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On the microbial transformation of α,β-unsaturated aryl ketones by the fungus *Beauveria bassiana*

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Abstract

α,β-unsaturated aryl ketones, **1a–12**, have been submitted to the action of the fungus *Beauveria bassiana* (ATCC 7159) in growing conditions. The saturation of the double bond strictly depends from the substituent α to the carbonyl group. The saturated ketone is then oxidised in a Baeyer-Villiger type reaction. This new oxidative capacity of the fungus has been studied and the adaptability of the micro organism towards structural modifications has been investigated. © 1998 Elsevier Science B.V.

Keywords: Microbial oxidation; *Beauveria bassiana*; Baeyer-Villiger; Raspberry ketone

1. Introduction

The microbial transformation of multifunctional substrates can give rise to mixtures of products due to the presence of numerous enzymatic activity in the whole cells biocatalyst. When the product of the first biotransformation is also the substrate for a second activity which is present in the microorganism or is stimulated by the presence of the substrate, a chain of reactions can occur. In some cases the end product can be obtained with high selectivity and yield. One such case is represented by the biotransformation of α,β-unsaturated carbonyl compounds which can give rise to the selective reduction of the C–C double bond, of the carbonyl group or to the saturated alcohol from the reduction of both functional groups. When prochiral substrates are employed, enantiomerically pure chiral compounds can be obtained. The potentiality of whole cells enzymatic systems in the reduction of unsaturated aldehydes has been exploited in our laboratory, for the preparation of biologically active chiral intermediates [1–3]. There the product arises from the reduction of both functional groups.

In this article we wish to report our observations on the microbial reduction of α,β-unsaturated ketones carried out by the fungus *Beauveria bassiana* in which the product obtained from the initial C–C double bond reduction, the saturated ketone, is oxidised in a Baeyer-Villiger (B.-V.) type oxidation and then

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hydrolysed to the primary alcohol containing 2 carbon atom less with respect to the starting material. The reaction occurs in good yields and is the consequence of at least 3 independent enzymatic activities displayed by the organism in the mentioned sequence. The reduction of the C–C double bond in α,β-unsaturated ketones can be of interest for applications to the synthesis of pharmaceuticals and for the production of natural aromas for the food industry [4–6]. In particular reduction of substrate **1a** has been exploited in the production of raspberry ketone **1b**, the impact flavour of raspberry fruits, starting from a natural precursor [4,5,7,8].

In this context we found that *Beauveria bassiana*, a micro organism well known for its ability to selectively reduce C–C double bonds in the presence of conjugated carbonyl groups [9] is also capable to perform a C_2 chain shortening through (i) insertion of oxygen in the α position of the carbonyl compound, (ii) migration of the more substituted carbon atom (Baeyer-Villiger type oxidation) and (iii) hydrolysis of the resulting ester **14** to give tyrosol **15**. The all sequence occurs in overall quantitative yield [10] (Scheme 1).

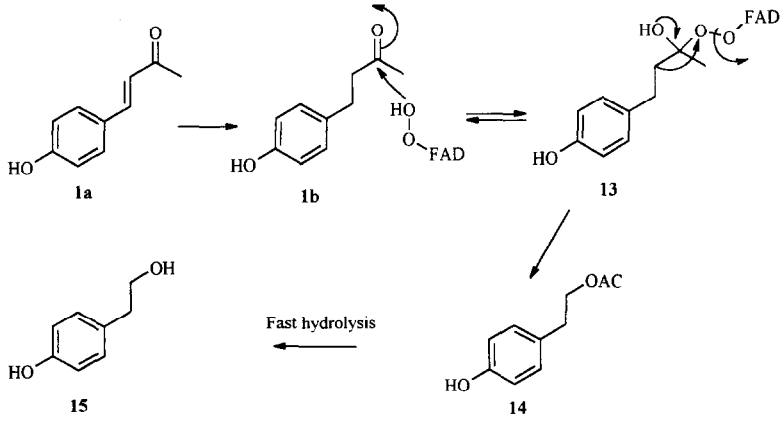
The microbial Baeyer-Villiger oxidation is a well known ability of different micro-organisms [11], but it has not been observed until now in *B. bassiana*. The oxidation is catalysed by a FAD depending mono oxygenase [12].

