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Oxidations by microbial NADH plus FMN-dependent luciferases from *Photobacterium phosphoreum* and *Vibrio fischeri*

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Abstract

The NADH plus FMN-dependent luciferase from *Photobacterium phosphoreum* NCIMB 844 has been shown to act as a Baeyer–Villiger monooxygenase able to perform regio-, and where relevant, enantioselective biotransformations of various xenobiotic aliphatic and alicyclic ketones by nucleophilic oxygenation. The useful lactone (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one was produced with high optical purity (> 95% ee). A similar biotransformation was recorded with the equivalent luciferase from *Vibrio fischeri* ATCC 7744.

Keywords: Biooxidations; Luciferase; Baeyer–Villiger monooxygenase; *Vibrio fischeri*; *Photobacterium phosphoreum*; Chiral lactones; Sulfoxidation

1. Introduction

Baeyer–Villiger monooxygenases are versatile NAD(P)H-dependent enzymes able to yield valuable synthons by two mechanistically different oxidations. The nucleophilic addition of an oxygen atom adjacent to a carbonyl group (ketone → lactone/ester) has been widely used to yield various useful chiral lactones [1–4]. The alternative electrophilic addition of oxygen to an organosulfide (sulfide → sulfoxide + / − sulfone) has been used less extensively to form chiral sulfoxides which can serve valuable synthetic roles as relay agents [5,6]. Because of considerations related both to the cost of the different nicotinamide nucleotides and the ease and convenience with which they can be recycled, there is much current interest in those Baeyer–Villiger monooxygenases that are dependent on NADH as the requisite coenzyme. Thus far, the most widely used NADH-dependent enzymes have been the two diketocamphane monooxygenase isozymes induced by the growth of *Pseudomonas putida* NCIMB 10007 on either enantiomer of camphor [3]. However, because of conflicting substrate specificities, the successful exploitation of these isozymic FMN-dependent linked enzyme systems requires the deployment of pure preparations of these particular biocatalysts [5,7].

One other known type of NADH-dependent Baeyer–Villiger monooxygenase whose potential for oxidative biotransformations has received scant attention to date is the luciferase (EC 1.14.14.3) present in some species of the bacterial genera *Vibrio* and *Photobacterium* [8]. With the exception of the reported ability of the luciferase from *P. phosphoreum* to yield sulfox-

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ides of undetermined enantiomeric excess from a related series of dialkyl sulfides including nonyl ethyl sulfide and decyl ethyl sulfide [9], there have been no prior reports of the ability of these otherwise well-studied FMN-dependent linked enzyme systems to undertake potentially useful oxidative biotransformations.

The purpose of this paper is to examine the ability of the luciferase present in whole-cell preparations of *Photobacterium phosphoreum* NCIMB 844 to undertake oxidative biotransformations with various alicyclic ketones and organosulfides. In addition, comparative tests have been conducted on the activity of a commercially-available partially purified preparation of luciferase from *V. fischeri* ATCC 7744.

2. Experimental

2.1. Microorganisms, maintenance and growth

Photobacterium phosphoreum NCIMB 844 was maintained on tryptone–yeast extract–glycerol–NaCl agar slopes. Cells were routinely cultured on a similarly-based liquid medium adjusted to pH 7.0 [10]. The inoculated flasks were grown in an orbital incubator (200 rpm) at 19°C. The cells were harvested at the time of maximum light emission which corresponded for both strains to the late log phase/early stationary phase of growth.

2.2. Measurement of emitted light

Light intensity was measured with a Perkin Elmer LS-5 fluorescence spectrophotometer operating in the luminescence mode.

