Monatshefte für Chemie 131, 673–685 (2000)

Monatshefte für Chemie
Chemical Monthly

Bacterial Biotransformation of Isoprene and Related Dienes

Derek R. Boyd,[1,*], **David Clarke**[1], **Marcel C. Cleij**[1], **John T. G Hamilton**[2], and **Gary N. Sheldrake**[1]

[1] School of Chemistry and QUESTOR Centre, The Queen's University of Belfast, Belfast BT9 5AG, UK
[2] Food Science Division, Department of Agriculture for Northern Ireland, Belfast BT9 5PX, UK

Summary. The bacterium *Pseudomonas putida* ML 2 was used in the oxidative biodegradation of the acyclic dienes isoprene, *trans*-piperylene, *cis*-piperylene, and 1,3-butadiene. Regioselective dioxygenase-catalyzed dihydroxylation of alkenes yielded vicinal diols in the preferred sequence monosubstituted > *cis*-disubstituted > *gem*-disubstituted > *trans*-disubstituted. The isolated diol metabolites had an excess of the *R* configuration (9–97% *ee*), and further diol oxidation was controlled by addition of propylene glycol as an inhibitor. Stereoselectivity using the ML2 strain resulted from both enzymatic asymmetric alkene dihydroxylation and kinetic resolution of diols. Enantioselective oxidation of the allylic secondary alcohol group of *R* configuration yielded the corresponding unsaturated ketoalcohol; the residual diol was recovered with a large excess ($\geq$ 93% *ee*) of the *S* configuration. In addition to the enzymatic diene oxidation steps yielding unsaturated diols and ketoalcohols, evidence was also found of enzymatic alkene hydrogenation to yield saturated ketoalcohols and diols.

Keywords. Asymmetric dihydroxylation; Chiral resolution; Dioxygenases; Enzymes; Isoprene.

Introduction

Methane and isoprene **1** are reported to be present in considerable quantities in the earth's atmosphere and are the most abundant volatile hydrocarbons in the environment [1,2]. Biogenic isoprene is produced in large quantities from plants (5×10^{14} g year^{-1}), particularly in the tropical rain forests and from the oceans [1–3]. It has been estimated that isoprene is responsible for *ca*. 30% of the total non-methane organic compounds (NMOCs) emitted into the environment in North America each year [1]. Isoprene can readily be involved in radical reactions under the influence of UV radiation and will strongly influence atmospheric chemistry. The isoprene radicals produced in the earth's atmosphere can thus undergo photochemical reactions with nitrogen oxides (NO, NO_2) from combustion sources resulting in the formation of ozone in the lowest layer of the atmosphere. Whereas the fate of isoprene in atmospheric chemistry has been studied extensively [4–6],

* Corresponding author

its effect on living systems appears to have been mainly confined to animals [7–10]. Isoprene is also produced in large quantities by the petrochemical industry as a monomer for *cis*-1,4-polyisoprene elastomer, and in this context the potential risk of human exposure to higher concentrations of anthropogenic isoprene than may normally occur in nature through biogenic isoprene has initiated toxicological studies [7–10]. These animal studies have shown that the first step in the metabolic pathway of isoprene degradation involves the monooxygenase-catalyzed formation of mutagenic epoxides. Liver microsomal cytochrome P-450 enzymes and dioxygen were found to be responsible for both *mono*- and *bis*-epoxidation of **1**. Hydrolysis of the isoprene monoepoxide metabolites gave the corresponding diols **2** and **3**. Apart from these earlier reports on the metabolism of **1** in eucaryotic cells [7–9], to our knowledge the metabolic fate of isoprene or related butadienes in procaryotic (bacterial) cells has not been reported[1]. Since both plants, a major biogenic source of isoprene, and bacteria, a major source of hydrocarbon-degrading enzymes, are found to co-exist within soil, metabolism by soil bacteria could play an important role as a biological sink controlling the concentration of biogenic isoprene released into the environment. This has indeed recently been established for the first time using both *in vitro* and *in vivo* studies of the microbial consumption of atmospheric isoprene by aerobic soil bacteria in a temperate forest soil [2]. However, this microbial consumption programme did not address the question of biodegradation pathways or the nature of metabolic intermediates and is thus complementary to the results obtained in the present study.

Dioxygenase-catalyzed *cis*-dihydroxylation of mono- and polycyclic aromatic compounds using *Pseudomonas putida* UV4 has been carried out in these laboratories [11–15]. Dioxygenase enzymes are also known to be present in two wild-type strains of *P. putida*, *i.e*. ML 2, a source of benzene dioxygenase (BDO), and NCIMB 8859, a source of naphthalene dioxygenase (NDO). UV4, a mutant strain of *P. putida* contains a dioxygenase (toluene diooxygenase, TDO), but is deficient in the *cis*-diol dehydrogenase enzyme which is responsible for the conversion of arene *cis*-dihydrodiols to catechols in the corresponding wild-type strain. Non-aromatic conjugated cyclic diene and triene substrates of different ring sizes have been reported to undergo stereoselective alkene *cis*-dihydroxylation in the presence of *P. putida* UV4 [15]. It has also been demonstrated that a cyclic aliphatic alkene can be dihydroxylated by toluene dioxygenase from *P. putida* F1 [17]. Dioxygenase-catalyzed asymmetric dihydroxylation of a series of volatile acyclic conjugated dienes, elucidation of the structure and stereochemistry of the resultant diols, and the metabolic sequence involved in the formation of other derived metabolites formed from this class of NMOCs are presented and discussed here.

