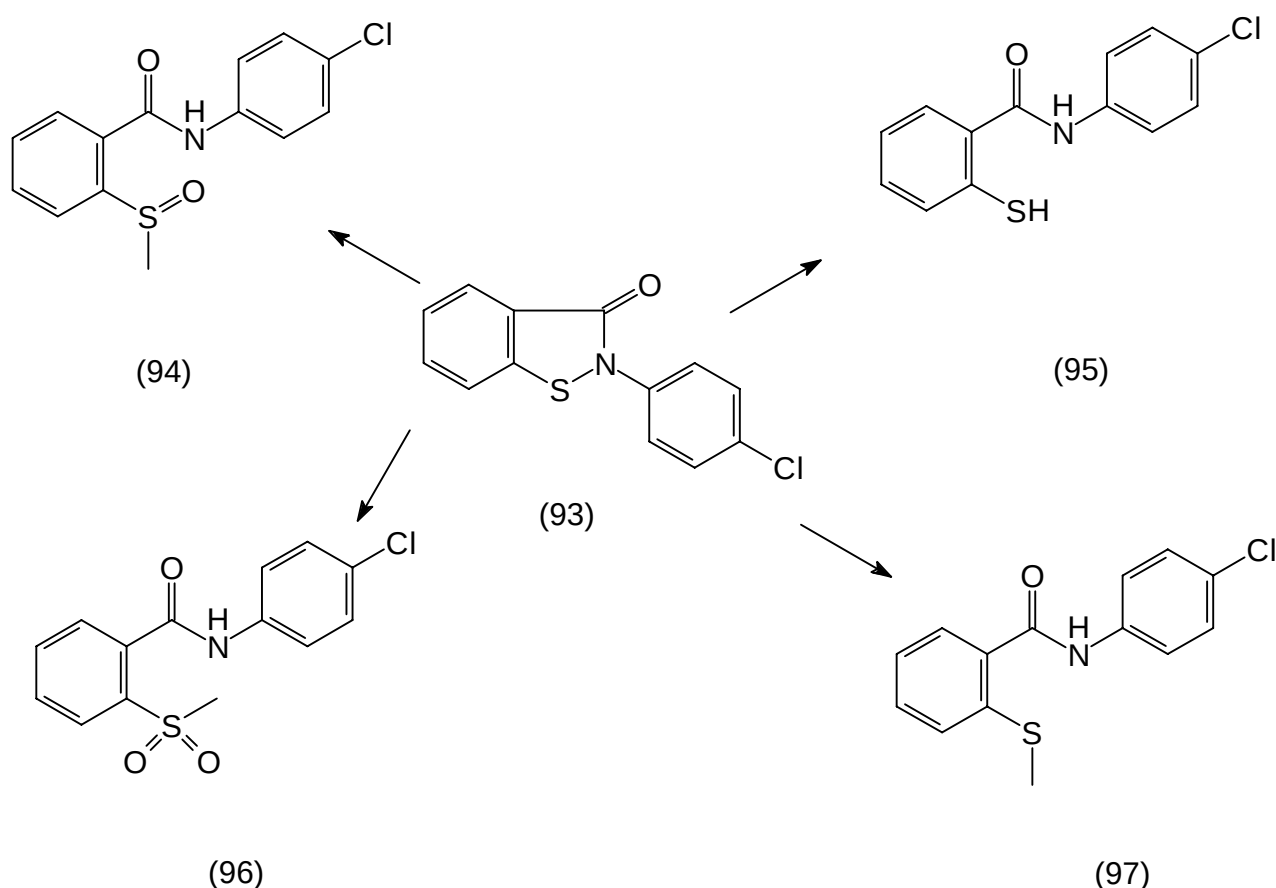


Figure 24: Biotransformation of *N*-(4-chlorophenyl)benzothiazolone

Species of the fungal genus *Curvularia* are known for their production of secondary metabolites including alkaloids like curvupallides⁵⁴ or spirostaphylotrichines.⁵⁵ *Curvularia fallax* formed 4-hydroxy- (98), 4-hydroxy-3-(3'-methyl-2-butenyl)- (99), 4-hydroxy-3-(3'-methyl-3'-buten-1'-inyl)benzoic acids (100), anofinic acid (101) and some derivatives thereof. It was assumed that 4-hydroxybenzoic acid is the precursor for the prenylated secondary metabolites and attempts were made to replace it by other aromatic acids. It turned out that the structural requirements for the prenylating enzymes were rather strict and 4-hydroxybenzoic acid could be replaced only by a few of the tested acids. One of the acids which could successfully replace 4-hydroxybenzoic acid was 4-aminobenzoic acid (102) (Figure 25). In the biotransformation only 4-amino-3-(3'-methyl-2-butenyl)benzoic acid (103) could be isolated and neither the butyne derivative nor the anofinic analogs could be detected.⁵⁶ The formation of 4-amino-3-prenylbenzoic acid is interesting both with regard to the biosynthesis of the metabolites and as a starting material for derivatives of novobiocin which contains in the natural antibiotic the 4-hydroxy-3-prenyl-

benzoic acid.