2. Results and discussion

In order to investigate the substrate specificity of each of these enzymes we first submitted to biotransformation a number of different compounds (**1a–12**), structurally related to **1a**. They differ for the ring substituents and the chain length (Fig. 1).

We also tested α,β-unsaturated esters, acids, or nitriles, compounds which are substrate for the enoate-reductase found in yeast [13], but they proved not to be transformed by this organism (data not reported).

We compared the analytical data obtained after 24 h incubation with the one obtained at 48 and 72 h (Table 1). There is a general trend consistent with an initial reduction of the double bond to the saturated ketone, while only for higher incubation time the latter compound is further reduced to the saturated carbinol. No formation of the unsaturated alcohol is observed. This behaviour is different from what occurs in the yeast reduction of α,β-unsaturated aldehydes, where the carbonyl is reduced very rapidly to an equilibrium mixture from which the activated C–C double bond of the remaining aldehyde is subsequently reduced, with a lower rate, to the saturated aldehyde and then to the corresponding alcohol [1–3]. Compounds **2** and **10** are significantly reduced in the short period to the saturated carbinols indicating



Scheme 1. Proposed mechanism of degradation of *p*-hydroxy-benzilydenacetone **1a** in growing cultures of *B. bassiana*.

Substrate	X	R	R'
1a	*p*-OH	Me	H
2	*p*-OH, *m*-OMe	Me	H
3	H	Me	H
4	*p*-F	Me	H
5	*o*-OH	*i*-Pr	H
6	*p*-OH	Ph	H
7	*p*-OH	Et	H
8	*p*-OH	Me	Me
9	*p*-OH	Me	Et
10	*p*-OMe	Me	H
11	*m*-CF_3	Me	H
12	*p*-OH	*n*-Bu	H

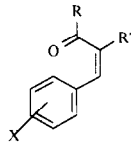
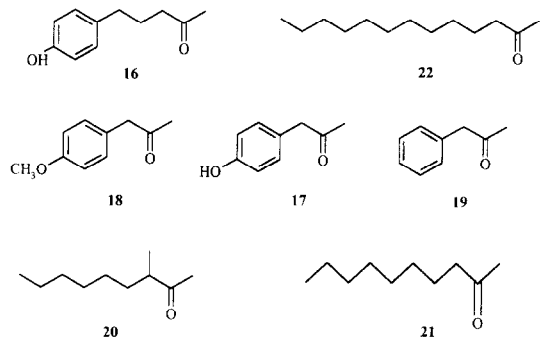


Fig. 1. Substrate submitted to the action of *B, bassiana* in growing conditions.



Scheme 2.

the prevalence of another enzymatic activity while **4** and **9** are only marginally transformed. The fact that at 48 h the product from the degradative oxidation is present in minor amounts while it is the major one in all cases at 72 h, proves that the substrates for the Baeyer-Villiger type oxidation are the saturated ketone as it is suggested in the mechanism of the degradation (Scheme 1). In fact when the same substrates are submitted to the biotransformation in the saturated carbonyl form, the rate of formation of the degradative product is enhanced. We also observed that the oxidation reaction takes place equally well when the saturated alcohol is used as a substrate. During this reaction the ketone is present in small percent (< 15%) in the mixture. The alcohol is presumably oxidised to the carbonyl form which is subsequently transformed by the monooxigenase type of enzyme to the rearranged ester and subsequently hydrolysed. Apparently only methyl ketones are the substrates of the Baeyer-Villiger type oxidation and only a methyl group is tolerated in the α-position. Differences between **1a**, **2** or **10** and **3** and **4** can be attributed to the distinct migrating attitude of the differently substituted aromatic moieties [14]. The hidden stereochemistry of the migrating carbon atom has been shown to proceed with retention of configuration from experiments with labelled compounds 15,16]. In order to broadly define the substrate specificity of this new enzymatic activity, we extended our study to structurally different ketones with varying distances between the carbonyl group and the aromatic ring, substrates **16–19**, or simply aliphatic