2.3. Whole-cell biotransformations

Cells harvested during late log phase were washed once with 50 mM phosphate buffer pH 7.0, and then resuspended to 1/10th of the volume of the growth medium using the same buffer. Resultant washed whole-cell suspensions were used immediately or stored at −20°C until required. Unless otherwise stated, 55 μg of the substrate predissolved in ethanol (10 mg ml^{-1}) was added to the cell suspension to give a total volume of 1 ml, and the reaction mixture placed in an orbital incubator (200 rpm) at 25°C. Resultant biotransformations were monitored by routine sampling of aliquots (200 μl) which were extracted with an equal volume of ethyl acetate and then analysed (1 μl) by GC (BP1 capillary column, injector temperature 250°C, detector temperature 250°C, carrier gas (He) flow rate 0.75 ml min^{-1}). Samples chosen for more detailed examination were analysed using a GC equipped with a Lipodex E chiral capillary column using authentic samples of chemically-synthesised standards [3].

2.4. Biotransformations with partially purified luciferase

A preparation of luciferase from *Vibrio fischeri* ATCC 7744 was obtained from Sigma (Poole, UK), and partially purified as described by Gunsalus-Miguel et al. [11]. The enzyme (200 μl) was tested in a reaction mixture containing 2 mg NADH, 0.4 mg FMN, 55 μg substrate predissolved in ethanol (10 mg ml^{-1}), and 2 mg BSA made up to a total volume of 1 ml with 50 mM phosphate buffer pH 7.0.

The reaction mixture was treated as above for whole-cell biotransformations.

3. Results and discussion

In order to maximise the titre of luciferase in the washed-cell preparations subsequently used to undertake oxidative biotransformations, a culture of *P. phosphoreum* NCIMB 844 was monitored to establish the optimum time to harvest the cells from the growth medium (Fig. 1). When grown in batch culture, luciferase appears to be an inducible enzyme that is only present for a period of about 10 h during the late log phase of growth. A similar profile of activity

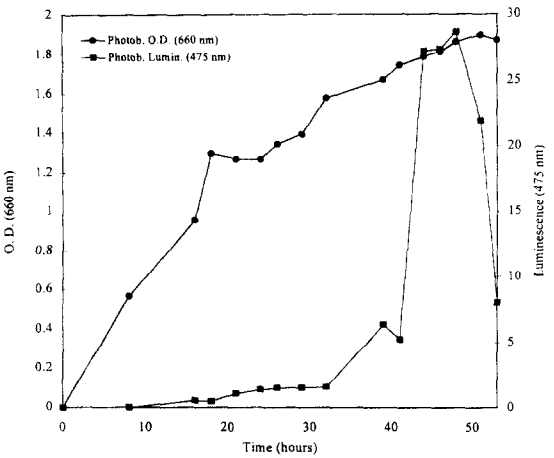

Fig. 1. Growth curve (O.D. 660 nm, —●—) and luminescence (475 nm, —■—) of *P. phosphoreum* NCIMB 844.

change has been recorded for other Baeyer–Villiger monooxygenases such as cyclohexanone monooxygenase in cyclohexanol-grown *Acinetobacter calcoaceticus* NCIMB 9871 [12] and the two diketocamphane monooxygenase isozymes in camphor-grown *Pseudomonas putida* NCIMB 10007 [7].

When challenged with dodecanal, the reported natural substrate of luciferase in *P. phosphoreum* [8], a washed-cell suspension of NCIMB 844 harvested in late log phase produced over 50% dodecanoic acid in 60 min. The reported ability of the enzyme to emit light [8] was not examined. In order to determine the activity of the enzyme with abiotic substrates, the initial test substrate chosen was 2-tridecanone because of its similarity to dodecanal. After 240 min, over 20% of the ketone had been metabolised, and an equivalent amount of dodecanoic acid was detected as the major product in the spent reaction mixture (Fig. 2a). This is interesting because, not only does it confirm for the first time that an aliphatic ketone such as 2-tridecanone can serve as an alternative substrate for the luciferase from *P. phosphoreum* NCIMB 844, but it implies that the enzymes has introduced an oxygen atom into the substrate between the carbonyl group and the terminal methyl group, the resultant methyl dodecanoate being a transitory intermediate that is subsequently hydrolysed by an esterase/lipase activity also known to be present in the whole-cell preparation. This implied regioselectivity of oxygenation by *P. phosphoreum* contrasts with previously reported Baeyer–Villiger monooxygenase-dependent biotransformations of 2-tridecanone by both *Pseudomonas aeruginosa*

