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Stereochemical and mechanistic aspects of dioxygenase-catalysed benzylic hydroxylation of indene and chromane substrates

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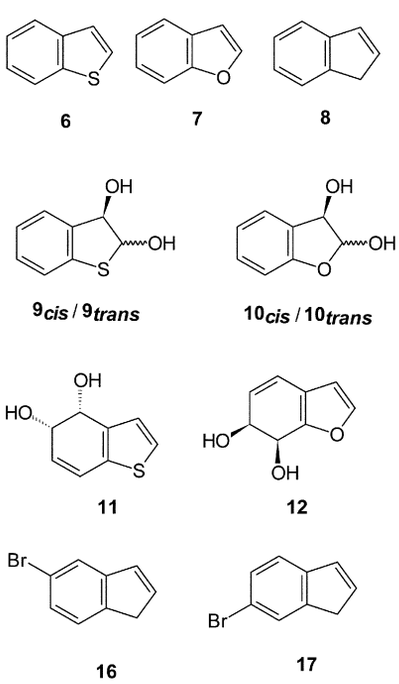
Toluene dioxygenase (TDO)-catalysed benzylic hydroxylation of indene substrates (**8**, **16** and **17**), using whole cell cultures of *Pseudomonas putida* UV4, was found to yield inden-1-ol (**14** and **22**) and indan-1-one bioproducts (**15** and **23**). The formation of these bioproducts is consistent with the involvement of carbon-centred radical intermediates. TDO-catalysed oxidation of indenes **8** and **16** also gave *cis*-diols **13** and **18** respectively. TDO and naphthalene dioxygenase (NDO), used as both whole-cell preparations and as purified enzymes, were found to catalyse the benzylic hydroxylation of chromane **30**, deuteriated (±)-chromane $\mathbf{30_D}$ and enantiomers (4*S*)-$\mathbf{30_D}$ and (4*R*)-$\mathbf{30_D}$ to yield (4*R*)- and (4*S*)-chroman-4-ols $\mathbf{31/31_D}$ respectively. The mechanism of benzylic hydroxylation of chromane $\mathbf{30/30_D}$ involves the stereoselective abstraction of a *pro-R* (with TDO) or a *pro-S* (with NDO) hydrogen atom at C-4 and a marked preference for retention of configuration.

Introduction

Ring hydroxylating dioxygenase enzymes (Rieske non-heme iron oxygenases)[1] catalyse a wide range of stereoselective oxygenations.[2–5] These include monooxygenation (*e.g.* benzylic hydroxylation[6–11] or sulfoxidation[12–15]), dioxygenation (*e.g. cis*-dihydroxylation[3,4] or bis-benzylic hydroxylation[10]), trioxygenation (*e.g.* benzylic hydroxylation/*cis*-dihydroxylation[11,16,17] or *cis*-dihydroxylation/sulfoxidation[15,18]) and tetraoxygenation (*e.g.* bis-*cis*-dihydroxylation[19,20]).

The mechanism of any of these dioxygenase-catalysed oxygenations remains to be elucidated. However, it is known that aromatic ring hydroxylating dioxygenase systems generally contain two or three components. In naphthalene dioxygenase (NDO, E.C.1.14.12) these are: (i) an iron–sulfur flavoprotein reductase, (ii) a Rieske [2Fe–2S] ferredoxin involved in electron transfer from NADP(H) and (iii) a catalytic oxygenase component with a mononuclear iron site.[1] Recent X-ray crystallographic studies of NDO have greatly increased our understanding of the structure of its catalytic domain.[21,22] The active site of NDO was found to contain several hydrophobic amino acids in a relatively flat and elongated cavity capable of accepting aromatic substrates. An X-ray crystal structure of NDO has also shown indole **1**, an aromatic substrate, bound at the active site as an Fe(III) peroxo species **2** linked at C-3, that may be an intermediate in the oxidation of indole **1** to indigo **5** (Scheme 1). The product, released from the binding site, is assumed to be the transient *cis*/*trans*-1,2-dihydrodiol metabolite of indole, $\mathbf{3}_{cis}/\mathbf{3}_{trans}$, that spontaneously dehydrates yielding indoxyl **4** and the autoxidation product indigo **5**.

Structures similar to the Fe(III) peroxo species **2** or involving an Fe(III)OOH species have been postulated as intermediates in the mechanism of mono- and di-oxygenation reactions of comparable substrates catalysed by dioxygenases.[21–23] Biotransformation studies of the indole isosteres, benzo[*b*]thiophene **6**, benzo[*b*]furan **7** and indene **8**, have been carried out in our laboratories.[6,24–27] In contrast with the transient equilibrating *cis*/*trans* diol metabolites of indole, $\mathbf{3}_{cis}/\mathbf{3}_{trans}$ shown in Scheme 1, the corresponding heterocyclic dihydrodiols, $\mathbf{9}_{cis}/\mathbf{9}_{trans}$ and $\mathbf{10}_{cis}/\mathbf{10}_{trans}$, proved to be sufficiently stable to isolate and assign stereochemically.[26,27] The carbocyclic *cis*-dihydrodiols **11** and **12**, derived from benzo[*b*]thiophene[26] and benzo[*b*]furan[27] respectively, and ring opened products from dihydrodiols, $\mathbf{9}_{cis}/\mathbf{9}_{trans}$ and $\mathbf{10}_{cis}/\mathbf{10}_{trans}$, were also isolated. Furthermore, dioxygenase-catalysed sulfoxidation was observed in benzo[*b*]thiophene substrates, yielding their sulfoxides and the derived cycloadducts.[26,27] Thus for mechanism studies, the indenes **8**, **16** and **17** were considered as simpler models for the dioxygenase-catalysed oxidation of indole **1**.