Results and Discussion

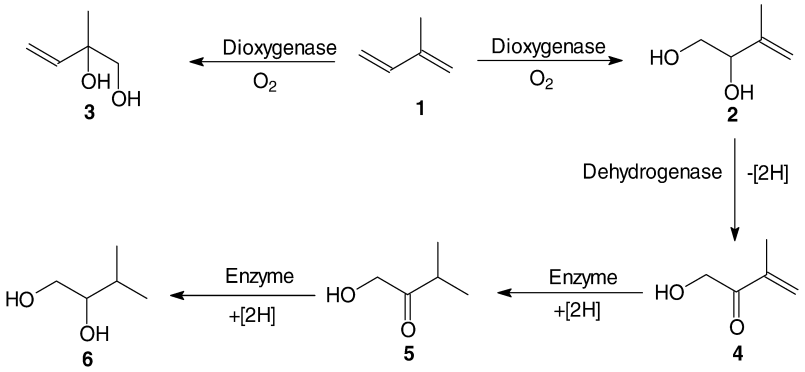
A major objective of this study was to elucidate the sequence of steps involved in the metabolism of dienes **1**, **7**, **15**, and **19** by different strains of the soil bacterium *P. putida* (Schemes 1–4) and to characterize and, where possible, to assign

[1] See note added in proof

stereochemistry to the metabolites. Due to the volatile nature of dienes **1** (b.p.: 34°C), **7** (b.p.: 42°C), **15** (b.p.: 44°C), and **19** (b.p.: –5°C) and their limited solubility in the aqueous culture medium, the isolated yields were expected to be quite low using our reported standard biotransformation conditions [11, 14, 16]. However, by modification of these biotransformation procedures and utilization of more sensitive assay methods, it was possible to identify and stereochemically assign the bioproducts. This was achieved by using a lower incubation temperature (4°C) and higher cell densities in shake flasks (optical density values of *ca*. 10 at 600 nm), by addition of a competitive inhibitor of the diol dehydrogenase (propylene glycol) during biotransformations, and by application of chiral stationary phase GC/FID, GC/MS, and GC/IR methods for analysis of culture supernatants. Despite these modifications the isolated yields of bioproducts were relatively low (< 10%) and thus currently of limited synthetic value.

Based on reported results using *P. putida* UV4 and a series of cyclic diene substrates where the corresponding *vic*-diols were obtained [15], diols **2**, **3**, **8**, **9**, **16**, **17**, and **20** were expected to be the initial metabolities from the acylic dienes **1**, **7**, **15**, and **19**. Authentic samples of these diols were prepared by osmylation (OsO_4) of the corresponding dienes **1**, **7**, **15**, and **19** and were identified by NMR spectroscopy, GC/MS, and GC/IR analysis and by comparison with literature data. Enantiomerically enriched (7–79% *ee*) samples of these diols were also obtained by catalytic asymmetric dihydroxylation using osmium tetroxide in the presence of a chiral ligand (AD-mix-β). This chemical method of asymmetric dihydroxylation has been studied extensively by *Sharpless et al*. [18, 19] using a range of alkenes and dienes, and a model has been developed which allows tentative assignments of the absolute configurations of the product diols to be made.

In view of its importance in the environment, the biotransformation of isoprene (**1**) was studied more rigorously than that of butadiene (**19**) or the diene isomers **7** and **15** (Table 1). Using the ML2 and 8859 strains of *P. putida* as sources of BDO and NDO, respectively, and **1** as substrate, diols **2** and **3** were isolated as initial metabolites. These diols could not be detected using GC/MS analysis and the TDO enzyme present in the UV 4 strain (Table 1). The regioselectivity of dioxygenase-catalyzed dihydroxylation was consistently found to favour attack at the monosubstituted alkene bond relative to the *gem*-disubstituted alkene bond



Scheme 1

Table 1. Concentrations of bioproducts **2**, **3**, **5**, and **6**, respectively, and enantiomeric excess values (*ee*) obtained from the biodegradation of isoprene (**1**) by the ML2, UV4, and 8859 strains of *P. putida*

	Propylene glycol %v/v	[**3**]/m*M*	*ee* (**3**)	[**2**]/m*M*	*ee* (**2**)	[**2**]/[**3**]	[**5**]/m*M*	[**6**]/m*M*
ML2[a]	0	1.5	40%	6.3	34%	4.2	2.0	0.2
ML2[a]	15	1.5	44%	6.4	16%	4.3	0	0
UV4[b]	0	0		0			0	0
UV4[b]	15	0.4	45%	0.9	9%	2.3	0	0
8859[c]	0	0.06	14%	0.2	12%	3.3	0	0
8859[c]	15	0.2	16%	0.6	12%	3.0	0	0

[a] BDO; [b] TDO; [c] NDO

(**2/3** = 3.3–4.2). The highest yields of diols **2** (6.3 m*M*) and **3** (1.5 m*M*) were obtained using the ML2 strain. Using the latter strain the yield of diol **2** was further diminished by oxidative metabolism under the catalytic influence of an alcohol dehydrogenase enzyme to yield ketoalcohol **4** which was further metabolized to yield the saturated ketoalcohol **5** and saturated diol **6**. Since the tertiary alcohol group in diol **3** was unaffected by the alcohol dehydrogenase, no further metabolites were found.

It had earlier been found that propylene glycol can act as a competitive inhibitor both for a diol dehydrogenase enzyme isolated from the wild-type ML2 strain (*ca*. 36% activity compared with the parent substrate, benzene *cis*-glycol) [20] and for a diol dehydrogenase present in an *E. coli* recombinant strain cloned from the ML2 strain (*ca*. 189% activity compared with the parent substrate, benzene *cis*-glycol) [21]. Hence, use of the ML2 strain and addition of propylene glycol was expected to reduce the rate of formation of ketoalcohol and to give a more reliable estimate of both regio- and stereoselectivity resulting from dioxygenase-catalyzed dihydroxylation. The effect of adding propylene glycol (15% v/v) to biotransformations of **1** with the three *P. putida* strains is evident from Table 1. Whereas the yields of diols **2** and **3** increased slightly using the wild-type ML2 and 8859 strains, ketoalcohol formation was eliminated when the ML2 strain was used with propylene glycol as additive. A more marked effect was observed with the UV4 strain where formation of **2** and **3** was increased to levels which were readily detectable (0.4 and 0.9 m*M*, respectively). This result suggests that the mutant UV 4 strain, although lacking the arene *cis*-dihydrodiol dehydrogenase enzyme, may contain a further type of dehydrogenase which can oxidize acyclic substrates like **2** and **3** and which is also effectively inhibited by propylene glycol.

