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CAMPHOR-GROWN PSEUDOMONAS PUTIDA, A MULTIFUNCTIONAL BIOCATALYST FOR UNDERTAKING BAEYER-VILLIGER MONOOXYGENASE-DEPENDENT BIOTRANSFORMATIONS

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Summary Both NADH- and NADPH-dependent Baeyer-Villiger monooxygenase activities with potential uses as biocatalysts for biotransformations are present to different extents throughout the growth of Pseudomonas putida NCIMB 10007 on (+)-camphor. The two activities give a different pattern of stereoselective oxygenations with various mono- and bicyclic ketone substrates.

Introduction

The use of various NADPH- and NADH-dependent Baeyer-Villiger monooxygenases with complementary coupled enzymes to promote *in situ* coenzyme recycling is now a well-established technique to provide valuable synthons for chemoenzymatic syntheses by stereoselective oxygenation of appropriate cyclic ketones (Abril *et al.*, 1989; Willetts *et al.*, 1991; Grogan *et al.*, 1992; Grogan *et al.*, 1993). The recently-developed use of the NADH-dependent diketocamphane monooxygenase from (+)-camphor-grown Pseudomonas putida NCIMB 10007 is an interesting departure from the more traditional use of the NADPH-dependent cycloalkanone monooxygenase from cyclohexanol-grown Acinetobacter calcoaceticus NCIMB 9871 because when tested with bicyclo(3.2.0)hept-2-en-6-one as substrate the two oxidative enzymes yield both 2-oxa and 3-oxa lactones that are in each case antipodes (Grogan *et al.*, 1992, 1993). The deployment of Pseudomonas putida NCIMB 10007 is interesting in another respect, because two Baeyer-Villiger monooxygenase activities can be obtained as discrete partially purified preparations from the bacterium after growth on camphor as the sole source of carbon. One is the already-tested NADH-dependent activity (MO1), known to be comprised of two isoenzymes (2,5-diketocamphane monooxygenase and 3,6-diketocamphane monooxygenase) involved in the oxygenation of bicyclic intermediates during the catabolism of (+)- and (-)-camphor respectively (Jones *et al.*, 1993). The other is a discrete NADPH-dependent activity (MO2) attributed to 2-oxo-Δ^3-4,5,5,-trimethylcyclopentylacetyl-CoA monooxygenase (Ougham *et al.*, 1983), involved in

the oxygenation of this monocyclic intermediate during the later stages of camphor metabolism. Although not conclusively demonstrated, it has been suggested that MO2 may also comprise enantiocomplementary isoenzymes to catabolise the two possible antipodes (Gunsalus et al., 1971).

The purpose of the present paper is three-fold. Firstly, in an attempt to make the use of the Baeyer-Villiger monooxygenases from P. putida NCIMB 10007 readily reproducible, the effect of growth conditions on the titre of MO1 and MO2 has been investigated. Secondly, the relative effectiveness of MO1 and MO2, enzymes evolved to oxygenate bicyclic and monocyclic ketones respectively, has been compared using both monocyclic and bicyclic carbonyl substrates. Thirdly, the effect, if any, of the length of an α-substituted alkyl side-chain (= lipophilic character of the substrate) has been examined using a series of cyclopentanones. There are now many precedents where variation of the chain length of a substituent has a significant influence on the stereochemistry of a biotransformation (Zhou et al., 1983; Ladner et al., 1984; Holland et al., 1985; Keinan et al., 1986; Colonna et al., 1988; Pan et al., 1990; Effenberger et al., 1991). This potentially influential factor has been previously examined with the NADPH-dependent Baeyer-Villiger monooxygenase known to be present in washed-cell suspensions of Acinetobacter calcoaceticus NCIMB 9871 and Acinetobacter TD63 (Alphand et al., 1990): in both cases the yield of the lactone product increased whereas the enantiomeric excess decreased as the length of the side-chain of a series of 2-substituted cyclopentanones was increased from C_5 to C_{11}.

Materials and Methods

Microorganisms, maintenance and growth. These were as previously described (Grogan et al., 1992).

Preparation of partially purified enzymes. For NADH-dependent diketocamphane monooxygenases (MO1) the procedure of Williams (1991) was used, whereas that of Ougham et al. (1983) was used to obtain NADPH-dependent 2-oxo-Δ^3-4,5,5-trimethylcyclopentylacetyl-CoA monooxygenase(s) (MO2).

Biotransformation conditions. These were as previously described (Willetts et al., 1991: Grogan et al., 1993).