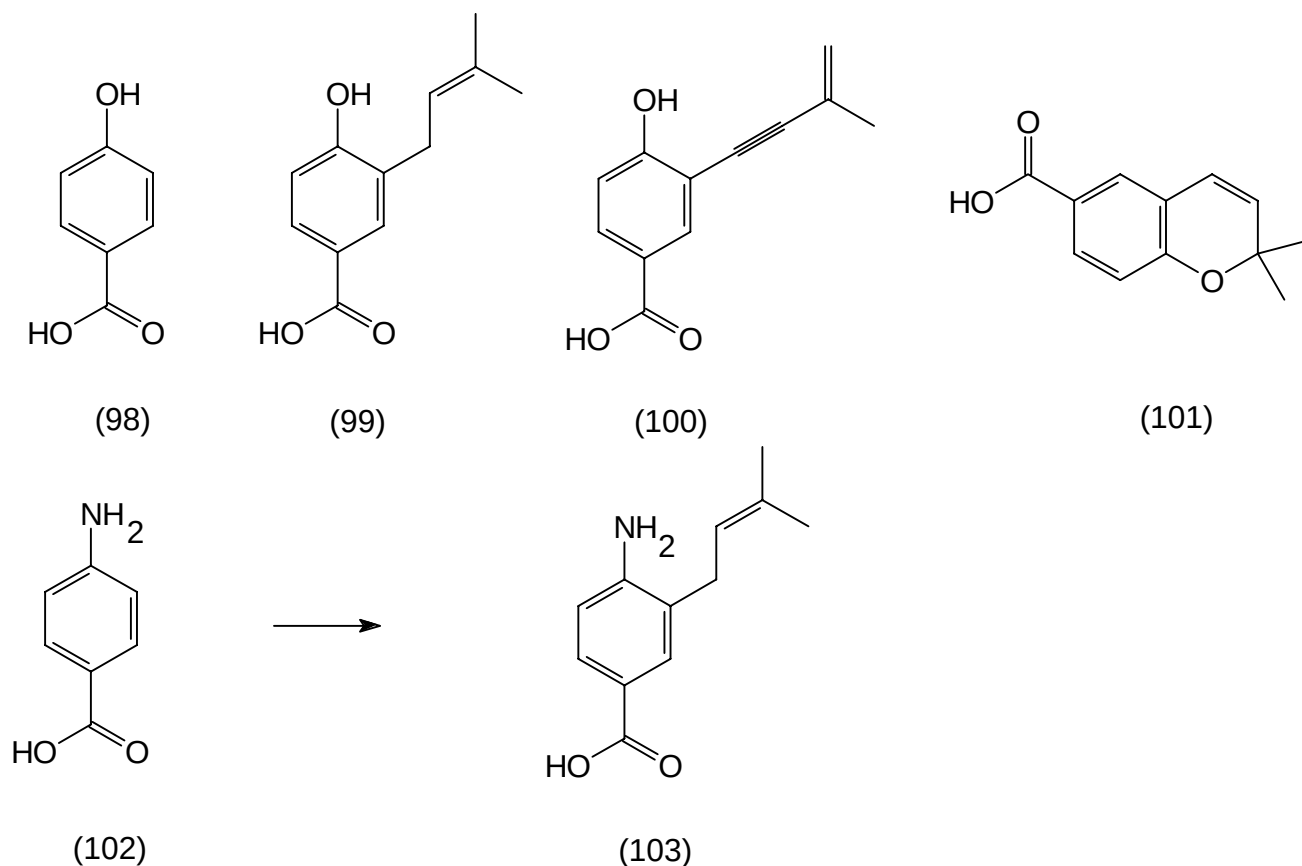


Figure 25: Secondary metabolites of *Curvularia fallax* and microbial prenylation of 4-aminobenzoic acid by the same strain

Fungal modification of selected drugs gave products with improved therapeutical properties. Lucanthone (**104**) was oxidized by *Aspergillus sclerotiorum* to hycanthone (**105**) (Figure 26) which is more active than lucanthone itself as a schistosomicidal agent in man when administered orally or intraperitoneally.⁵⁷

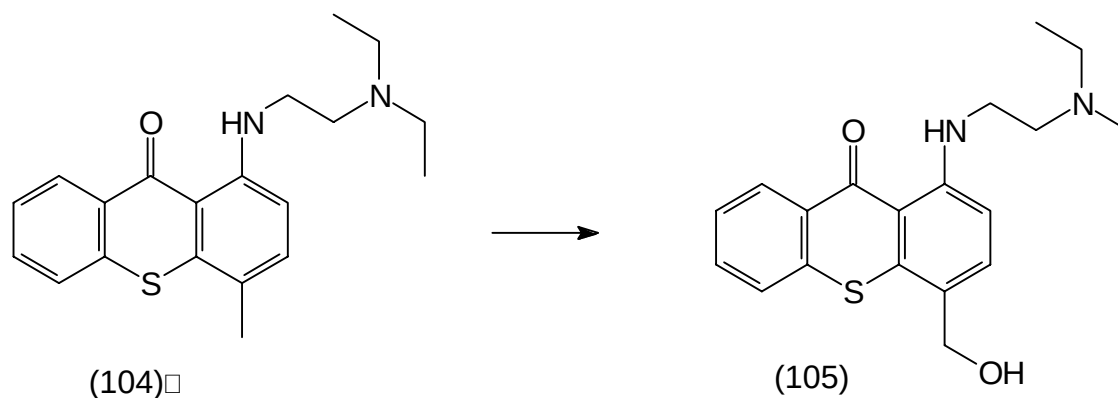


Figure 26: Microbial oxidation of lucanthone

OUTLOOK AND POSSIBLE FUTURE DEVELOPMENTS

The biotransformations of a number of very different alkaloids have been reported using a wide range of different microorganisms, both fungi and bacteria. Until recently the best strains were identified by a random screening of easy to handle microorganisms. However, in a systematic search for microorganisms with related biotransformation capabilities it was found that biotransformation reactions are often coupled to the phylogenetic position of the species.⁵⁸ This has been successfully demonstrated for the biotransformation of monoterpenoids⁵⁹ and sesquiterpenoids.⁶⁰ From these studies an approach has been developed demanding for a given biotransformation first a pre-screen with strains covering the whole width of phylogenetic lineages. Such a pre-screen will identify the most active phylogenetic groups of microorganisms and in the second step a screen with strains selected from these active groups will be executed.⁶¹ The discovery that the different lineages of bacteria and fungi show different biotransformation preferences as well can be used to speed the process of screening. Some strains produce alkaloids as secondary metabolites and the substrate spectra of the involved enzymes can be exploited for epoxidation reactions but usually the ranges are narrow.