The stereoselectivity of the enzyme-catalyzed dihydroxylation process was determined by use of a chiral stationary phase (CSP) GC column to resolve the enantiomers followed by identification using mass and IR spectroscopy. CSPGC/FID analysis was used to determine *ee* values of the samples of diols **2**, **3**, **8**, **9**, **16**, **17**, and **20** obtained using both chemical (OsO_4 and AD-mix-β) and biological (dioxygenase) asymmetric dihydroxylation catalysts. The absolute configurations were tentatively assigned on the basis of (*i*) the preferred configurations of diols produced using the β-form of AD-mix and the model developed by *Sharpless* for

Table 2. Enantiomeric excess, absolute configuration, and ratio of diol isomers **2**, **3**, **8**, **9**, **16**, **17**, and **20** obtained by asymmetric dihydroxylation using AD-mix-β

Diol	*ee* (%)	Abs. config.	Isomer ratio
3	21	2*R*	
2	64	2*R*	2.0 (**2/3**)
9	79	2*R*, 3*R*	
8	65	2*R*	0.36 (**8/9**)
17	7	2*R*, 1*S*	
16	44	2*R*	1.8 (**16/17**)
20	45	2*R*	

predicting the enantiofacial selectivity of the dihydroxylation process for different alkene types [18, 19] (Table 2) and (*ii*) the elution sequence observed using cyclodextrin-based CSPGC columns. The late eluting enantiomer of each diol was assigned the *R* configuration at the allylic chiral centre.

The enantiomeric excess values observed for the diols **2**, **3**, **8**, **9**, **16**, **17**, and **20** obtained by asymmetric dihydroxylation using β-AD-mix (H_2O:*t*-BuOH, 20°C) were found to be in the range of 7–79% with the lowest value being associated with dihydroxylation of a *cis*-disubstituted alkene bond as predicted from earlier studies [18, 19]. Using the BDO enzyme (from ML2), **1** was asymmetrically dihydroxylated to yield diols **2** (16% *ee* in the presence of propylene glycol) and **3** (44% *ee* in the presence of propylene glycol) with an excess of the *R* enantiomer. This enantiomeric excess increased (34% *ee*) for diol **2** when the diol dehydrogenase enzyme was present. When the TDO (from UV4) and NDO (from 8859) enzymes were used, the stereoselectivity was again generally low for diols **2** (9–12%) and **3** (14–45%) but also showed a preference for the *R* configuration.

When the TDO- and NDO-containing strains of *P. putida* (UV4 and 8859) and **1** as substrate were studied, the diols **2** and **3** were formed without evidence of further metabolism to the corresponding ketoalcohol **4** or their derivatives. Similarly, when the BDO-containing strain (ML2) was used, only a trace of ketoalcohol **4** was detected by GC/MS and GC/IR analysis; however, in this case significant quantities of the saturated ketoalcohol **5** (2.0 m*M*) and diol **6** (0.2 m*M*) were identified. The unexpected formation of the latter two metabolities was confirmed by addition of an authentic sample of the unsaturated ketoalcohol **4** to ML2 cultures. The alkene bond in compound **4** was found to be rapidly hydrogenated to yield the ketol **5** which was in turn slowly reduced to diol **6**. Further evidence for the metabolic sequence alkene dihydroxylation → allylic alcohol oxidation → alkene hydrogenation → saturated ketone reduction (Scheme 1) was obtained from a time-course study over 24 h which showed the unsaturated diol **2** to be the most abundant metabolite during the biotransformation, whereas the saturated diol **6** was only detected after 12 h (Fig. 1).

A time-course study of the biotransformation of the racemic *cis*-diol metabolites of isoprene (**2** and **3**) using the ML2 strain over a 24 h period again showed that the concentration of **3** remained constant, whereas the proportion of **2** decreased as the amounts of **5** and **6** increased (Fig. 2). CSPGC along with both FID and MS detection demonstrated that the *R* enantiomer of **2** was selectively

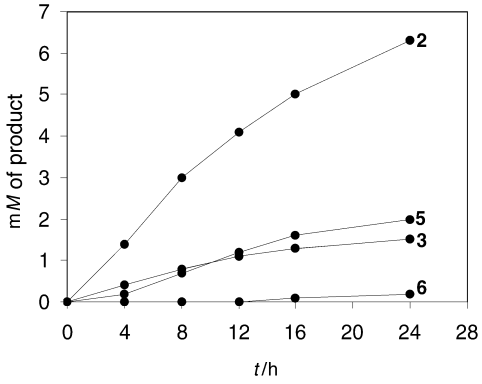


Fig 1. Time-course study of the formation of metabolites **2**, **3**, **5**, and **6** from isoprene (**1**) using *P. putida* ML2

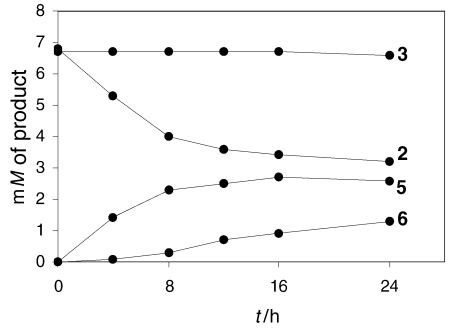
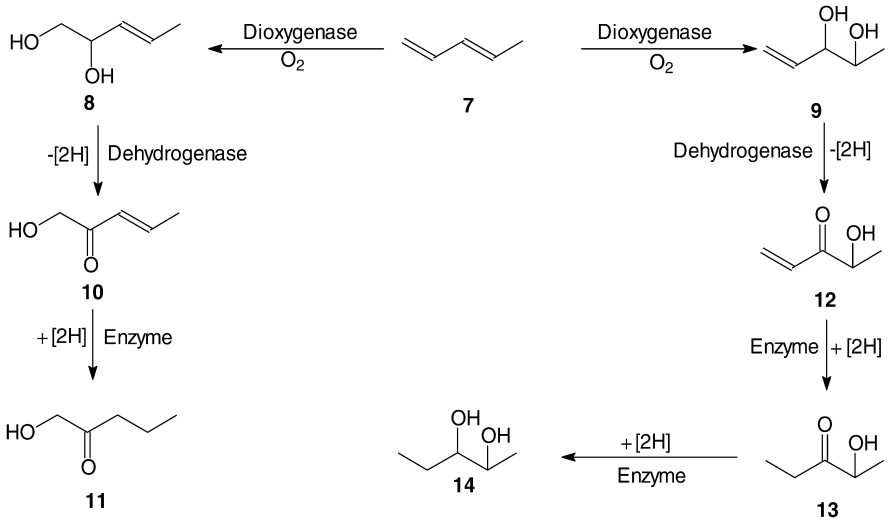