Analytical methods. Bicyclic lactone products were characterised and quantified by chiral capillary GC as described previously (Carnell et al., 1992). Identification of lactone peaks was confirmed by co-chromatography with authentic chemically synthesised standards. As reported by Alphand et al. (1990), the absolute configuration of the optically active monocyclic lactones was ascertained by comparison of the sign of rotation

with literature values, and the optical purity assessed by conversion into the acetals followed by GC analysis. The enantiomeric ratio of lactone products (E_p) was calculated after the method of Chen et al. (1982).

Results

It has already been established that (+)-camphor-grown P. putida NCIMB 10007 contains substantial amounts of an NADH-dependent bicyclic ketone oxygenating Baeyer-Villiger monooxygenase activity (MO1 = 2,5-diketo-camphane-1,2-monooxygenase plus 3,6-diketocamphane-1,6-monooxygenase, Jones et al., 1993) that can be precipitated from crude cell-free extracts by 60-75% saturation with $(NH_4)_2SO_4$ (Grogan et al., 1992). Equivalent experiments confirmed that the same cell-free extracts additionally contained significant amounts of an NADPH-dependent Baeyer-Villiger mono-oxygenase (MO2) able to oxygenate bicyclic ketones that was also precipitated by 60-75% saturation with $(NH_4)_2SO_4$ (Grogan et al., 1993). As with the equivalent NADH-dependent activity MO1, no enzyme activity for MO2 could be recorded in the absence of exogenous coenzyme. In order to optimise the yields of MO1 and MO2, and possibly to help facilitate separation, the time-dependent change in the specific activity of both biocatalysts was determined using partially purified enzymes prepared from cells harvested at different times during growth of P. putida NCIMB 10007 on (+)-camphor (Figure 1). These data, obtained by measuring the rate of oxidation of the respective nicotinamide coenzyme in the presence of bicyclo(3.2.0)hept-2-en-6-one, suggest that MO1 and MO2 are not coordinately controlled, but that the titre of the enzymes evolved to catabolise bicyclic substrates resulting from camphor catabolism peaks before that of the enzyme(s) evolved to catabolise monocyclic substrates. This observation may help to explain the previously-reported transitory accumulation of established pathway intermediates in (+)-camphor-catabolising P. putida (Hedegaard et al., 1965).

Although the data obtained with bicyclo(3.2.0)hept-2-en-6-one serves to show the relative distribution of MO1 and MO2 throughout the growth curve, the absolute values obtained with the NADPH-dependent activity MO2 are probably a poor reflexion of the effectiveness of this biocatalyst with compounds more related to its natural monocyclic substrate. This was substantiated by a comparative study of the specific activity of both partially purified MO1 and MO2 with 2-hexylcyclopentanone and the

previously-tested bicyclic ketone which confirmed that the effective ratio of MO1:MO2 was 1:2.0 with the monocyclic substrate rather than 1:0.3 with the bicyclic substrate.

In order to assess more subtle aspects of the specificity of MO1 and MO2, both the regio- and stereospecificity of the two partially purified activities were probed with a number of representative racemic synthetic bicyclic and monocyclic analogue substrates (Table 1). In each case the products were characterised and quantified by comparison with literature values and/or appropriate GC techniques using authentic chemically synthesised standards. For comparative purposes, the results of equivalent biotransformations previously obtained with the partially-purified NADPH-dependent Baeyer-Villiger monooxygenase from cyclohexanol-grown Acinetobacter calcoaceticus NCIMB 9871 are also included. Regarding regio-specificity, it is apparent that whereas both the NADH- and NADPH-dependent activities from (+)-camphor-grown NCIMB 10007 are highly specific for the tested monocyclic and bicyclic (2.2.1) ketones, both of the newly-tested biocatalysts are not regiospecific for the bicyclic (3.2.0) ketones tested. The data obtained clearly supports the stereoselective nature of both oxygenating activities from P. putida. Interestingly, with some substrates such as bicyclo(3.2.0)hept-2-en-6-one and bicyclo(2.2.1)heptan-2-one, the NADH- and NADPH-dependent Baeyer-Villiger monooxygenases from NCIMB 10007 consistently gave enantiocomplementary lactone products, whereas with other substrates such as 7,7-dimethylbicyclo(3.2.0)hept-2-en-6-one and 2-hexyl-cyclopentanone, these two biocatalysts yielded the same lactone products. It was also apparent that the NADPH-dependent activity from P. putida NCIMB 10007, which is probably predisposed by evolution to metabolise monocyclic ketone substrate(2), is noticeably more stereoselective with the synthetic monocyclic substrate than the equivalent NADH-dependent activity which is similarly predisposed towards bicyclic ketone substrate(2). The converse is true for the relative stereoselectivity of these two activities with all the synthetic bicyclic substrates tested. It remains to be confirmed whether the outcome of the biotransformation of the two bicyclic (3.2.0) ketones obtained with both MO1 and MO2 results in each case from the combined activity of two enantiocomplementary isoenzymes with divergent regio- and stereospecificities. If this is so, then both MO1 and MO2 must each contain one isoenzyme with a higher degree of specificity than its partner.