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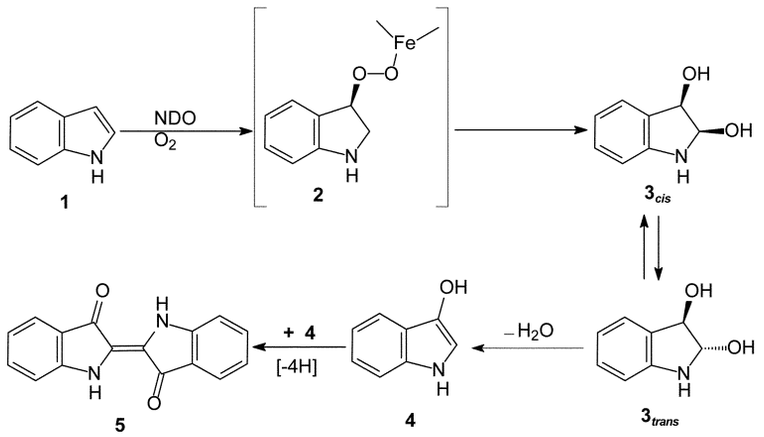
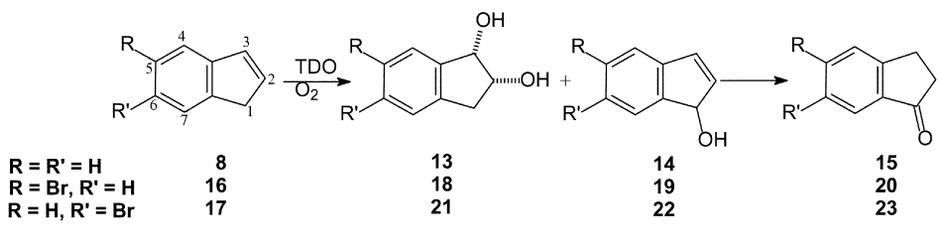
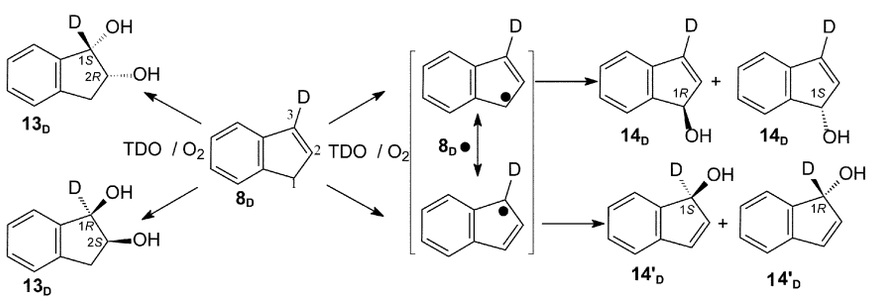


Scheme 1



Scheme 2



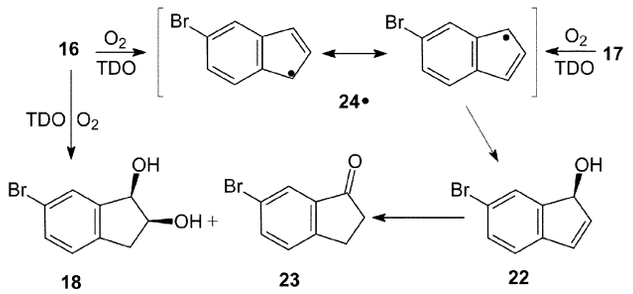
Scheme 3

Results and discussion

Initial studies with indene **8** were carried out using whole cell mutant strains of bacteria containing TDO (*P. putida* 39/D[6] and *P. putida* UV4[7,25]). The *cis*-dihydrodiol dehydrogenase enzyme, responsible for the desaturation of the substituted benzene *cis*-dihydrodiol metabolites to the corresponding catechols, was blocked in the UV4 and 39/D strains thus allowing the *cis*-dihydrodiol metabolites to accumulate. With indene substrate **8**, both strains were found to yield *cis*-1,2-dihydroxyindane **13** (*cis*-dihydroxylation product), inden-1-ol **14** (benzylic hydroxylation product) and indan-1-one **15** (rearrangement product of inden-1-ol **14**) as major bioproducts (Scheme 2).