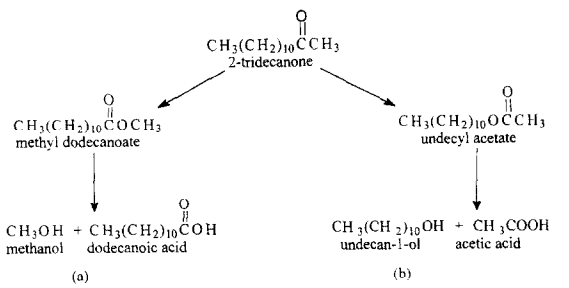

Fig. 2. Pathway of biotransformation of 2-tridecanone in (a) *P.phosphoreum* and (b) *Pseudomonas aeruginosa* and *Pseudomonas cepacia*.

Table 1
Biotransformation of ketone substrates by washed-cell preparations of *P. phosphoreum* NCIMB 844

Time	2 h		24 h	
Ketone substrate	% Ketone	% Lactone	% Ketone	% Lactone
Bicyclo[3.2.0]heptan-2-one [a]	81.4	9.7	72.6	13.8
Bicyclo[2.2.1]heptan-2-one	100	–	100	–
Camphor	100	–	100	–
2-Propylcyclobutanone	95.2	4.8	92.6	7.4
2-Hexylcyclobutanone	94.9	5.1	90.6	9.4
Tricyclo[4.2.1.$0^{3,9}$]nonan-2-one	100	–	100	–
Dihexylketone	100	–	100	–

[a] Remainder of the reaction mixture was the equivalent alcohol(s).

[13] and *Pseudomonas cepacia* [14], in which the major characterised metabolite was undecan-1-ol resulting from the alternative pattern of regioselective sub-terminal oxygenation of the ketone (Fig. 2b). This ability of the luciferase to promote migration of the smaller methyl group in this particular biotransformation may be related to the evolved role of this enzyme in promoting hydrogen migration during oxygenation of the natural substrate dodecanal.

Washed-cell suspensions of NCIMB 844 were then tested with a range of other aliphatic and alicyclic ketones (Table 1). Whereas no evidence of oxygenation was recorded with a symmetrical aliphatic ketone, plus tricyclic and bicyclic [2.2.1] ketones, some lactone formation was recorded with two substituted small-ring monocyclic ketones, and a bicyclic [3.2.0] ketone. More detailed analysis of the latter biotransformation (Table 2) provided the first evidence that *P. phosphoreum* NCIMB 844 possesses an active Baeyer–Villiger monooxygenase that exhibits elements of enantio- as well as regioselectivity, albeit this facet of specificity is only evident in yielding the bridgehead lactone (−)-(1*S*,5*R*)-2-oxabicyclo[3.3.0]oct-6-en-3-one. This result is similar to that obtained with the same substrate using whole-cell preparations of some dematiaceous fungi of the genera *Dreschlera* and *Curvularia* [15]. A commercially-available partially-purified luciferase from another bacterium, *Vibrio fischeri* ATCC 7744 was then tested with the same bicyclic ketone to examine aspects of the regio- and enantioselectivity of this type of Baeyer–Villiger monooxygenase. The result obtained (Table 2) is very similar to that obtained with *P. phosphoreum* NCIMB 844, providing indirect evidence to support the role of this enzyme the whole-cell experiments. The small enantiomeric excess recorded with the (−)-(1*R*,5*S*)-3-oxabicyclo[3.3.0]oct-6-en-2-one formed by the isolated enzyme may reflect either a minor difference between the two luciferases, or a difference in the time course of the biotransformation.