Even with a large collection of microbes our access to the biodiversity available in the environment is very limited. The reason for this is the fact that most bacteria and probably also a number of fungi presently cannot be cultivated as pure cultures in the laboratory.⁶² The bacteria which can be grown as pure cultures are not randomly distributed and whole phyla withstand completely our cultivation efforts. In such a situation the diversity and biotransformation potential available in the environment cannot be assessed but is probably very large. To get our hands on the diversity of the unculturable microorganisms a number of approaches have been developed. In the area of genome sequencing the field is wide open to use the genetic information of these strains. Promising approaches are the cloning of gene clusters into cosmid, bacterial or yeast artificial chromosomes (BAC or YAC) libraries and the screening of these libraries by high through-put screens for activities of interest. These developments are only at an early stage but have already brought new antibiotics.

The main problem of biotransformation, which is to find the strain with the fastest reaction, highest yields and highest tolerated substrate concentrations, can also be overcome by the use of genetically engineered microorganisms. The use of plasmids can usually not be recommended because of the instability often observed with such systems. Integration of the gene(s) into the chromosome results in stable genetically engineered microorganisms. A step further is the construction of new enzymes by site directed mutagenesis to yield mutants with increased accessibility to the active site, higher regio- and enantioselectivity, higher turnover rates or higher turnover numbers. For such an approach examples have been described in biotransformations with other substrates.⁶³ A consequent development of this would be the application of combinatorial chemistry or the application of phage display systems and a selection of the mutants by a high efficient screening system. Such approaches have been summarized under the term “evolutionary biotechnology” but no application to biotransformations have been

published. The application of the various tools of genetic engineering to achieve regioselective oxidations will close the gap between the traditional biotransformation reactions using bacterial or fungal strains and the more chemical approach using isolated enzymes. With all these new methods in hand, and new ones on the horizon, more and more novel biotransformation reactions will become available and hopefully will help to improve and broaden the biotransformation of alkaloids.

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REFERENCES

- ¹ K. Kieslich, *Pharm. Ztg.*, 1985, **130**, 2310.
- ² B. Sikyta, *Cesk. Farm.*, 1985, **34**, 237.
- ³ H. L. Holland, *Adv. Appl. Microbiol.*, 1997, **44**, 125.
- ⁴ K. Kieslich, *Microbial Transformations of Non-Steroid Cyclic Compounds*, John Wiley, London, 1976.
- ⁵ O. K. Sebek, *Mycologia*, 1983, **75**, 383.
- ⁶ D. L. Lister, G. Kanungo, D. A. Rathbone, and N. C. Bruce, *FEMS Microbiol. Lett.*, 1999, **181**, 137.
- ⁷ N. C. Bruce, C. E. French, A. M. Hailes, M. T. Long, and D. A. Rathbone, *Trends Biotechnol.*, 1995, **13**, 200.
- ⁸ N. C. Bruce, C. J. Wilmot, K. N. Jordan, A. E. Trebilcock, L. D. Gray Stephens, and C. R. Lowe, *Arch. Microbiol.*, 1990, **154**, 465.
- ⁹ A. M. Hailes, C. E. French, D. A. Rathbone, and N. C. Bruce, *Ann. N. Y. Acad. Sci.*, 1996, **799**, 391.
- ¹⁰ D. A. Kunz, *Abstr. Annu. Meet. Am. Soc. Microbiol.*, 1985, 85th Meet., 247.
- ¹¹ R. England, S. S. Abe Ali, and C. J. Soper, *DECHEMA-Biotechnol. Conf.*, 1988, 6th Meet., Vol. 2, 163.
- ¹² M. Gibson, T. M. Jefferies, and C. J. Soper, *Analyst*, 1987, **112**, 1667.
- ¹³ K. M. Madyastha, G. V. B. Reddy, H. Nagarajappa, and G. R. Sridhar, *Indian J. Chem., Sect. B: Org. Chem. Incl. Med. Chem.*, 2000, **39B**, 377.
- ¹⁴ K. Iizuka, M. Yamada, J. Suzuki, I. Seki, K. Aida, S. Acadia, T. Alai, and K. Tsuga, *Chem. Pharm. Bull.*, 1962, **10**, 67.
M. Yamada, *Chem. Pharm. Bull.*, 1963, **11**, 356.
- ¹⁵ G. Spassov, W.-R. Abraham, K. Kieslich, H. Schmidhammer, A. Brossi, G. Snatzke, and R. Vlahov, *Appl. Microbiol. Biotechnol.* 1986, **23**, 211.
- ¹⁶ V. Kren, *Adv. Biochem. Eng. Biotechnol.*, 1991, **44**, 123.
- ¹⁷ M. M. Cien and J. P. Rosazza, *Drug Metabolism Disposition*, 1979, **7**, 214.
- ¹⁸ M. M. Cien and J. P. Rosazza, *J. Nat. Prod.*, 1979, **42**, 643.
- ¹⁹ P. Formisyn, C. Tran Minh, and J. Bourgois, *Lett. Appl. Microbiol.*, 1994, **19**, 244.
- ²⁰ R. P. Borris and G. A. Cordell, *J. Nat. Prod.*, 1983, **46**, 211.
- ²¹ W.-R. Abraham and H.-A. Arfmann, *Appl. Microbiol. Biotechnol.*, 1989, **32**, 295.