The earlier report[6] on the metabolism of [3-^{2}H]- or [3-D]-indene $\mathbf{8_D}$ using TDO (*P. putida* F 39/D) showed that (−)-*cis*-diol $\mathbf{13_D}$ was formed with an enantiomeric excess (ee) favouring the (1*S*,2*R*) configuration (30% ee) (Scheme 3). Diol $\mathbf{13_D}$ also had the ^{2}H atom located exclusively at the C-1 position while the (+)-(1*S*)-inden-1-ol (26% ee) showed the ^{2}H atom to be distributed between positions C-3 (60% ^{2}H, $\mathbf{14_D}$) and C-1 (40% ^{2}H, $\mathbf{14'_D}$). A comparative study, again with [3-^{2}H]-indene **8** and TDO (whole cells of *P. putida* UV4), gave comparable results. Thus, the ^{2}H atom was found only at C-1 in (−)-(1*S*,2*R*)-diol $\mathbf{13_D}$ (38% ee) while inden-1-ol showed the ^{2}H atom to be located at positions C-3 (43% ^{2}H, $\mathbf{14_D}$) and C-1 (57% ^{2}H, $\mathbf{14'_D}$) by ^{1}H-NMR analysis. A preference for the opposite (−)-(1*R*) configuration of inden-1-ol $\mathbf{14_D/14'_D}$ (28% ee) was, however, observed using *P. putida* UV4. Earlier studies with unlabelled indene **8** and this strain had shown that (−)-(1*R*)-inden-1-ol **14** was enantiopure (> 98% ee). This change in ee value may have been due to asymmetric destruction of one enantiomer of inden-1-ol and/or a primary kinetic isotope effect. Stereoselective removal of one enantiomer of indan-1-ol had been observed earlier[10] with *P. putida* UV4. Spontaneous rearrangement of the ^{2}H-labelled samples of inden-1-ol $\mathbf{14_D}$ and $\mathbf{14'_D}$ gave both labelled [3-^{2}H] and unlabelled forms of indan-1-one **15** (*cf*. Scheme 2). The equilibration of the ^{2}H atom between the C-1 and C-3 positions in indene $\mathbf{8_D}$ is evident from inden-1-ol metabolites $\mathbf{14_D/14'_D}$ found using UV4 or 39/D mutant strains of *P. putida*. This is consistent with the intermediacy of a stabilised allylic/benzylic carbon-centred radical $\mathbf{8_D^{\bullet}}$ (Scheme 3).

Further evidence for the involvement of delocalised carbon-centred radicals similar to $\mathbf{8^{\bullet}}$ during TDO-catalysed benzylic hydroxylation of 5-bromoindene **16** and 6-bromoindene **17** substrates was sought (Scheme 2). The possible indane *cis*-diol (**13**,**18**,**21**), inden-1-ol (**14**,**19**,**22**) and indan-1-one (**15**,**20**,**23**) metabolites, from the corresponding indene substrates (**8**,**16**,**17**), are shown in Scheme 2. In practice, the (+)-(1*R*,2*S*)-dihydrodiol metabolite **18** (46% ee) was isolated from 5-bromoindene **16** as the major (42% yield) metabolite (Scheme 4). This result is comparable to that found earlier using indene substrate $\mathbf{8_D}$ where *cis*-1,2-dihydroxyindane $\mathbf{13_D}$ of opposite configuration (1*S*,2*R*, 38% ee) was obtained. The (1*R*,2*S*) absolute configuration of *cis*-diol **18** was determined by circular dichroism (CD) comparison with (1*S*,2*R*)-indene *cis*-diol metabolite **13** where the strongest CD absorption peaks (196–203 nm) were of opposite signs. The ee value of *cis*-diol **18** was



Scheme 4

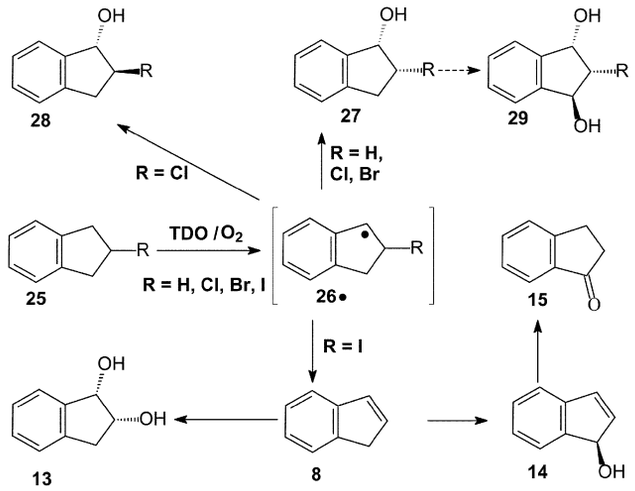
estimated by ^{1}H-NMR analysis of the (−)-(*S*)-[2-(1′-methoxyethyl)benzene]boronic acid (MEBBA) derivative.[28]

A minor (4% yield) metabolite from 5-bromoindene **16** was identified as 6-bromoindan-1-one **23**; it was assumed to have been obtained by spontaneous rearrangement of the relatively unstable 6-bromoinden-1-ol intermediate **22** (Scheme 4). This isolation of metabolite **23** is significant since it provides further evidence of an equilibrating allylic/benzylic radical (**24**$^{\bullet}$). In this case abstraction of a hydrogen atom from C-1 in 5-bromoindene **16** was followed by double bond migration and hydroxylation at the original C-3 position; no evidence of either inden-1-ol **19** or ketone **20** was found.

Biotransformation of 6-bromoindene **17** using *P. putida* UV4 involved benzylic hydroxylation with an exclusive preference for oxidation at the C-1 position to yield 6-bromoinden-1-ol **22** (16% yield). The derived 6-bromoindan-1-one **23** (28% yield) was also isolated; no evidence was found of the dihydroxylation product, *cis*-diol **21**. The observed benzylic hydroxylation of both 5-bromoindene **16** and 6-bromoindene **17** to yield the same indan-1-one product **23** appears to be the result of one of the resonance forms of the allylic/benzylic radical **24**$^{\bullet}$ being preferred for hydroxyl-group-transfer at the active site. The absolute configuration of 6-bromoinden-1-ol **22** was determined as (1*S*) by comparison of its CD spectrum with (−)-inden-1-ol **14** (from indene **8** with *P. putida* UV4), which had the opposite (1*R*) absolute configuration. Benzylic alcohol **22** was found to have an ee value of > 98% by chiral stationary phase high-pressure liquid chromatography (CSPHPLC). The examination of relative contributions of enzyme-catalysed asymmetric synthesis, or stereoselective removal of one enantiomer, on the absolute configurations and ee values of *cis*-diol **18** and inden-1-ol **22** was not carried out.