Table 1
Biotransformation of substrates **1a–12** in growing cultures of *B. bassiana*

Entry [a]	Saturated ketone			Saturated carbinol			Degradative oxidation			Yield [b]
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	72 h
1a	70	23	1	16	71	2	2	4	95	98
2	4	10	< 1	94	59	< 1	1	30	98	99
3	81	85	46	18	14	51	—	< 1	2	99
4	20	8	15	3	18	11	—	—	—	26
7	69	74	70	3	2	4	—	—	< 1	75
8	5	5	5	2	2	4	< 1	1	30	39
9	4	7	6	8	5	6	—	—	< 1	12
10	39	38	< 1	47	34	< 1	11	24	98	98

[a] Substrates **5**, **6**, **11** and **12** are not accepted by the fungus for the biotransformation and they are recovered totally unreacted.
[b] The amount is expressed in mole percent, the recovery is practically quantitative and is calculated by GC with internal standard.

Table 2
Biotransformation of substrates **8–22** in growing cultures of *B. bassiana*.

Entry [a]	Saturated carbinol			Degradative oxidation			Yield [b]
	24 h	48 h	72 h	24 h	48 h	72 h	72 h
8 [c]	17	27	1	18	18	59	60
9 [c]	5	7	7	—	—	< 1	7
16	31	38	37	9	14	15	55
17	< 1	7	8	—	—	—	8
18	30	45	29	< 1	12	28	57
19	18	42	52	17	18	70	
20	—	—	< 1	—	—	—	—
21	—	—	1	—	—	—	1
22	8	14	15	—	—	—	15

[a] Substrates **5**, **6**, **11** and **12** (once chemically saturated) are not accepted by the fungus for the biotransformation and they are recovered totally unreacted.
[b] Calculated by GC with internal standard.
[c] Chemically saturated (H_2, 10% Pd on charcoal, EtOH).

straight chain ketones, substrates **20–22** (Scheme 2).

While the latter were not transformed in significant amounts into the corresponding alcohols with two carbon less in the chain, the aromatic compounds were transformed (Table 2). Compound **17** was a notable exception. Cyclohexanone, the substrate of cyclohexanone mono-oxygenase from several bacterial species, was metabolised but we were unable to isolate any lactone from the mixture. We do not know whether this might be attributed to the high hydrolysing properties of the fungus.

In conclusion we have studied a new oxidative activity found in *B. bassiana* and we have broadly defined the structural requirements for the substrates of this enzymatic system.

3. Experimental

3.1. Analytical procedures: GLC

The samples were analysed by GLC using a 30 mt × 0.25 mm i.d. fused silica capillary column (J&W, USA) coated with 0.25 μm layer of cross-linked DB5. Analysis were carried out in a DANI apparatus (model 8610) equipped with PTV injector and FID detector. Helium was used as carrier gas at 0.9 bar. Temperature program for all the compounds: 80°C for 1 min, 10°C/min, 155°C for 1 min, 1°C/min, 165°C for 2 min, 15°C/min, 250°C; injector temperature 250°C, detector temperature 250°C.