- ²² W.-R. Abraham, L. Ernst, and B. Stumpf, *Phytochemistry*, 1990, **29**, 115.
- ²³ W.-R. Abraham, L. Ernst, and H.-A. Arfmann, *Phytochemistry*, 1990, **29**, 757.
- ²⁴ Z. Shen, W. Eisenreich, and T. M. Kutchan, *Phytochemistry*, 1998, **48**, 293.
- ²⁵ E. L. Patterson, W. W. Andres, E. F. Krause, R. E. Hartman, and L. A. Mitscher, *Arch. Biochem. Biophys.*, 1963, **103**, 117.
- ²⁶ T. L. Lemke, R. A. Johnson, H. C. Murray, D. J. Duchamp, C. G. Chidester, J. B. Hester, and R. V. Heinzelman, *J. Org. Chem.*, 1971, **36**, 2823.
- ²⁷ L. So, R. White, B. Arison, B. Petuch, M. S. Schwartz, H. Cheng, R. Monaghan, and T. S. Chen, *J. Ind. Microbiol.*, 1995, **15**, 108.
- ²⁸ K. A. el Sayed, *J. Nat. Prod.*, 1998, **61**, 149.
- ²⁹ L. C. Vining, "Economic Microbiology: Microbial Enzymes and Bioconversions", Vol. 5, ed. by A. H. Rose, Academic Press, New York, 1980, pp. 523-530.
J. P. N. Rosazza and M. W. Duffel, "Alkaloids: Chemistry and Pharmacology", Vol. 27, ed. by A. Brossi, Academic Press, New York, 1986, pp. 391-392.
- ³⁰ K. K. Bhutani, R. N. Thakur, and C. K. Atal, *Indian J. Pharm. Sci.*, 1983, **45**, 60.
- ³¹ K. Y. Orabi, E. Li, A. M. Clark, and C. D. Hufford, *J. Nat. Prod.*, 1999, **62**, 988.
- ³² K. Y. Orabi, A. M. Clark, and C. D. Hufford, *J. Nat. Prod.*, 2000, **63**, 396.
- ³³ L. Ray and S. K. Majumdar, *Indian J. Exp. Biol.*, 1991, **29**, 282.
- ³⁴ F. M. Eckenrode, *J. Nat. Prod.*, 1984, **47**, 882.
- ³⁵ D. Abate, W.-R. Abraham, and H. Meyer, *Phytochemistry*, 1997, **44**, 1443.
- ³⁶ S. Siebers-Wolff, H.-A. Arfmann, W.-R. Abraham, and K. Kieslich, *Biocatalysis*, 1993, **8**, 47.
- ³⁷ W.-R. Abraham and H.-A. Arfmann, *Phytochemistry*, 1990, **29**, 1025.
- ³⁸ *GBF Wissenschaftlicher Ergebnisbericht* 1984, ed. by J.-H. Walsdorf, Braunschweig, 1985, 71.
- ³⁹ *GBF Wissenschaftlicher Ergebnisbericht* 1985, ed. by J.-H. Walsdorf, Braunschweig, 1986, 76.
- ⁴⁰ J. P. Schaumberg and J. P. Rosazza, *J. Nat. Prod.*, 1981, **44**, 478.
- ⁴¹ A. Goswami, J. P. Schaumberg, M. W. Duffel, and J. P. Rosazza, *J. Org. Chem.*, 1987, **52**, 1500.
- ⁴² J. P. Rosazza, M. W. Duffel, F. S. Sariaslani, F. M. Eckenrode, and Filippelli, *Dev. Ind. Microbiol.*, 1985, **26**, 157.
- ⁴³ J. P. Rosazza, M. W. Duffel, S. el Marakby, and S. H. Ahn, *J. Nat. Prod.*, 1992, **55**, 269.
- ⁴⁴ L. Canonica, G. Galliani, B. Rindone, S. Tollari, V. Andreoni, and E. Galli, *Experientia*, 1983, **39**, 1273.
- ⁴⁵ L. K. Hoover, M. MooYoung, and R. L. Legge, *Biotechnol. Bioeng.*, 1991, **38**, 1029.
- ⁴⁶ K. A. El Sayed, *Phytochemistry*, 2000, **53**, 675.
- ⁴⁷ K. M. Kerr and P. J. Davis, *J. Org. Chem.*, 1986, **51**, 1741.
- ⁴⁸ P. J. Davis and J. P. Rosazza, *Bioorg. Chem.*, 1981, **10**, 97.
P. J., Davis and R. E. Talaat, *Appl. Environm. Microbiol.*, 1981, **41**, 1243.
- ⁴⁹ G. Spassov, W.-R. Abraham, K. Kieslich, R. Vlahov, D. Krikorian, S. Parushev, M. Chinova, and G. Snatzke, *Appl. Microbiol. Biotechnol.*, 1986, **23**, 206.
- ⁵⁰ R. Vlahov, D. Krikorian, G. Spassov, M. Chinova, I. Vlahov, S. Parushev, G. Snatzke, L. Ernst, K. Kieslich, W.-R. Abraham, and W. S. Sheldrick, *Tetrahedron*, 1989, **45**, 3329.
- ⁵¹ M. Glace and F. Lindens, *Appl. Microbiol. Biotechnol.*, 1987, **25**, 334.
- ⁵² T. Furumoto, N. Asano, Y. Kameda, and K. Matsui, *J. Antibiot.*, 1989, **42**, 1302.
- ⁵³ B. Stumpf, W.-R. Abraham, and K. Kieslich, *Appl. Microbiol. Biotechnol.*, 1986, **23**, 215.

