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MICROBIAL TRANSFORMATIONS . PART 4(1). REGIOSELECTIVE PARA HYDROXYLATION OF AROMATIC RINGS BY THE FUNGUS Beauveria sulfurescens THE METABOLISM OF ISOPROPYL N-PHENYL CARBAMATE (PROPHAM®) .

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Summary : A specific para hydroxylation of the aromatic ring of isopropyl N-phenyl carbamate 1 by the fungus Beauveria sulfurescens has been achieved. Studies of the deuterium labelled compound show high retention (NIH shift) for this transformation, a fact which indicates that the mechanism implied is consistent which the intermediate formation of an arene oxide. Also, formation of a glucoside conjugate of the phenol has been observed.

Introduction. - Selective hydroxylation of aromatic compounds is a very difficult task in preparative organic chemistry (2). Therefore, in the course of our work concerning the ability of the fungus Beauveria sulfurescens to achieve hydroxylation of non actived carbon atoms (1), it was of interest for us to check whether this fungus would also be able to hydroxylate aromatic rings.

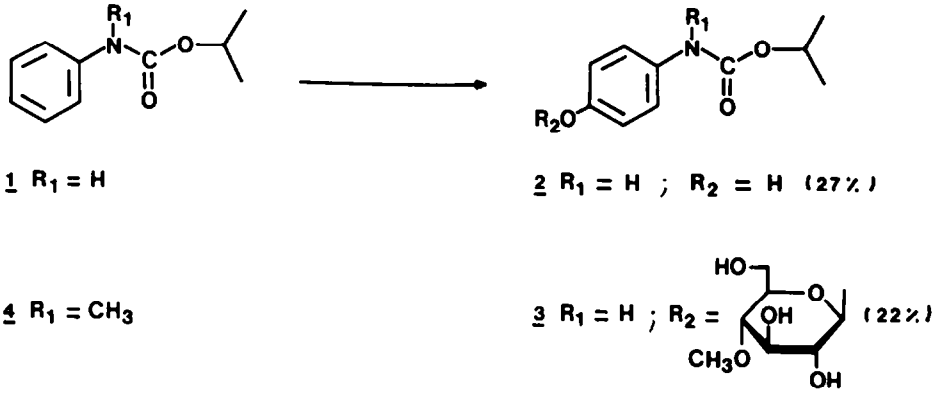
In fact, microbiological hydroxylation of aromatic rings has to date turned up far less of synthetic value than has the hydroxylation of non activated carbons. One plausible reason for this situation is the fact that, once hydroxylated, the aromatic compounds ordinarily become extremely susceptible to further oxidation, usually leading to ring cleavage, a process which can in turn be very usefull in order to get rid of unwanted aromatic pollutants (3).

Owing to our previous experience on hydroxylations achieved by this fungus, we have studied the bioconversion of an aromatic carbamate derivative, i.e. Propham 1, a product which, some years ago , has been widely used as a preemergence herbicide applied to soil for the control of weed grasses.

Results. - When added to a 48 hours old culture of Beauveria sulfurescens in a shaken flask culture, 1 is transformed (47% conversion) after 72 hours into two major metabolites. These have been isolated using high performance liquid chromatography and their structures have been determined by way of ^{1}H and ^{13}C NMR spectroscopy as being phenol 2 and its conjugated form with 4 O-methyl, β glucoside 3 (respective yields 27 and 22%)(Figure 1). It is interesting to note that this type of synthetic reaction , which leads to the formation of highly polar water soluble products, is only rarely observed with enzymes or microorganisms (4-7) , whereas it is a very common process in plants (8,9).

Owing to the fact that the conversion of 1 appears not to be complete, we decided to study the behaviour of the N-methylated compound 4 which, being more lipophilic than 1, could be expected to be more extensively metabolized. Indeed, this methylation leads to a much higher toxicity of the compound towards the fungus, a fact which necessitates to considerably diminish the substrate concentration for the bioconversion. This, however, allows to highly improve the conversion yield, since one observes complete desappearance of the starting compound (100% conversion), leading to three metabolites. The structures of these compounds have been determinated as being 1, 2 and 3 (respective yields 50, 11 and 39%) (Figure 1). Acid hydrolysis of 3 by a refluxing 2N sulfuric acid solution leads back to the aglycon 2.