The results obtained using TDO (*P. putida* UV4) as biocatalyst with [3-^{2}H]-indene **8**, 5-bromoindene **16** and 6-bromoindene **17** are compatible with the initial formation of equilibrating radical intermediates (**8**$^{\bullet}$ or **24**$^{\bullet}$) shown in Schemes 3 and 4. The hydrogen-atom-abstraction process is facilitated by the relatively weak allylic/benzylic C–H bond in substrates **8**, **16** and **17** and by the resulting delocalisation of a single electron between C-1 and C-3 in intermediate **8**$^{\bullet}$ or **24**$^{\bullet}$. It is evident that the precise regioselectivity of dioxygenase-catalysed oxidation at the indene ring would be determined by orientation of the substrate within the active site; this would, in turn, depend upon the relative positions of the indene substituent and the radical intermediate.

Earlier studies of the metabolism of indane **25** (R = H),[6,7,29] 2-substituted indanes **25** (R = Cl, Br, I),[10] and indanone substrates[30] also lend support to the concept of stereoselective TDO- and NDO-catalysed monohydroxylation *via* a benzylic radical *e.g.* **26**$^{\bullet}$ (Scheme 5). The biotransformation pathways (*P. putida* UV4) of indanes **25** (R = H, Cl, Br, I) include benzylic mono-hydroxylation to yield monols **27** (R = H, Cl, Br) and **28** (R = Cl) and benzylic bis-hydroxylation to yield 1,3-diols **29** (R = H, Cl, Br).[10] The enantiopure indan-1-ol products **27**–**29** obtained had identical configurations at the benzylic stereogenic centres (Scheme 5), and could have resulted from either asymmetric oxidation or stereoselective asymmetric



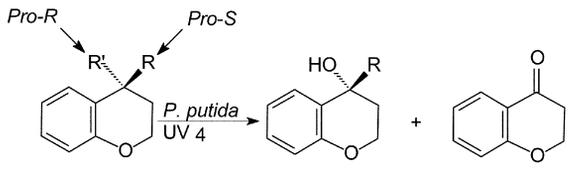
Scheme 5

destruction.[10] The configuration of the minor metabolite (+)-**28** (R = Cl) shown in Scheme 5 was earlier[10] incorrectly assigned as (1*R*,2*R*) instead of (1*S*,2*S*).

Biotransformation of 2-iodoindane **25** (R = I) was of particular interest in the context of radical intermediate formation during dioxygenase-catalysed benzylic hydroxylation; inden-1-ol **14** (13% yield) and *cis*-diol **13** (20% yield) were obtained along with traces (< 1% yield) of the anticipated benzylic hydroxylation products **27** and **29** (R = I). The former two major metabolites were structurally and stereochemically indistinguishable from the metabolites of indene **8** (Scheme 2); their formation can be explained by the initial generation of a benzylic radical **26**$^{\bullet}$ followed by the loss of an iodine atom (Scheme 5). This rapid homolytic cleavage of an iodine atom β to a carbon-centred radical intermediate, *e.g.* **26**$^{\bullet}$, is well precedented. Thus, the rate of homolysis of an iodoethyl radical to yield propene is 700 times faster than the ring opening of the ethylcyclopropyl radical to yield the methylallyl radical, a favoured process widely used to detect radicals.[31]

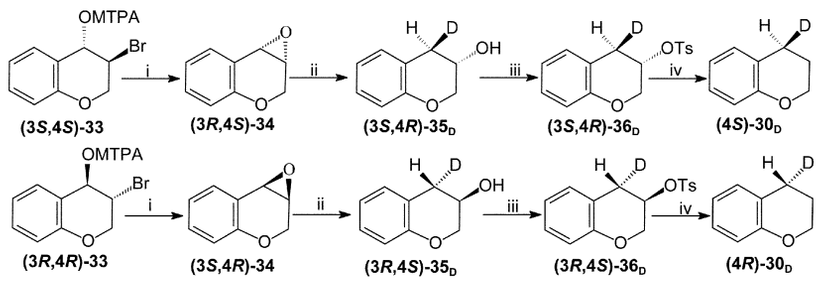
The instability of inden-1-ol bioproducts, **14** and **22**, obtained from indenes **8** and **17** respectively (Schemes 2 and 4), and the range of oxidation products from indanes **25** (R = H, Cl, Br and I, Scheme 5), rendered them less desirable substrates for dioxygenase mechanism studies. 1,2-Dihydrobenzo[*b*]-thiophene and 1,2-dihydrobenzo[*b*]furan have bicyclic structures similar to indenes, and contain only one benzylic site with a heteroatom at the other benzylic position. However, with TDO (*P. putida* UV4) as biocatalyst, benzylic hydroxylation, sulfoxidation, desaturation and *cis*-dihydroxylation reactions occur and hence a mixture of bioproducts was also found with these substrates.[26,27,32]