Fig 2. Time-course study of the formation of metabolites **5** and **6** from racemic diols **2** and **3** using *P. putida* ML2

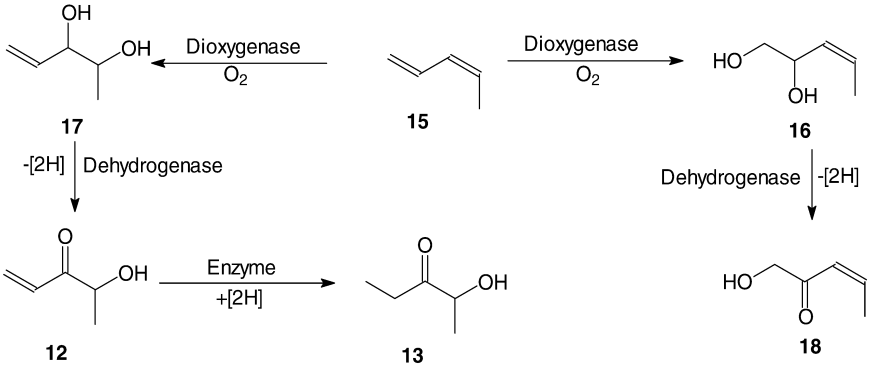
oxidized to ketoalcohol **4**, and after 24 h the residual diol was mainly of the *S* configuration (95% *ee)*. The enantiopurity of **2** obtained during the biotransformation of **1** is thus due to the combined effect of asymmetric dihydroxylation and kinetic resolution each favouring the *R* configuration and is an example of enantiocomplementarity. It has been established from metabolic studies of arene substrates [20] that the ML2 strain of *P. putida* contains both benzene dioxygenase and *cis*-diol dehydrogenase enzymes which could in the present context account for the formation of diols **2** and **3** and the ketoalcohol **4**. The nature of ML2 enzymes responsible for the final two steps, *i.e*. hydrogenation to yield ketol **5** and ketone reduction to yield diol **6**, has not been established. Formation of the saturated ketoalcohol **5** and saturated diol **6** indicates that enzymatic hydrogenation can occur on the *gem*-disubstituted alkene.

Based upon the higher yields of diol metabolites obtained and the unexpected results resulting from both oxidation (**2** → **4**) and reduction steps (**4** → **5** → **6**) using the ML2 strain with **1** as substrate, the related diene substrates *trans*-piperylene (**7**), *cis*-piperylene (**15**), and 1,3-butadiene (**20**) were also examined using this strain (Schemes 2–4). In accordance with expectations, the *trans* isomer **7** was found to yield the corresponding diol metabolites **8** (0.6 m*M*) and **9** (0.3 m*M*) with regioselectivity favouring the monosubstitued alkene bond, *i.e*. the rate of



Scheme 2

formation of diol **8** was *ca*. five-fold faster compared with diol **9**. In the presence of propylene glycol, which was added to inhibit further oxidative metabolism, both diols were found to be formed in relatively low enantiomeric excess (**8**, 38% *ee*; **9**, 33% *ee*). Using the methods discussed earlier, the absolute configurations were tentatively assigned as 2*R*, 3*R*, and 2*R* for diols **9** and **8**, respectively. In the absence of propylene glycol, diol **8** was rapidly metabolized in a stereoselective manner to yield the unsaturated ketoalcohol **10** (0.5 m*M*) and the saturated ketoalcohol **11** (1.6 m*M*). The residual diol **8** was found to be of high enantiopurity (93% *ee)* and of the 2*S* configuration. In a similar manner, diol **9** was further biotransformed but at a slower rate (presumably *via* the ketoalcohol **12** which was undetected) to the saturated ketoalcohol **13** (0.1 m*M*) and the diol **14** (0.1 m*M*). The formation of the saturated ketoalcohols **11** and **13** and of the saturated diol **14** indicates that the enzyme-catalyzed hydrogenation can occur on both monosubstituted and *trans*-disubstituted alkenes. The remaining diol **9** was found to be present with an excess of the 2*S*, 3*S* enantiomer (15% *ee*). Evidence in support of the metabolic sequences **7** → **8** → **10** →**11** and **7** → **9** → **12** → **13** → **14** was obtained by addition of the

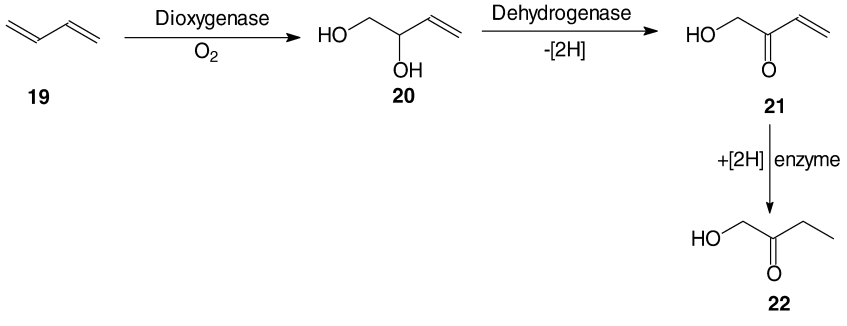


Scheme 3

racemic diols **8** and **9** individually as substrates and the detection of the corresponding metabolites **10**, **11**, **13**, and **14**. The formation of both diols **8** and **9** with an excess of the *R* allylic alcohol chiral centre from asymmetric dihydroxylation (in the presence of an inhibitor) and the preferential oxidation of this enantiomer to leave mainly the *S* enantiomer after kinetic resolution again provides an example of enzymatic enantiocomplementarity, *i.e*. the use of enzymes to produce either enantiomer.