3.2. Microbial transformations

These conditions are the ones suggested by Professor Veschambre together with the gift of the strain of *Beauveria*. We change the media (MPGB) for the biotransformation because we found to work better with our substrates. Similar conditions are reported in literature [17]. Following are reported the culture conditions. 5 ml of T1 medium were seeded with the microorganism and incubated for 4 days at 30°C. The biomass was suspended in 4 ml of T3 medium and 2 ml of this suspension was inoculated in 50 ml of the same medium and shaken at 180 rpm for 24 h at 30°C. 5 ml of this culture was inoculated in 50 ml of fresh T3 medium and incubated for 3 days in the same conditions. 3 ml of the content of the flask was transferred in 50 ml of MPGB medium and shaken at 180 rpm at 30°C for 24 h. At this point 50 mg of solid substrates **1a–22** were added and the mixture stirred at 180 rpm for 24/72 h at 30°C. The incubation mixture was extracted with 2 × 25 ml of ethyl acetate. The separated organic phase, once dried, was evaporated under vacuum to give a crude extract which was used directly for GLC analysis. Composition of the media: T1, corn step atomised 12 g/l, D-glucose 10 g/l, agar 30 g/l, pH 5.5. T3, bacto-triptone 10 g/l, K_2HPO_4 1 g/l, D-glucose 30 g/l, $FeSO_4 \cdot 7H_2O$ 0.01 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, $ZnSO_4 \cdot 7H_2O$ 0.3 g/l, KCl 0.5 g/l, pH 7.2. MPGB, D-glucose 20 g/l, peptone 5 g/l, malt 20 g/l.

3.3. General methods

Compounds **3** and **23** were purchased from Aldrich. Product **21** is of commercial origin (Fluka). All the substituted benzaldehydes, sub-

stituted acetones and phenyl acetic acids were of commercial origin.

3.4. Preparation of α,β-unsaturated alkyl aryl ketones (benzylidenacetone type) 1a–12

The appropriate substituted benzaldehyde and a substituted acetone of commercial source were reacted together in an aqueous sodium hydroxide solution. The procedures is described in the literature [18] for the synthesis of **1a**. With this procedures substrates **2**, **4**, **5**, **6**, **7**, **10**, **11** and **12** have been synthesised. All the compounds have been purified by column chromatography using mixtures of hexane and ethyl acetate as eluent; solids were crystallised from toluene. Yields ranged between 68 and 85%. Anal. Calcd.: **2** (Found C, 68.63; H, 6.32. $C_{11}H_{12}O_3$ requires C, 68.74; H, 6.29); **4** (Found C, 73.28; H, 5.55. $C_{10}H_9FO$ requires C, 73.16; H, 5.53); **5** (Found C, 75.83; H, 6.32. $C_{12}H_{14}O_2$ requires C, 75.76; H, 6.29); **6** (Found C, 80.23; H, 5.32. $C_{15}H_{12}O_2$ requires C, 80.34; H, 5.39); **7** (Found C, 74.83; H, 6.92. $C_{11}H_{12}O_2$ requires C, 74.96; H, 6.86); **10** (Found C, 74.90; H, 6.89. $C_{11}H_{12}O_2$ requires C, 74.96; H, 6.86); **11** (Found C, 61.73; H, 4.25. $C_{11}H_9F_3O$ requires C, 61.69; H, 4.24); **12** (Found C, 76.33; H, 7.82. $C_{13}H_{16}O_2$ requires C, 76.44; H, 7.90).

3.5. 4-(4-hydroxy-phenyl)-3-methyl-but-3-en-2-one 8 and 3-ethyl-4-(4-hydroxy-phenyl-but-3-en-2-one 9

0.1 mol of *p*-hydroxy benzaldehyde and 0.2 mol of methyl ethyl ketone or methyl propyl ketone were mixed together and HCl gas was flushed in the mixture at 0°C for 10 min. The mixture was left under stirring overnight at 25°C, then poured into ice and extracted with ethyl acetate. The organic phase was washed with dilute aqueous solution of $NaHCO_3$ and dried over Na_2SO_4. The solvent was removed at reduced pressure and the solid residue was crystallised from toluene to obtain **8** yield 68%, (Found C, 74.98; H, 6.88. $C_{11}H_{12}O_2$ requires C, 74.96; H, 6.86); and **9** (Found C, 75.83; H, 7.48. $C_{12}H_{14}O_2$ requires C, 75.76; H, 7.42).