Figure 1. Growth on (+)-camphor and changes in the specific activity of key catabolic enzymes in Pseudomonas putida NCIMB 10007

Specific activities of diketocamphane monooxygenase (●) and 2-oxo-Δ^3-4,5,5-trimethylcyclopentylacetyl-CoA monooxygenase (○) are expressed as a function of the optical density (= growth) of the culture

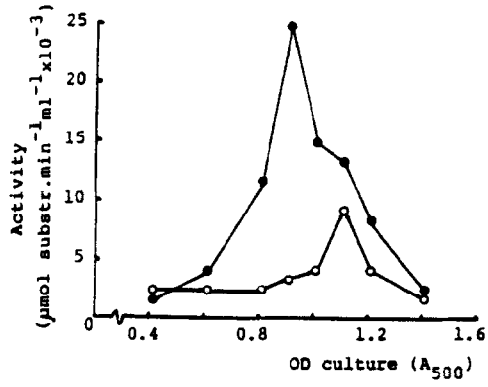

Table 1. Biotransformation of bicyclic and monocyclic ketones by various partially purified monooxygenases

	ketone substrates							
	lactone products[1]		lactone products[1]		lactone products[2]		lactone products[2]	
Biocatalyst	3-oxa	2-oxa	3-oxa	2-oxa	3-oxa	2-oxa	3-oxa	2-oxa
MO1(NADH)10007	1S,5R 99%ee	1R,5S 89%ee	1R,5S 80%ee	1S,5R 95%ee	nd	1R,5S 53%ee	nd	2S;$[\alpha]_D^{29}$= -26°(c 1.02,$CHCl_3$)[3] 74%ee
MO2(NADPH)10007	1R,5S 95%ee	1S,5R 35%ee	1R,5S 60%ee	1S,5R 95%ee	nd	1S,5R 14%ee	nd	2S;$[\alpha]_D^{29}$= -29°(c 1.03,$CHCl_3$)[3] 92%ee
MO(NADPH)9871	1R,5S 95%ee	1S,5R 92%ee	nd	1R,5S 29%ee	nd	racemic	nd	2S;$[\alpha]_D^{25}$= -40.2°(c 1.66,TMF)[4] 90%ee

1 = reaction taken to 100% substrate biotransformed

2 = reaction taken to 50% substrate biotransformed

3 = tentative assignment of absolute configuration

4 = data for heptyl homologue; all C_{odd} homologues tested C_5-C_{11} reported to give (2S0-lactones (Alphand et al., 1990)

nd = not detectable

Table 2. Biotransformation of 2-alkylcyclopentanones by partially purified monooxygenases from Pseudomonas putida NCIMB 10007

	2-alkylcyclopentanone substrates			
	R = C_4H_9	R = C_6H_{13}	R = C_8H_{17}	R = $C_{10}H_{21}$
Biocatalyst	(-)-lactone	(-)-lactone	(-)-lactone	(-)-lactone
MO1(NADH)10007	E_p= 4.7±0.1	E_p= 10.4±0.4	E_p= 23.0±0.6	E_p= 20.3±0.5
MO2(NADPH)10007	E_p= 104.0±21	E_p= 52.0±5	E_p= 63.0±4	E_p= 7.0±0.3

The effect, if any, of an α-substituted alkyl side-chain on the outcome of oxidative biotransformations by both the NADH- and NADPH-dependent Baeyer-Villiger monooxygenase activities partially purified from (+)-camphor-grown P. putida NCIMB 10007 was tested using a homologous series of 2-alkyl cyclopentanones (C_4-C_{10}). With both biocatalysts, all tested

substrates were biotransformed in a regio- and stereoselective manner (Table 2). The results, when expressed in terms of the recorded enantiomeric ratio (E_p) of the (-)-lactone products, suggested that whereas for the NADPH-dependent activity that could be expected to be predisposed towards monocyclic ketone substrates the effect of increasing the length of the alkyl side chain (= increasing lipophilic character) resulted in a progressive decrease in the enantioselectivity of the biocatalysts, the converse was true for the NADH-dependent activity that could be expected to be less predisposed towards monocyclic ketone substrates. In the only other precedent so far examined, the enantiomeric excess of the lactone products formed decreased as the length of the alkyl side-chain increased (C_5-C_{11}) using washed-cell preparations of two cyclohexanol-grown bacteria known to contain NADPH-dependent Baeyer-Villiger monooxygenases (Alphand et al., 1990).

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