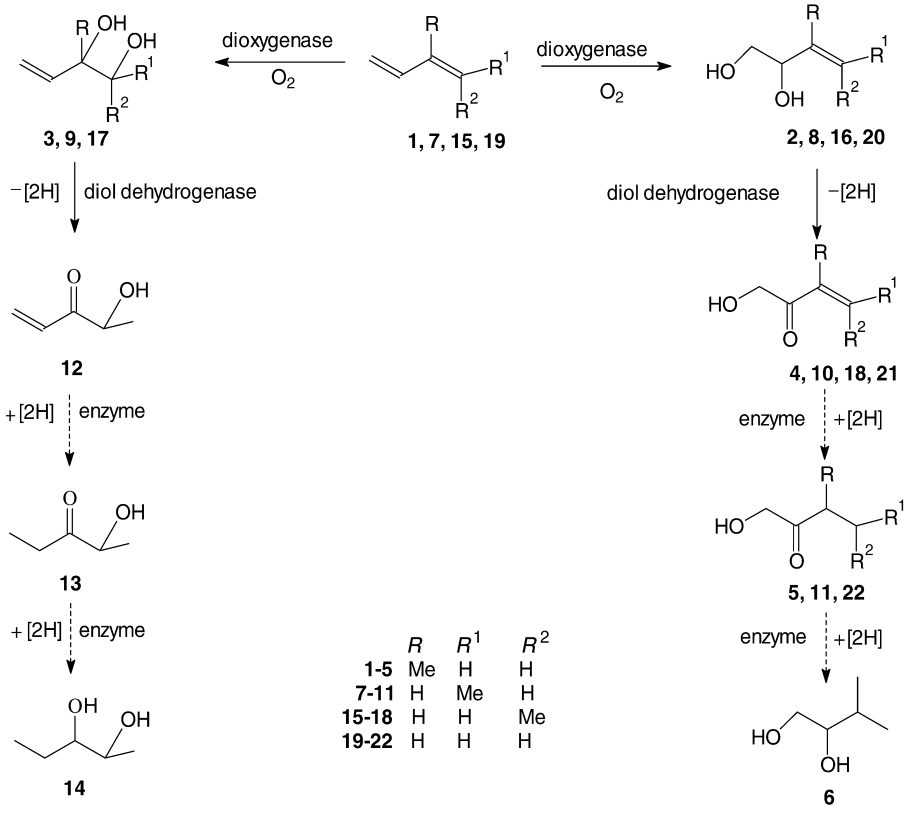
The biotransformation of *cis*-1,3-pentadiene (**15**) using the ML 2 strain in the presence of propylene glycol yielded the expected diol metabolites **16** (1.1 m*M*; 74% *ee*) and **17** (1.2 m*M*; 70% *ee*). As before, the dioxygenase-catalyzed dihydroxylation appeared to favour diol formation at a monosubstituted alkene bond over a disubstituted alkene bond (*ca*. 3-fold rate difference) and of an enantiomer having an *R* configuration at the allylic chiral centre. Preferential removal of the allylic *R* enantiomer from diols **16** and **17** was again observed in the absence of propylene glycol inhibitor, leading to a residual sample of diol **16** (26% *ee*, 2*R*) and diol **17** (97% *ee*, 2*S*, 3*R*). Diol **16** proved to be a particularly good substrate yielding only the unsaturated ketoalcohol product **18** (4.4 m*M*), whereas diol **17** yielded the saturated ketoalcohol **13** (1.0 m*M*), probably *via* reduction of the saturated ketoalcohol **12**. It is noteworthy that while enzymatic hydrogenation was found on the monosubstituted alkene group in the ketoalcohol **12**, the *cis*-disubstituted alkene group in metabolite **18** was not hydrogenated.

On account of its volatility, butadiene (**19**) proved to be more difficult to study experimentally using the ML 2 strain of *P. putida*. However, by bubbling gaseous **19** into heptamethylnonane (0°C) and adding the resulting solution to the culture medium (4°C), dioxygenase-catalyzed dihydroxylation was found to yield diol **20** (0.2 m*M*) with a relatively low preference for the 2*R* enantiomer (25% *ee*). In the absence of the diol dehydrogenase inhibitor the 2*R* enantiomer of diol **20** was selectively oxidized to yield the saturated ketoalcohol **22** (0.3 m*M*). The unreacted diol **20** was found to be mainly of 2*S* configuration (95% *ee*). Thus, the enantiocomplementarity previously observed during the biotransformations of dienes **1**, **7**, and **15** to yield the corresponding chiral diols was again observed for diene **19**. It is noteworthy that the biotransformation of monosubstituted benzene and naphthalene substrates by *P. putida* mutant strains has been reported [22] to yield a single *cis*-dihydrodiol enantiomer and that the closely coupled *cis*-diol dehydrogenase was found to act exclusively on the same enantiomer.



19 20 21 22

Scheme 4



Scheme 5

Conclusions

Bacterial biodegradation of isoprene (**1**) and related diene substrates **7**, **15**, and **19** using three different strains of the soil bacterium *P. putida* has generally been found to proceed *via* the dioxygenase-catalyzed asymmetric dihydroxylation of an alkene group to yield all of the possible diols **2**, **3**, **8**, **9**, **16**, **17**, and **20**, each with an excess of the allylic *R* enantiomer. The rate of dihydroxylation appeared to be faster on monosubstituted rather than disubstituted alkene bonds. Further diol dehydrogenase-catalyzed oxidation at the secondary allylic hydroxyl group to yield unsaturated ketoalcohols **4**, **10**, **12**, **18**, and **21** was found to occur preferentially on the allylic *R* enantiomer, thus leading to the recovery of diols having an excess of the allylic *S* configuration. The unsaturated ketoalcohol bioproducts were further metabolized through a reductive process involving enzymatic hydrogenation to yield saturated ketoalcohols **5**, **11**, **13**, and **22** and ketone reduction to yield diols **6** and **14**. A general biodegradation pathway to account for the formation of all metabolites from dienes **1**, **7**, **15**, and **19** is shown in Scheme 5.

Experimental

NMR analyzis was carried out using both Bruker Avance DPX-300 (300 MHz) and DRX-500 (500 MHz) instruments. All NMR samples were run in $CDCl_3$ using internal *TMS* as reference.