- ⁵⁴ W.-R. Abraham, Holger Meyer, and D. Abate, *Tetrahedron*, 1995, **51**, 4947.
- ⁵⁵ W.-R. Abraham, H.-P. Hanssen, and H.-A. Arfmann, *Phytochemistry*, 1995, **38**, 843.
- ⁵⁶ W.-R. Abraham and H.-A. Arfmann, *Phytochemistry*, 1990, **29**, 2641.
- ⁵⁷ D. Rosi, G. P. Peruzzotti, E. W. Denis, D. A. Berberian, H. Freele, B. F. Tullar, and S. Archer, *J. Medic. Chem.*, 1967, **10**, 867.
- ⁵⁸ W.-R. Abraham, *World J. Microbiol. Biotechnol.*, 1994, **10**, 88.
- ⁵⁹ W.-R. Abraham, *Zeitschr. Naturforsch.*, 1994, **49c**, 553.
- ⁶⁰ W.-R. Abraham, A. Riep, and H.-P. Hanssen, *Bioorganic Chem.*, 1996, **24**, 19.
- ⁶¹ W.-R. Abraham, In: *Bioorganic Chemistry. Highlights and New Aspects*, ed. by U. Diederichsen, T. K. Lindhorst, B. Westermann, and L. A. Wessjohann, Wiley-VCH, Weinheim, 1999, pp. 121-126.
- ⁶² P. Hugenholtz, B. M. Goebel, and N. R. Pace, *J. Bacteriol.*, 1998, **180**, 4765.
- ⁶³ W.-R. Abraham, In: *Stereoselective Biocatalysis*, ed. R. Patel, Marcel Dekker, New York, 2000, pp. 181-203.