3.6. Preparation of α,β-unsaturated alkyl aryl ketones 17 and 18

A mixture of 0.1 mol of *p*-hydroxy or methoxy phenyl acetic acid, and 0.11 mol of sodium acetate in 25 ml of acetic anhydride were refluxed for 18 h. At the end of the reaction the mixture was quenched with 30% aqueous NaOH and extracted with ethyl ether. The organic solvent once dried over Na_2SO_4 was evaporated at reduced pressure and the residue distilled at 25 mm Hg to give pure **17**, (b.p. 150°C), yield 45% (Found C, 71.95; H, 6.65. $C_9H_{10}O_2$ requires C, 71.98; H,6.71) and **18** (b.p. 145°C) yield 57% (Found C, 73.18; H, 7.32. $C_{10}H_{12}O_2$ requires C, 73.15; H, 7.37).

3.7. Synthesis of methyl alkyl ketones 20 and 22

In a three necked flask with stirrer, dropping funnel and nitrogen inlet, 0.03 mol of methyl dithiane were dissolved in 100 ml of anhydrous THF. The mixture was cooled to −60°C and 20 ml of 1.6 M *n*-BuLi were added dropwise. After 5 h at −20°C the reaction vessel was cooled at −60°C and 0.03 mol of 2-bromo-octane or 1-bromo-undecane were added dropwise. The mixture was stirred at 25°C for 16 h, then quenched with a 1:1 mixture of MeOH:acetone. The crude mixture was extracted with ethyl acetate, the organic solvent separated and dried over Na_2SO_4. The crude oily residue was chromatographed on silica gel using mixture of hexane and ethyl acetate as eluent, to give pure ditianyl derivative (yield 84%). To a solution of 5 mmol of dithianyl derivative in 75 ml of THF/water 70/30 and 10 mmol of HgO were added in one portion 10 mmol of BF_3 etherate. The mixture was stirred for 2 h, then poured into an equal volume of ethyl ether. The organic solvent was separated, washed with an aqueous solution of $NaHCO_3$, dried and evaporated. The crude oily residue

was purified by column chromatography to give pure **20** and **22**, respectively (yield 65%). **20** (Found C, 76.91; H, 12.77. $C_{10}H_{20}O$ requires C, 76.86; H, 12.90); **22** (Found C, 78.74; H, 13.28. $C_{13}H_{26}O$ requires C, 78.72; H, 13.21) crystal.

3.8. 5-(4'-hydroxyphenyl)-pentan-2-one ***16***

3-(4-hydroxy-phenyl)-propionic acid (Aldrich), treated with MeOH/HCl gas, 25°C 24 h gave 3-(4-hydroxy-phenyl)-propionic acid methyl ester in 94% yield. This was then reacted with NaH in DMF followed by addition of $PhCH_2Cl$, to give after 24 h at 25°C 3-(4-phenoxy-phenyl)-propionic acid methyl ester in 73% yield; this was reduced to 3-(4-phenoxy-phenyl)-propan-1-ol with THF/$LiAlH_4$ in 87% yield. Bromination of the latter compound with NBS, Ph_3P, CH_2Cl_2 gave the corresponding 3-(4-phenoxy-phenyl)-1-bromo-propane, in 85% yield (Found C, 62.78; H, 5.58. $C_{16}H_{17}BrO$ requires C, 62.96; H, 5.61). From this point ahead the procedure is the same as described for the preparation of methyl–alkyl ketones. In this manner we obtained pure **16**, yield 65%, (Found C, 74.18; H, 7.91. $C_{11}H_{14}O_2$ requires C, 74.13; H, 7.92).

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