Chromane **30** was next adopted as a model for further mechanism studies, initially using whole cells of *P. putida* UV4. Chroman-4-ol **31** (13% yield) and chroman-4-one **32** (5%) were isolated as the sole metabolites (Scheme 6, **a**). The more polar alcohol **31**, isolated by PLC (R_f 0.28, 50% Et_2O in hexane), was found to have a large excess of the (4*R*) enantiomer (95% ee) by CSPHPLC analysis. While this ee value could be due to selective substitution of the *pro-R* benzylic hydrogen atom during hydroxylation, the possible role of stereoselective destruction of one enantiomer (kinetic resolution) occurring during further oxidation to ketone in *P. putida* UV4 must also be considered. This process was indeed observed using racemic chroman-4-ol **31** as substrate. Chroman-4-one **32** was formed (18% yield) and the residual alcohol **31** (21% recovered yield) was found to have an excess of the (4*R*) enantiomer (84% ee). Kinetic resolution involving the selective oxidation of the (4*S*) enantiomer of alcohol **31** to yield ketone **32** is assumed to have arisen from an alcohol dehydrogenase enzyme present in whole cells of *P. putida* UV4. Since the (4*R*) enantiomer of chroman-4-ol

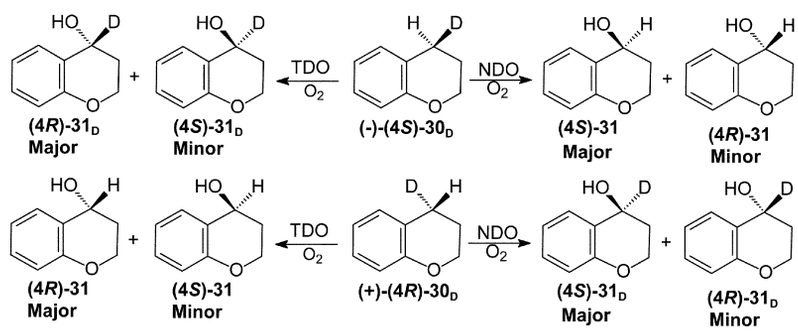


(a) R = R'= H	30	(4*R*)-31	32
(b) R = R' = H or D	(4*R*/4*S*)-30_D	(4*R*)-31_D and (4*R*)-31	
(c) R = D, R' = H	(4*S*)-30_D	(4*R*)-31_D	
(d) R = H, R' = D	(4*R*)-30_D	(4*R*)-31	

Scheme 6



Scheme 7



Scheme 8

31 was preferentially formed, using either chromane **30** or racemic chroman-4-ol **31** as substrates, the relative contributions of asymmetric synthesis and kinetic resolution during the whole cell biotransformation could not be estimated.

Racemic and enantiopure forms of 4-deuteriated chromane $\mathbf{30_D}$ were required as substrates to investigate further aspects of the dioxygenase-catalysed benzylic hydroxylation mechanism (Scheme 6). These were synthesised by $LiAl^2H_4$ reduction of racemic, (−)-(3*R*,4*S*)- and (+)-(3*S*,4*R*)-3,4-epoxychromane **34** to yield chroman-3-ol $\mathbf{35_D}$ (Scheme 7). Reaction of alcohol $\mathbf{35_D}$ with *p*-toluenesulfonyl chloride to yield the tosylate $\mathbf{36_D}$ and reductive cleavage with $LiAlH_4$ gave the mono-deuteriated racemic, (4*S*)- and (4*R*)-forms of chromane $\mathbf{30_D}$. The 3,4-epoxychromane enantiomers (3*R*,4*S*)-**34** ($[a]_D$ −71) and (3*S*,4*R*)-**34** ($[a]_D$ + 69), obtained earlier from their 3-bromo-4-methoxy-(phenyl)trifluoromethylacetate (bromo-MTPA) esters (3*S*,4*S*)-**33** and (3*R*,4*R*)-**33**,[33] gave the corresponding [4-^{2}H]-chromane enantiomers (4*S*)-$\mathbf{30_D}$ ($[a]_D$ −1.7 ± 0.5) and (4*R*)-$\mathbf{30_D}$ ($[a]_D$ + 1.0 ± 0.5) respectively. Enantiocomplementary CD spectra with maximum absorption at *ca.* λ 228 nm were also obtained for each enantiomer.

Chromane enantiomers (4*S*)-$\mathbf{30_D}$ and (4*R*)-$\mathbf{30_D}$ were used, as substrates, to address the stereochemical/mechanistic question of whether dioxygenase-catalysed benzylic hydroxylation occurs with retention or inversion of configuration (Schemes 6 and 8). Biotransformation (*P. putida* UV4) of racemic [4-^{2}H]-chromane (4*R*/4*S*)-$\mathbf{30_D}$ (Scheme 6, **b**) gave enantiopure deuteriated (4*R*)-chroman-4-ol $\mathbf{31_D}$ (7% yield) and non-deuteriated (4*R*)-chroman-4-ol **31** (4% yield) and chroman-4-one **32** (7% yield). The slight excess of deuteriated (4*R*) enantiomer $\mathbf{31_D}$ over non-deuteriated (4*R*)-chroman-4-ol **31** may be due to a primary kinetic isotope effect operating during the enzyme-catalysed hydroxylation and/or ketone formation. The presence of deuterium at a benzylic position had earlier been found to exert a marked influence on the ratio of bioproducts obtained from TDO-catalysed (*P. putida* UV4) monohydroxylation of benzyl cyanide and was attributed to a primary kinetic isotope effect.[11]