Despite the above results, when presented with a range of dialkylsulfides (decyl methyl-, dodecyl methyl-, nonyl ethyl-) and alkyl aryl sulfides (methyl phenyl-, ethyl phenyl-, methyl tolyl-, isopropyl tolyl-) for up to 24 h, washed-cell preparations of *P. phosphoreum* NCIMB 844 and the commercially-available luciferase from *V. fischeri* ATCC 7744 both failed to form any detectable sulfoxides or sulfones, the anticipated products from the electrophilic oxygenation of these heteroatom-containing compounds. This contrasts with the previously reported ability of cells of an unnamed species of *P. phosphoreum* to produce sulfoxides from dialkyl sulfides, including nonyl ethyl sulfide, one of the substrates specifically tested with NCIMB 844.

In conclusion, this is the first evidence that FMN plus NADH-dependent luciferases can perform regio- and enantioselective biotransfor-

Table 2
Biotransformation of racemic bicyclo[3.2.0]hept-2-en-6-one by luciferases from *Photobacterium phosphoreum* NCIMB 844 and *Vibrio fischeri* ATCC 7744

	Washed-cells of *P. phosphoreum*	Part pure luciferase from *V. fischeri*
Ketone substrate		
[S] $\mu g\ ml^{-1}$	55	55
Reaction time h	24	20
% remaining	88.3	83.5
Lactone products		
% formed	11.7	16.5
ee_p %	(−)-2-oxa, > 95	(−)-2-oxa, > 95
	3-oxa, racemic	(−)-3-oxa, 15

mations of abiotic carbonyl-containing substrates. However, at present the potential value of this type of Baeyer–Villiger monooxygenase in undertaking biotransformations to yield useful chiral synthons appears to be limited both by the low activity and narrow substrate range of the enzymes tested to date.

References

[1] V. Alphand, A. Archelas and R. Furstoss, Biocatalysis 3 (1990) 73.
[2] A.J. Carnell, S.M. Roberts and A.J. Willetts, J. Chem. Soc., Perkin Trans., 1 (1991) 2385.
[3] R. Gagnon, G. Grogan, S.M. Roberts and A.J. Willetts, Chem. Soc., Perkin Trans., 1 (1995) 1505.
[4] B. Adger, T.M. Bes, G. Grogan, R. McCague, S. Pedragosa-Moreau, S.M. Roberts, R. Villa, P.W.H. Wan and A. Willetts, J. Chem. Soc., Chem. Commun., (1995) 1563.
[5] G. Carrea, B. Redigolo, S. Riva, S. Colonna, N. Gaggero, E. Battistel and D. Bianchi, Tetrahedron Asymm., 3 (1992) 1063.
[6] J. Beecher, P. Richardson, S. Roberts and A. Willetts, Biotechnol. Lett., 17 (1995) 1069.
[7] R. Gagnon, G. Grogan, M. Levitt, S.M. Roberts, P.W.H. Wan and A. Willetts, J. Chem. Soc., Perkin Trans., 1 (1994) 2537.
[8] J.W. Hastings and K.H. Nealson, Ann. Rev. Microbiol., 31 (1977) 549.
[9] F. McCapra and D. Hart, J. Chem. Soc., Chem. Commun., (1976) 273.
[10] D. Riendeau and E. Meighen, J. Biol. Chem., 254 (1979) 7488.
[11] A. Gunsalus-Miguel, E.A. Meighen, M.Z. Nichol, K.H. Nealson and J.W. Hastings, J. Biol. Chem., 247 (1972) 398.
[12] H. Sandey and A. Willetts, Biotechnol. Lett., 14 (1992) 1119.
[13] J.W. Forney and A.J. Markovetz, J. Bacteriol., 102 (1970) 281.
[14] A.C. Shum and A.J. Markovetz, J. Bacteriol., 118 (1974) 890.
[15] A.J. Carnell and A.J. Willetts, Biotechnol. Lett., 14 (1992) 17.