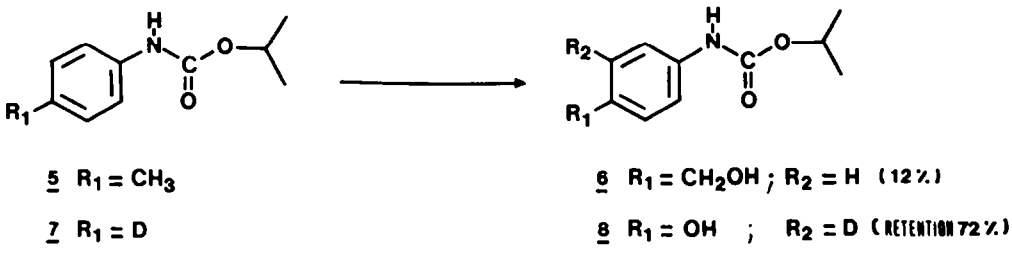
Figure 1 : Bioconversion of Propham and of N-methyl Propham.



These results suggest that the first step in the bioconversion of the N-methyl, N-isopropylphenyl carbamate 4 by this particular fungus involves demethylation of the nitrogen atom, which presumably occurs via hydroxylation of the methyl group, followed by hydrolysis of the aminoacetal thus formed (10,11) . The second step of the process then implies hydroxylation of the aromatic ring to lead to 2, the third step being conjugation of the thus formed phenol with the carbohydrate moiety, to yield 3.

As has been widely described, the mechanism generally involved for the monohydroxylation of various aromatic rings implies the so called NIH shift, claimed as being due to intermediate formation of an arene oxide specie (12) . In order to get some more insight into the hydroxylations we observed , we decided to check whether the NIH shift process does also occur in the coarse of this biotransformation. Therefore, we studied the bioconversion of the corresponding para methylated and para deuterated derivatives 5 and 7 (Figure 2). The obtained results show that, in spite of a conversion ratio of over 30%, no aromatic hydroxylation is observed in the case of compound 5, the only product formed being the benzyl alcohol 6 (12%). This indicates that no NIH shift has occured in this case. On the other hand, however, conversion of para deuterated Propham 7 (64% deuterium content as measured by MS), leads to the corresponding para hydroxylated derivative 8, showing a 46% deuterium content. This means that an NIH shift effectively has occured, in this case, with a (corrected) retention ratio of deuterium of about 70%. This is quite surprising since it is known that the presence of an electron releasing group on the aromatic ring generally leads to extensive loss of the deuterium label, due to intermediate formation of conjugated systems (13) .

Figure 2 : Bioconversion of substituted Propham derivatives.



Discussion. - The results we have obtained throughout this study are interesting from different points of vue. First, one has to emphasize the fact that, to the contrary of all the previously described bioconversions achieved with Beauveria sulfurescens, including those of urethane derivatives (2,14) we do observe a hydroxylation process of the aromatic ring. Interestingly enough, this process appears to be highly specific for the para position, a fact which has never been observed for this particular fungus (15,16). This is quite interesting, as far as synthesis is concerned, since it is well known that chemical hydroxylation of aromatic rings is a very difficult process, which is never achieved with complete para regioselectivity. This biochemical method which, to the best of our knowledge, is one of the very rare examples of this type of microbial transformation, could therefore be of value for specific synthesis of phenol derivatives.

Secondly, quite striking is the fact that no hydroxylation of the alkyl group of 1 does occur. This is also a surprizing fact since the distances between the oxygen atom or the nitrogen atom and the isopropyl methyl groups are, respectively, of about 4.5 and 2.5 Å, values which are in agreement with the required distances between these atoms (14) . No satisfactory explanation of this puzzling fact is available at the moment. However, one hypothesis could be that the presence of an urethane moiety (instead of an amide group) on the molecule, would result in a different positioning of the substrate on the enzymatic site, as already proposed elsewhere for similar results (17) (Figure 3). A more obvious explanation would, of coarse, implie the existence of different enzymes for this two types of substrates.

Figure 3 : Proposed differently oriented positionings of amides and urethanes on the hydroxylating enzyme.

A third point of interest is the fact that the NIH shift observed for the para deuterated compound indicates that the hydroxylation process is consistent with the intermediate formation of an arene oxide. This is an interesting observation since, to our knowledge, nothing is known by now about the nature and mechanism of the various hydroxylations performed by this particular fungus.