Addition of (−)-(4*S*)-chromane $\mathbf{30_D}$ to whole cells of *P. putida* UV4 gave (+)-(4*R*)-chroman-4-ol $\mathbf{31_D}$ (14% yield, > 98% ee) with the deuterium atom retained at C-4 (> 98% ^{2}H, by ^{1}H- and ^{2}H-NMR analysis) and 4-chromanone **32** (9% yield) (Scheme 6, **c**). Similarly (+)-(4*R*)-chromane $\mathbf{30_D}$ gave (+)-(4*R*)-chroman-4-ol **31** (> 98% ee and 14% yield) with almost total removal of the benzylic ^{2}H atom (< 2% ^{2}H by ^{1}H- and ^{2}H-NMR analysis) and chroman-4-one **32** (9% yield) (Scheme 6, **d**). These results are consistent with a mechanism involving the benzylic hydroxylation of chromane **30** through exclusive abstraction of the *pro-R* benzylic hydrogen atom followed by complete retention of configuration during insertion of the hydroxyl group. However, if the formation and subsequent stereoselective loss of the (−)-(4*S*)-enantiomer of alcohol **31** were to occur, it would challenge this explanation since chroman-4-one **32** was obtained as a significant metabolite (9–14% yield). In order to reduce the role of further oxidation of chroman-4-ol **31** to chroman-4-one **32**, biotransformations with purified TDO (*P. putida* 39/D) were carried out on substrates (−)-(4*S*)-chromane $\mathbf{30_D}$ and (+)-(4*R*)-chromane $\mathbf{30_D}$ (Scheme 8).

As anticipated, with a purified form of TDO, benzylic hydroxylation of racemic (4*S*/4*R*)-, and enantiopure (4*S*)- and

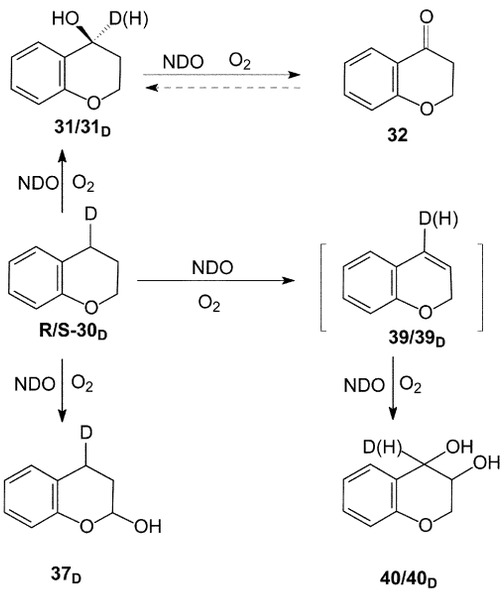
Table 1 Relative ratio of (4*R*)-chroman-4-ol **31/31$_D$** and chroman-2-ol **37$_D$** hydroxylation products from pure TDO- and NDO-catalysed hydroxylation of chromane **30$_D$**

Substrate **30$_D$**	Enzyme	(4*R*)-Chroman-4-ol **31/31$_D$** (*R* or *S*, % ee)	Chroman-2-ol **37$_D$**
(*R*/*S*)-**30$_D$**	TDO	100 (*R*, 72)	
(*S*)-**30$_D$**	TDO	100 (*R*, 68)	
		100 (*R*, 75)[a]	
(*R*)-**30$_D$**	TDO	100 (*R*, 47)	
		100 (*R*, 65)[a]	
(*R*/*S*)-**30$_D$**	NDO	51 (*S*, 92)[b]	49[b]
(*S*)-**30$_D$**	NDO	46 (*S*, 88)[b]	54[b]
		48 (*S*, 80)[c]	52[a]
(*R*)-**30$_D$**	NDO	55 (*S*, 99)[b]	45[b]
		48 (*S*, 96)[c]	52

[a] Repeat experiment using a larger quantity of substrate **30$_D$**. [b] Accompanied by a minor amount (3–9%) of 2,3-dihydroxychromane **40/40$_D$**. [c] Repeat experiment using a larger quantity of substrate and with little evidence of 2,3-dihydroxychromane **40/40$_D$** formation.

(4*R*)-chromane **30$_D$** was observed without evidence of further oxidation to ketone **32** (Scheme 8, Table 1). In all cases chroman-4-ol **31** or **31$_D$** was enriched (47–75% ee) in the (+)-(4*R*) enantiomer based on CSPHPLC analysis. This observation confirmed that a significant proportion of the ee was due to a TDO-catalysed asymmetric hydroxylation process. GC-MS analysis of the individual (4*R*)- and (4*S*)-enantiomers of chroman-4-ol **31$_D$**, obtained from substrate (−)-(4*S*)-chromane **30$_D$** (separated by CSPHPLC), showed that the ^{2}H atom at C-4 was retained (> 90% ^{2}H) in each enantiomer. Conversely when (+)-(4*R*)-chromane **30$_D$** was added as substrate to purified TDO, the resulting (4*R*)- and (4*S*)-enantiomers of chroman-4-ol **31** showed loss of the ^{2}H-atom at C-4 (> 90% H). The experimental results obtained using purified TDO (Scheme 8) with (−)-(4*S*)-chromane **30$_D$** and (+)-(4*R*)-chromane **30$_D$** confirm the earlier observation using *P. putida* UV4 whole cells (Scheme 6), *i.e.* the *pro*-*R* benzylic hydrogen atom is cleaved in a highly stereoselective manner. The foregoing results support the premise that the formation of benzylic hydroxylation bioproduct with a strong preference (74–87%) for the (*R*) enantiomer means that the hydroxylation has largely occurred with the retention of configuration. A summary of results obtained using pure TDO with racemic and enantiopure forms of chromane **30$_D$** is presented in Table 1.