Coupling constants are expressed in Hz. GC/MS analyses were carried out using a Hewlett Packard 6890 gas chromatograph directly linked to a Hewlett Packard 5973 Mass Selective Detector (MSD). Mass spectroscopic data were obtained by operating the MSD in the electron impact scanning mode measuring ion currents between $m/z = 30$ and 400. The instrument was also operated in the chemical ionization mode using methane as reagent gas (CIMS) and measuring ion currents between $m/z = 65$ and 400 to determine the molecular ion for each compound. GC/IR analysis was completed using a Hewlett Packard 5890A gas chromatograph/5965B – infrared detector/mass selective detector (GC/IRD/MSD) system with the IRD/MSD in the series configuration. The IRD scanned the range of 750–4000 cm^{-1}. The gas chromatographs which were fitted with Supelco fused silica capillary columns (30 m × 0.25 mm i.d. with a 0.25μm Beta Dex-120 phase) were equipped with autosamplers. The ovens were programmed at 65°C for 1 min and then ramped at 10°C min^{-1} to 220°C and held at this temperature for 5 min. Samples (1 mm^3) were injected in the splitless mode into the injector port which was maintained at 250°C. Helium was employed as the carrier gas at a flow rate of 1 $cm^3 \cdot min^{-1}$. Quantification of analytes was performed using gas chromatography with flame ionisation detection (GC/FID). Chromatographic separations for FID were performed using the following conditions: system A: 10 m Chrompak CP-Chirasil-DEX CB fused silica column (3.3 psi, isothermal at 100°C); system B: 10 m Chrompak CP-Chirasil-DEX CB fused silica column (3.3 psi, isothermal at 110°C); system C: 30 m Supelco-β-DEX 225 column (35 psi, isothermal at 100°C); system D: 30 m Supelco-β-DEX 225 column (19 psi; isothermal at 100°C).

The dienes **1**, **7**, **15**, and **19** (> 97% purity) were purchased from Aldrich and used as substrates without further purification. Diol **20** in racemic form was available from Fluka.

Authentic samples of the known diol metabolites **2**, **3**, **8**, **9**, **16**, and **17** were obtained by osmylation. This hydroxylation procedure proved to be rather difficult due to the reactivity of the initially formed diols in the presence of OsO_4 and hence resulted in relatively low yields. In a typical general procedure a solution of diene (50 m*M*) and N-methylmorpholine-N-oxide (55 m*M*) in a mixture of acetone (50 cm^3) and water (1.5 cm^3) was added to a catalytic amount of OsO_4. After stirring overnight at ambient temperature the solution was concentrated *in vacuo*. The residual oil was dissolved in CH_2Cl_2, dried over $MgSO_4$, concentrated, and purified by flash chromatography (silica gel) starting with CH_2Cl_2 (100%) as eluent and gradually increasing the acetone concentration to 10% v/v. The product was in each case an oil containing a racemic mixture of the two isomeric diols (**2/3 8/9**, **16/17**). The yield of diols obtained were in the range of 20–30%. The structures of the individual diol metabolites were confirmed by comparison of IR, ^{1}H NMR, and MS data comparison with those of authentic samples. The ML 2 strain of *P. putida* was obtained from University of Warwick (Prof. *H. Dalton*); the NCIMB 8859 strain was available from the Collection of Industrial and Marine Bacteria Ltd., Aberdeen, UK. Since authentic samples of the *cis*-diols **2/3**, **8/9**, **16/17**, and **20** were available and since these were always the initial (and often the major) metabolites, a summary of the relevant spectroscopic and chromatographic data for each of these known racemic compounds is provided [4, 8].

(2R/2S)-1,2-Dihydroxy-2-methyl-3-butene (**3**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.3 (3H, s, Me), 3.45 (2H, d, $J = 11.0$, CH*H*OH), 3.50 (1H, d, $J = 11.0$, C*H*HOH), 3.73 (2H, bs, OH), 5.2 (1H, dd, $J = 10.4$, 1.0, =CH*H*), 5.35 (1H, dd, $J = 17.6$, 1.0, =C*H*H), 5.9 (1H, dd, $J = 10.4$, 17.6, –CH=) ppm; GC/IR: $\nu = 1644$, 3095 (H_2C=CH), 3626 (OH) cm^{-1}; GC/CIMS: $MH^+ = 103$; GC/EIMS: $m/z =$ 71 (100), 43 (82), 41 (27), 31 (18), 87 (3); CSPGC/FID: system A: 2*S*-**3** (7.8 min), 2*R*-**3** (8.1 min).

(2R/2S)-1,2-Dihydroxy-3-methyl-3-butene (**2**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.75 (3H, s, Me), 3.55 (1H, dd, $J = 11.0$, 7.3, *H*HCOH), 3.67 (1H, dd, $J = 11.0$, 2.8, H*H*COH), 4.18 (1H, dd, $J = 7.3$, 2.8, C*H*OH), 4.96 (1H, d, $J = 0.8$, =CH*H*), 5.06

(1H, d, $J = 0.8$, =C*H*H) ppm; GC/IR: $v = 1649$, 3085 (C=CH_2), 3636 (OH) cm^{-1}; GC/EIMS: $m/z = 102$ (M^+, 0.5), 71 (100), 43 (92), 41 (59), 39 (43), 31 (25), 87 (1); CSPGC/FID: system A: 2*S*-**2** (15.1 min), 2*R*-**2** (16.3 min).

(2S,3S/2R,3R)-2,3-Dihydroxy-4-pentene (**9**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.1 (3H, d, $J = 6.3$, Me), 3.0 (2H, bs, 2×OH), 3.65 (1H, dq, $J = 6.3$, 6.3, C*H*OHMe), 3.7 (1H, dd, $J = 7.0$, 6.3, C*H*OH), 5.24 (1H, d, $J = 10.4$, =CH*H*), 5.36 (1H, d, $J = 17.0$, =C*H*H), 5.85 (1H, ddd, $J = 17.0$, 10.4, 6.3, H_2C=C*H*) ppm; GC/IR: $v = 1644$, 3090 (H_2C=CH), 3618 (OH) cm^{-1}; GC/EIMS: $m/z = 45$ (100), 58 (78), 57 (56), 43 (28), 31 (14); CSPGC/FID: system B: 2*S*,3*S*-**9** (4.84 min), 2*R*-3*R*-**9** (5.23 min).