Finally, another point of interest is the fact that the obtained para hydroxylation mimics the mammalian hydroxylation pathway of detoxication. Indeed, it has been observed that, whereas ortho orientation is usually predominant in fungi, para hydroxylation is the common process in hepatic systems (18). For instance it has been shown that Propham is converted in the rat into the sulfate of isopropyl N-parahydroxyphenyl carbamate (19) whereas numerous studies with pure cultures in vitro have established that the major route of phenylcarbamate metabolism and detoxication is by hydrolysis to the free aniline, carbon dioxide and the corresponding alcohol (20). Therefore, it appears that the use of the fungus Beauveria sulfurescens may be a good way to achieve preparation of various metabolites of aromatic bioactive xenobiotics (21). Work is in progress in our laboratory in order to check whether this aromatic hydroxylation is a general process for several structurally related carbamates.

Experimental part

General : The ^{1}H and ^{13}C-NMR spectra have been realized on a Bruker AM 200 apparatus (Department of Pharmacology, Aix Marseille II University, Marseille, France). Chemical shifts are given in ppm relative to TMS as internal standart. IR spectra were recorded using a Beckman Acculab 4 spectrometer. Elemental analyses of C,H,N were performed by the Service Central d'Analyse du CNRS (Vernaison, France).

Microorganism : the strain used in the present work is Beauveria sulfurescens ATCC 7159 (originally purchased as Sporotrichum sulfurescens).

Medium composition : The medium used is constituted by 20g of corn steep liquor and 10g of glucose per liter (tap water, adjusted to pH 4.85 with natrium hydroxide).

General procedure : The sterilized medium is inoculated by a 48 hours old starter culture and incubated with shaking on a reciprocating horizontal shaker at 28°C in erlenmeyer flasks. After 48 hours growth, a solution of the substrate in ethyl alcohol (1g/10 ml) is added to the culture. After an additional 72 hours period of incubation, the mycelium is separated by filtration, and washed with water. The combined aqueous layers are saturated with NH_4Cl , acidified with aqueous HCl and the filtrate is continuously extracted (24 hrs) with chloroform. This organic phase was dried over $MgSO_4$, and the solvent stripped under vacuum.

Analysis and isolation of products : the crude residue was analysed by TLC (Merck 60 F_{254} ; 0.2 mm)(Et_2O or Et_2O/MeOH). The products where purified by HPLC.

Propham 1 : a solution of phenyl isocyanate (6.7 g, 5.6 x 10^{-2}mol.) in dry carbon tetrachloride (10 ml) was added dropwise to a solution of 3.4 g (5.7 x 10^{-2}mol) of isopropyl alcohol in dry carbon tetrachloride (50 ml). After addition, the solution was stirred overnight at room temperature. The solution was washed with water and dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by recrystallization from hexan to yield pure 1 (9.7g, 97%). m.p. 88°C. IR($CHCl_3$) ν = 3440, 1725, 1520, 1440, 1100 cm^{-1} .^{1}H-NMR($CDCl_3$,60 MHz): 6.8-7.5(m, 5H); 5.0(sept.1H); 1.3(d,J=6Hz,6H). ^{13}C-NMR($CDCl_3$) : 153.3(CO); 138.1(C_{Ar}); 129.0(CH_{Ar}); 123.2(CH_{Ar}); 118.6(CH_{Ar}); 68.7(CH); 22.1(CH_3). MS (70eV): 179($M^{+\cdot}$,54) ; 137(47); 119(60); 93(100); 43(60). Exact mass : calc. for $C_{10}H_{13}NO_2$: 179.094; found : 179.095.

Metabolites of Propham : Propham 1 (400 mg/l) was subjected to the action of a culture of Beauveria sulfurescens. After work up as previously described, two metabolites are obtained.

4 hydroxy Propham 2 : After extraction and purification the yield of metabolite 2 was 27%. White solid. m.p. 109°C. IR ($CHCl_3$) ν = 3440, 1720, 1520, 1110 cm^{-1}. ^{1}H-NMR ($CDCl_3$,200 MHz): 7.2 (d,J=8.3 Hz, 2H) ; 6.7 (d,J=8.3 Hz, 2H); 6.5 (s, 1H); 5.0 (sept, 1H); 1.3 (d, J=6.3 Hz, 6H). ^{13}C-NMR($CDCl_3$): 154.6(CO); 152.8(C_{Ar}); 130.1(C_{Ar}); 122.0(CH_{Ar}); 115.9(CH_{Ar}); 69.0 (CH); 22.1 (CH_3). MS (70 eV) : 195($M^{+\cdot}$,11); 153(99); 109(100); 43 (87). Exact mass : calc. for $C_{10}H_{13}NO_3$: 195.0895; found : 195.0885.