A whole cell biotransformation (*P. putida* 9816/11 containing NDO) of racemic substrate [4-^{2}H]-chromane **30$_D$** was also carried out (Scheme 9). This NDO-catalysed hydroxylation was mainly found to occur at C-4 to give (4*S*)-chroman-4-ol **31/31$_D$** (40% relative yield) and chroman-4-one **32** (53% relative yield) metabolites based on GC-MS and ^{1}H-NMR analysis. (4*S*)-Chroman-4-ol **31/31$_D$** was found to have lost *ca.* 75% of the original deuterium atom. It could be accounted for by a metabolic sequence involving: (i) direct enzyme-catalysed hydroxylation of [4-^{2}H]-chromane **30$_D$** to (4*S*)-chroman-4-ol **31/31$_D$**; (ii) oxidation of a portion of chroman-4-ol **31/31$_D$** to chroman-4-one **32**; and (iii) reduction of chroman-4-one **32** to yield unlabelled chroman-4-ol **31**. The relatively low ee value found for chroman-4-ol **31/31$_D$** (24% compared with > 90% ee obtained later using the purified NDO, Table 1) is consistent with its reformation by reduction of chroman-4-one **32**. A minor metabolite of chromane **30$_D$** (7% relative yield) showed similar GC-MS characteristics to an authentic sample of *cis*-3,4-dihydroxychromane **40/40$_D$**. A significant proportion of the original deuterium atom present in [4-^{2}H]-chromane **30$_D$** was found to be retained at C-4 in *cis*-3,4-dihydroxychromane **40$_D$** (64% ^{2}H). Direct spectral comparison with an authentic sample of chroman-2-ol **37$_D$** indicated that it was not present among the monohydroxylated bioproducts.

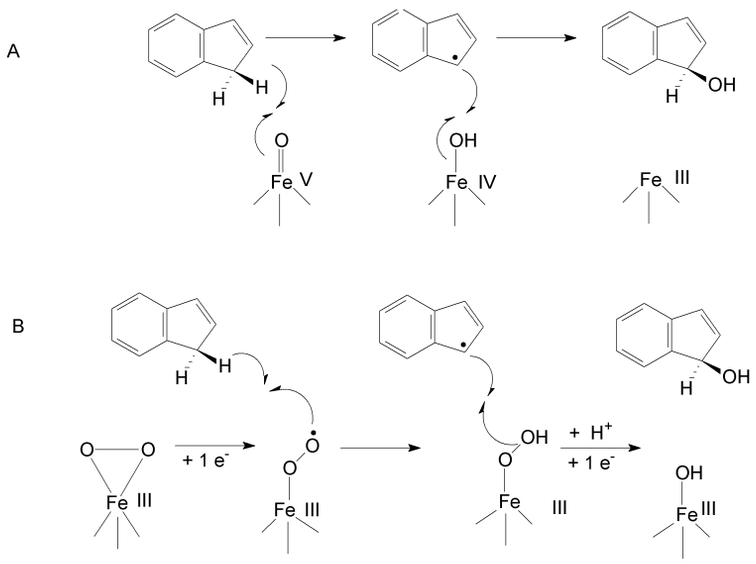


Scheme 9

Finally, a comparative study was conducted using purified NDO with racemic and enantiopure (4*R*)- and (4*S*)-chromane substrates **30$_D$** (Scheme 8). GC-MS analysis indicated that in each case chroman-4-ol **31** or **31$_D$** was formed as a major metabolite (46–55% relative yield, Table 1). As expected from other benzylic hydroxylations catalysed by NDO,[5,34] a marked preference (> 80%) was found for the (*S*)-configuration of chroman-4-ol **31/31$_D$**. The absolute configurations of the major chroman-4-ol enantiomers **31/31$_D$** formed with enzymes TDO (*R*) and NDO (*S*)[5–11] were found to be enantiocomplementary.

While a single bioproduct, chroman-4-ol **31/31$_D$**, was obtained with pure TDO, the use of pure NDO resulted in the formation of an additional monohydroxylation product; it was identified as chroman-2-ol **37$_D$** (45–54% relative yield) by GC-MS comparison with an authentic sample (Scheme 9). A precedent for NDO-catalysed hydroxylation on a carbon adjacent to an oxygen atom was observed during earlier studies with methoxybenzene and ethoxybenzene substrates. Dealkylation of the latter substrates was found as a result of the initial hydroxylation of the activated methyl and methylene groups adjacent to the oxygen atom.[34] A third metabolite, *cis*-3,4-dihydroxychromane **40/40$_D$** (< 10% relative yield), was also detected by GC-MS analysis using pure NDO. This NDO bioproduct had been found earlier in the whole cell study (*P. putida* 9816/11). A major proportion of the original deuterium at C-4 (73% ^{2}H from (4*R*)- and 72% ^{2}H from (4*S*)-chromane **30$_D$**) was retained in *cis*-3,4-dihydroxychromane **40/40$_D$**. The formation of *cis*-diol **40/40$_D$** from chromane **30$_D$** using both whole cells and purified NDO could be accounted for by NDO-catalysed desaturation to yield chromene **39/39$_D$** followed by *cis*-dihydroxylation (Scheme 9). The retention of a similar major proportion of deuterium at C-4 from both (4*R*)- and (4*S*)-chromane **30$_D$** substrates during this desaturation process was unexpected. The mechanism may involve a kinetic isotope effect rather than stereoselective removal of one benzylic hydrogen atom. The NDO enzyme system has previously been found to catalyse the desaturation of indane **25** (R = H) to yield indene **8**,[5] conversion of chromane **30$_D$** to chromene **39/39$_D$** may have occurred *via* a similar mechanism.