Trans-(2R/2S)-1,2-Dihydroxy-3-pentene (**8**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.7 (3H, d, $J = 6.5$, Me), 2.3 (2H, bs, 2×OH), 3.3 (1H, dd, $J = 11.0$, 7.7, CH*H*), 3.7 (1H, dd, $J = 5.3$, 11.0, C*H*H), 4.2 (1H, ddd, $J = 8.4$, 7.0, 3.5, C*H*OH), 5.48 (1H, ddq, $J = 12.6$, 6.9, 15.5, *H*C=CHMe), 5.8 (1H, dq, $J = 15.5$, 6.5, =C*H*Me) ppm; GC/IR: $v = 1672$, (C=C), 3635 (OH) cm^{-1}; GC/EIMS: $m/z = 102$ (M^+, 0.5), 71 (100), 43 (42), 41 (40), 39 (26), 53 (26), 31 (21), 87 (0.5); CSPGC/FID: system B: *S*-**8** (7.4 min), *R*-**8** (8.2 min).

(2R3S/2S3R)-2,3-Dihydroxy-4-pentene (**17**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.15 (3H, d, $J = 6.4$, Me), 2.15 (2H, bs, 2×OH), 3.88 (1H, dq, $J = 6.4$, 3.6, C*H*Me), 4.09 (1H, dd, $J = 6.3$, 3.6, C*H*OH), 5.27 (1H, ddd, $J = 10.5$, 1.2, 1.2, H*H*C=), 5.35 (1H, ddd, $J = 17.2$, 1.2, 1.2, *H*HC=), 5.91 (1H, ddd, $J = 17.2$, 10.6, 6.5, =CH) ppm; GC/IR: $v = 1644$, 3089 (H_2C=CH), 3632 (OH) cm^{-1}; GC/EIMS: $m/z = 45$ (100), 58 (83), 57 (60), 43 (19); CSPGC/FID: system C: 2*S*,3*R*-**17** (9.2 min), 2*R*,3S-**17** (9.5 min).

cis-(2R/2S)-1,2-Dihydroxy-3-pentene (**16**; $C_5H_{10}O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 1.71 (3H, d, $J = 7.0$, Me), 2.15 (2H, bs, 2×OH), 3.50 (1H, dd, $J = 11.2$, 7.9, C*H*HOH), 3.59 (1H, dd, $J = 11.2$, 3.7, CH*H*OH), 4.59 (1H, ddd, $J = 8.4$, 8.4, 3.6, C*H*OH), 5.39 (1H, m, =C*H*Me), 5.65 (1H, m, HC=) ppm; GC/IR: $v = 1658$ (C=C), 3636 (OH) cm^{-1}; GC/EIMS: $m/z = 71$ (100), 43 (41), 41 (41), 39 (27), 53 (27), 31 (22), 87 (1); CSPGC/FID: system C: 2*S*-**16** (14.6 min) 2*R*-**16** (15.6 min).

(2R/2S)-1,2-Dihydroxy-3-butene (**20**; $C_4H_8O_2$)

^{1}H NMR (500 MHz, δ, $CDCl_3$): 3.50 (1H, dd, $J = 11.0$, 7.5, CH*H*OH), 2.95 (2H, bs, 2×OH), 3.67 (1H, dd, $J = 11.4$, 3.4, C*H*HOH), 4.26 (1H, m, C*H*OH), 5.23 (1H, ddd, $J = 10.6$, 1.4, 1.4, H*H*C=), 5.35 (1H, ddd, $J = 17.3$, 1.5, 1.5, *H*HC=), 5.85 (1H, ddd, $J = 17.0$, 10.4, 5.6), ppm GC/IR: $v = 1648$ (C=C), 2887, 2938, 3091 (CH), 3641 (OH) cm^{-1}; GC/EIMS: $m/z = 57$ (100), 58 (65), 31 (62), 39 (44), 70 (37), 42(35); CSPGC/FID: system D: 2*S*-**20** (10.6 min) 2*R*-**20** (11.0 min).

Metabolites **5**, **6**, **10**, **11**, **13**, **14**, **18**, and **22** were identified from IR, EIMS and CIMS data obtained directly after GC separation.

1-Hydroxy-3-methyl-2-butanone (**5**; $C_5H_{10}O_2$)

GC/IR: $v = 3530$ (OH), 1727 (C=O) cm^{-1}; GC/EIMS: $m/z = 102$ (M^+, 1), 31 (31), 43 (100), 71 (35), 41 (48); GC/CIMS: $m/z = 103$ (MH^+).

3-Methyl-1,2-butanediol (**6**; $C_5H_{12}O_2$)

GC/IR: $v = 3644$ (OH), 2968, 2892 (CH), 1058 cm^{-1}; GC/EIMS: $m/z = 73$ (100), 55 (70), 43 (60), 61 (40), 31 (39), 41 (25); GC/CIMS: $m/z = 87$ ($MH\text{-}H_2O^+$).

(E)-1-Hydroxy-3-penten-2-one (**10**; $C_5H_8O_2$)

GC/IR: $v = 3525$ (OH), 1702 (α,β-unsatd. C=O), 1643, 966 (C=C) cm^{-1}; GC/EIMS: $m/z = 69$ (100), 41 (98), 39 (54); 31 (19); GC/CIMS: $m/z = 101$ (MH^+).

1-Hydroxy-2-pentanone (**11**; $C_5H_{10}O_2$)

GC/IR: $v = 3530$ (OH), 1728 C=O) cm^{-1}; GC/EIMS: $m/z = 102$ (M^+, 6), 43 (100), 71 (51), 31 (34); GC/CIMS: $m/z = 103$ (MH^+).

2-Hydroxy-3-pentanone (**13**; $C_5H_{10}O_2$)

GC/IR: $v = 3535$ (OH), 1728 C=O) cm^{-1}; GC/EIMS: $m/z = 102$ (M^+, 2), 45 (100), 57 (36), 31 (9); GC/CIMS: $m/z = 103$ (MH^+).

2,3-Pentanediol (**14**; $C_5H_{12}O_2$)

GC/EIMS: $m/z = 59$ (100), 31 (68), 45 (60), 41 (31), 75 (14); GC/CIMS: $m/z = 87$ ($MH\text{-}H_2O^+$).

(Z)-1-Hydroxy-3-penten-2-one (**18**; $C_5H_8O_2$)

GC/IR: $v = 3525$ (OH), 1701 (α,β-unsatd. C=O), 1631 (C=C) cm^{-1}; GC/EIMS: $m/z = 100$ (M^+, 2), 69 (100), 41 (79), 39 (52), 31 (21); GC/CIMS: $m/z = 101$ (MH^+).