4 O-methyl β glucoside 3 **of para hydroxy Propham** : The yield of this metabolite was 22%. White solid : m.p. 213°C(dec.); IR(KBr) ν = 3400, 2950, 2900, 1680, 1510, 1220, 1080, 1050 cm^{-1}. ^{1}H-NMR (pyr, 200 MHz): 7.9 (d, J=8.8 Hz, 2H); 7.4 (d, J=8.8 Hz, 2H); 5.5 (d, J =7.3 Hz, 1H); 5.1 (sept, 1H); 4.2-4.5 (m, 4H); 3.8-4.0 (m,5H); 1.2 (d, J=6.3 Hz, 6H). ^{13}C-NMR (CD_3OD) : 154.0 (CO); 153.1 (C_{Ar}); 133.2 (C_{Ar}); 119.6 (CH_{Ar}); 116.5 (CH_{Ar}); 101.0 (CH); 78.9 (CH); 76.2 (CH); 75.4 (CH); 73.3 (CH); 67.6 (CH); 60.3 (CH_2); 59.1 (CH_3); 20.6 (CH_3). MS (70 eV): 371($M^{+\cdot}$); 195 (100); 153 (87.8); 135 (34); 109 (41); 43 (36). Exact mass : calc. for $C_{17}H_{25}NO_8$: 371.1580; found 371.1553.

N-methyl Propham 4 : A solution of Propham 1 (1.5 g, 8.38 mM) in DMSO (54ml) was added dropwise under stirring to a solution of potassium hydroxide (1.88 g) in DMSO (30ml). Methyl iodide (2.4g, 1.05 ml) was then added dropwise to the mixture. After 30 minutes stirring, water and ethyl acetate were added to the medium. The aqueous phase was extracted three times with ethyl acetate, the combined organic phases were washed twice with brine and dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by distillation (118°C, 10^{-2} mm Hg) to yield pure product (1.6 g, 97%). IR($CHCl_3$) ν = 3000, 1680, 1600, 1370, 1160, 1100, 690 cm^{-1} ^{1}H-NMR ($CDCl_3$, 60 MHz) : 7.2 (s,5H); 4.9 (sept, 1H); 3.3 (s,3H); 1.3 (d, J=6.3 Hz, 6H). ^{13}C-NMR ($CDCl_3$) : 155.2 (C); 143.6 (CH_{Ar}); 128.7 (CH_{Ar}); 125.6 (CH_{Ar}); 125.5 (CH_{Ar}); 69.0 (CH); 37.4 (CH_3); 22.1 (CH_3).

para methyl Propham 5 : 4-methyl N-phenyl isocyanate (3.5g, 2.6 10^{-2}mol.) was added dropwise to dry isopropyl alcohol under stirring. The solution was stirred overnight at room temperature. The solution was washed with water and dried over $MgSO_4$. The solvent was removed under vacuum and the residue was purified by flash chromatography to yield 5g of pure 5 (100%). White solid, m.p.

54°C. IR($CHCl_3$) ν = 3440, 2980, 1720, 1520, 1310, 1105, 1050 cm^{-1}. ^1H-NMR ($CDCl_3$, 200 Mz) : 7.3 (d, J=8.1, 2H) ; 7.1 (d, J=8.1 Hz, 2H) ; 6.7 (s,1H) ; 5.0 (sept. 1H) ; 2,3 (s, 3H) ; 1,3 (d, J=6.3 Hz, 6H). ^{13}C-NMR ($CDCl_3$): 153.5 (CO); 135.6 (C_{Ar}); 132.7 (C_{Ar}); 129.5 (CH_{Ar}); 118.9 (CH_{Ar}); 68.6 (CH); 22.1 (CH_3); 20.7 (CH_3).