1-Hydroxy-2-butanone (**22**; $C_4H_8O_2$)

GC/IR: $v = 3536$ (OH), 1733 (C=O) cm^{-1}; GC/EIMS: $m/z = 88$ (M^+,13), 57 (100), 31 (36); GC/CIMS: $m/z = 89$ (MH^+).

Substrates were metabolized on a small scale using shake flask cultures of the constitutive mutant strain (UV4) of *P. putida* and the wild type *P. putida* NCIMB 8859 according to reported methods [15, 16, 20]. The wild type *P. putida* ML2 was grown on benzene as the sole carbon source. To obtain biomass, a 10 litre Biolab fermenter containing rich media (Luria Broth) was inoculated with a 1% inoculum of benzene grown cells. The cells were harvested by centrifugation (10000 g for 15 min) during the late exponential phase of growth and resuspended in potassium phosphate buffer (100 m*M*, $pH = 7.5$) to an optical density of 10 at 600 nm. These cell suspensions were used for the bioconversions. A typical procedure involved incubation of diene substrates **7**, **15**, and **19** (60 mm^3 in 3 cm^3 of culture medium containing the bacterial cells, $OD = 10$ at 600 nm) in shake flasks (10 cm^3) at a temperature of +4°C and $pH = 7.5$ for 24 h. As dienes **7**, **15**, and **19** had a very low solubility in water, a two-phase system was formed with the aqueous phase being saturated with the diene. In the case of 1,3-butadiene (**19**) the substrate was passed as a gas into heptamethylnonane to yield a saturated solution at 0°C. Subsequently, a portion (200 mm^3) of this solution was added to the bacterial cell solution. Bioconversions with the chemically synthesized mixtures of racemic diols **2**, **3**, **8**, **9**, **16**, **17**, and **20** as substrates for *P. putida* ML2 were carried out at a diol concentration of 2.0 $g.dm^{-3}$. The metabolites were obtained by rigorous extraction of the culture medium with ethyl acetate after saturation with salt. The extract was concentrated and analyzed by chiral stationary phase FID, MS, and IR detection. The structures of the *cis*-diol metabolites **2**, **3**, **8**, **9**, **16**, **17**, and **20** were established by comparison with authentic samples. The remaining ketoalcohols (**5**, **10**, **11**, **13**,

18, and **21**) and diols (**6** and **14**) were identified on the basis of comparison with literature data and from MS and IR spectra.

Note Added in Proof

In a recent report [23] the metabolism of isoprene in a *Rhodococcus* strain was found to occur *via* a monooxygenase-catalyzed epoxidation. No evidence of diene epoxidation was obtained using the *P. putida* strains in the present study.

Acknowledgements

We wish to thank Professor *H. Dalton* for the *P. putida* ML2 strain, Dr *N. I. Bowers* for the preliminary studies on *cis*-piperylene, and the Technology Development Programme of the European Regional Development Fund and the IRTU for financial support (*MC*, *DC*).

References

[1] Atkinson R, Arey J (1998) Acc Chem Res **31**: 574
[2] Cleveland CC, Yavitt JB (1998) Appl Environ Microbiol **64**: 172
[3] Konig G, Brunda M, Puxbaum H, Hewitt CN, Duckham SC, Rudolf J (1995) J Atmos Environ **29**: 861
[4] Becker KH, Barnes I, Ruppert L (1994) Physico-Chemical Behaviour of Atmospheric Pollutants **1**: 54
[5] Peeters A, Ectors A, Boullart W (1994) Physico-Chemical Behaviour of Atmospheric Pollutants **1**: 61
[6] Madronich S, Calvert JG (1990) J Geophys Res **95**: 5697
[7] Bleasdale C, Small RD, Watson WP, Wilson J, Golding BT (1996) Toxicology **113**: 290
[8] Wistuba D, Weigand K, Peter H (1994) Chem Res Toxicol **7**: 336
[9] Longo V, Citti L, Gervasi PG (1985) Toxicology Letters **29**: 33
[10] Gervasi PG (1985) Mutat Res **156**: 77
[11] Allen CCR, Boyd DR, Dalton H, Sharma ND, Brannigan I, Kerley NA, Sheldrake GN, Taylor ST (1995) J Chem Soc Chem Commun 117
[12] Allen CCR, Boyd DR, Hempenstall F, Larkin MJ, Sharma ND (1999) Appl and Environ Microbiol **65**: 1335
[13] Boyd DR, Sharma ND, Agarwal RA, Resnick SM, Schocken MJ, Gibson DT, Sayer JM, Yagi H, Jerina DM (1997) J Chem Soc Perkin Trans 1, 1715
[14] Boyd DR, Sharma ND, Evans TA, Groocock MG, Malone JF, Stevenson PJ, Dalton H (1997) J Chem Soc Perkin Trans 1, 1879
[15] Bowers NI, Boyd DR, Sharma ND, Kennedy MA, Sheldrake GN, Dalton H (1998) Tetrahedron Asymm **9**: 1831
[16] Boyd DR, Sharma ND, Haughey SA, Kennedy MA, McMurray BT, Sheldrake GN, Allen CCR, Dalton H, Sproule K (1998) J Chem Soc Perkin Trans 1, 1929
[17] Lange CC, Wackett LP (1997) J Bacteriology **179**: 3858
[18] Kolb HC, VanNiewenhze MS, Sharpless KB (1994) Chem Rev **94**: 2483
[19] Wang ZM, Kakiuchi K, Sharpless KB (1995) J Org Chem **59**: 6897
[20] Allen CCR, Boyd DR, Dalton H, Sharma ND, Walker CE (in preparation)
[21] Fong KPY, Goh CBH, Tan, H-M (1996) J Bacteriol **178**: 5592
[22] Allen, CCR, Boyd DR, Dalton H, Sharma ND, Brannigan I, Kerley NA, Sheldrake GN, Taylor SC (1994) J Chem Soc Chem 117
[23] van Hylckama Vlieg JE, Leemhuis H, Spelberg JH, Janssen DB (2000) J Bacteriol **182**: 1956

Received December 20, 1999. Accepted (revised) February 7, 2000