para hydroxy methyl Propham 6 . The biotransformation of 5 (400 mg/l of culture) leads to one single metabolite 6 (12%). IR($CHCl_3$) ν = 3440, 2980, 2860, 1720,1520, 1105 cm^{-1}.^1H-NMR ($CHCl_3$, 200 MHz): 7.3 (q, 4H); 6.7 (s, 1H); 5.0 (sept, 1H); 4.6 (s, 2H); 1.3 (d, J=6.3 Hz, 6H). ^{13}C-NMR ($CDCl_3$): 153.4 (C); 137.7 (C_{Ar}); 135.9 (C_{Ar}); 128.0 (CH_{Ar}); 118.9 (CH_{Ar}); 68.9 (CH); 65.0 (CH_2); 22.1 (CH_3). MS (70 eV): 209($M^{+\cdot}$,58); 167 (69); 138 (37); 43 (100). Exact mass. calc. for $C_{11}H_{15}NO_3$ 209.1052; found 209.1054.

para deutero Propham 7 :

para deutero toluene : A solution of parabromotoluene (37.6g, 0.22 mol) in 250 ml of dry THF was added dropwise under stirring to magnesium (46.5 g, 0,27 mol.) and some iodide crystals in dry THF . The solution of bromide was added to keep a gentle reflux. After the addition, the solution was heated under reflux for 30 min. D_2O (15 ml, 0.75 mol.) was added slowly to the chilled solution . The solution was filtered and the toluene was distilled yielding 6g of product (30%) .

para deutero benzoic acid : The previously obtained toluene (5g, 5.38 10^{-2} mole) was added to a solution of 2.5g of Na_2CO_3 in 400 ml boiling water. Crushed $KMnO_4$ (20g) was poured slowly into the solution. The mixture was kept under reflux until the purple colour has disappeared. The chilled solution was acidified with H_2SO_4 and heated again (30 mn). Sodium bisulfite was added to the cooled solution, the medium was filtered, extracted with ether and dried over $MgSO_4$. The solvent was stripped under vacuum yielding 3.7g (56%) of benzoic acid.

para deutero Propham 7 : A solution of para deutero benzoic acid (2.07g, 0.017 mol) in 30 ml of dry acetone was cooled at 0°C. Triethylamine (0.02 mol) in 8ml of dry acetone was then added under stirring. After addition, a solution of ethyl chloroformate in 8 ml of dry acetone was added dropwise. After stirring for 30 mn at 0°C, a solution of NaN_3 (0.03 mol) in 6 ml of H_2O was added slowly. After stirring for 1 hr, the mixture was poured on ice water (100ml). The azide was extracted with toluene (3x15 ml) cooled at 0°C. The combined organic phases were dried over $MgSO_4$ and then over P_2O_5. The solution was stored overnight at 0°C.

A three necked flask equiped with a thermometer and a reflux condenser was heated at 90°C in an oil bath. The solution of azide in toluene was added dropwise in the flask under stirring. After addition the mixture was heated under reflux for 1 hr, then isopropyl alcohol (1.1g, 0.018 mol) was added dropwise to the mixture. After cooling, the organic phase is successively washed with aqueous NaOH 3%, HCl 3% with H_2O and dried over $MgSO_4$. After stripping off the solvent the crude residue was purified by flash chromatography and the product recrystallized from hexan to give 2g of (66%).

White solid . m.p. 87°C.^1H-NMR ($CDCl_3$, 200 MHz): 7.4 (d, J=8.5 Hz, 2H); 7.3 (d, J=8.5Hz, 2H); 6.7 (s, 1H); 5.0 (sept, 1H); 1,3 (d, J=6.3 Hz, 6H). ^{13}C-NMR ($CDCl_3$): 153.3 (CO) ; 138.2 (C_{Ar}) ; 128.9 (CH_{Ar}) ; 123.2 (CH_{Ar}) ; 118.7 (CH_{Ar}) ; 68.7 (CH) ; 22.1 (CH_3) : MS (70 eV): deuterium incorporation 63,9%.

meta deutero para hydroxy Propham 8 : The bioconversion of 7 has been realized using the same culture conditions as for 1. The product has been analysed as follows. IR($CHCl_3$) ν = 3440, 1700, 1510, 1100 cm^{-1}.^1H-NMR ($CDCl_3$, 200 MHz) : 7.2 (d, J=8.3 Hz, 2H); 6.7 (d, J=8.3 Hz, 2H); 6.5 (s, 1H); 5.0 (sept, 1H); 1.3 (d, J=6.3 Hz, 6H). MS (12 eV); 54% d_0; 46% d_1.

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