When (4*S*)-chromane **30$_D$** was used as substrate with purified NDO (Scheme 8), the deuterium atom from the upper face was selectively removed (> 90% ^{2}H loss, equivalent to the benzylic *pro*-*S* hydrogen atom at C-4 in chromane **30**, Scheme 6). Addition of the hydroxyl radical was again found to occur mainly (> 90%) from the upper face to yield (4*S*)-chroman-4-ol **31/31$_D$** (80–88% ee, Table 1). Use of (4*R*)-chromane **30$_D$** led to



Scheme 10

preferential cleavage of the benzylic hydrogen atom from the upper face (> 90% ^{2}H retention) and hydroxyl radical addition almost exclusively (> 98%) from the same face to yield (4*S*)-chroman-4-ol **31/31$_D$** (96–99% ee).

Conclusion

The TDO-catalysed formation of benzylic monohydroxylation products **14** and **22**, using whole cells of *P. putida* UV4 and indene substrates **8** and **17** respectively, is consistent with the intermediacy of delocalised allylic/benzylic radicals. Using TDO and NDO from both whole cell and purified enzyme sources, benzylic hydroxylation of chromane **30** to yield chroman-4-ol **31** was found to occur mainly with retention of configuration (74–87% *R* with TDO and 90–100% *S* with NDO). This was accompanied by an initial stereospecific abstraction at a benzylic C–H bond favouring either the *pro-R* (TDO) or *pro-S* (NDO) hydrogen atom. A similar result (72–78% retention of configuration) was recently obtained during the monohydroxylation of specifically labelled ethane and butane substrates using methane monooxygenase containing a diiron centre.[35] NDO-catalysed oxidation of chromane **30** also resulted in non-benzylic hydroxylation to yield chroman-2-ol **37** and *via* desaturation/*cis*-dihydroxylation to yield *cis*-3,4-dihydroxychromane **40**.

The mechanism of dihydroxylation, the nature of the active oxygen species, and the intermediates in the catalytic cycle are still unknown for the *cis*-dihydroxylating dioxygenases. One of the earliest mechanism studies confirmed that both atoms from $^{18}O_2$ were incorporated during the *cis*-dihydroxylation of arenes.[44] The involvement of Rieske [2Fe–2S] clusters in catalysis implies that one-electron transfers are involved, which has been demonstrated experimentally by single turnover studies on NDO.[45] The isolation of monohydroxylated products, reported previously[6–11] and in this work, implies that the dihydroxylation reaction occurs by stepwise insertion of two oxygen atoms.

The hydroxylation reactions catalysed by cytochrome P-450 monooxygenases[46] and α-ketoglutarate-dependent dioxygenases[47] proceed *via* retention of configuration, as has been observed in this study. For P-450 monooxygenases[46] and α-ketoglutarate-dependent dioxygenases,[47,48] a high-valent iron–oxo species has been proposed as the active hydroxylating agent. Thus, a possible explanation of the observed data is the intermediacy of an iron–oxo species during *cis*-diol formation (*e.g.* Scheme 1).

Recent work on site-directed mutants of cytochrome P-450 monooxygenase Δ2B4 have led Vaz *et al*. to propose that an iron(III) hydroperoxide intermediate could function as an electrophilic oxidant for P-450-catalysed epoxidation and hydroxylation.[49,50] Therefore, a second alternative is that the initial oxidant in the *cis*-dihydroxylation mechanism might be an electrophilic iron hydroperoxide species. This hypothesis is supported by the observation of an indole hydroperoxide in the active site of naphthalene dioxygenase by X-ray crystallography.[51] The interpretation of the single turnover experiments,[45] and recent results showing that hydrogen peroxide can replace the requirement for oxygen and a reduced Rieske [2Fe–2S] cluster,[52] also suggest the formation of a hydroperoxo intermediate.

The observed monohydroxylations can be explained by reaction of a carbon-centred radical with an activated oxygen species, which might be either a high-valent iron–oxo intermediate (Scheme 10, **A**), or an iron hydroperoxide species (Scheme 10, **B**), which presumably is an intermediate in the *cis*-dihydroxylation reaction. Recent crystallographic studies with NDO have identified oxygen bound in a side-on position near iron, as found in a cyclic peroxide of the type shown in Scheme 10B.[53] This species could be readily converted *via* a one electron reduction process into its more reactive hydroperoxide. Either species could be responsible for the monohydroxylation shown in Scheme 10B and for the diverse range of mono- and poly-oxygenation reactions catalysed by the Rieske dioxygenases.

Experimental

^{1}H-NMR spectra were recorded using Bruker Avance DPX-300 and DPX-500 instruments and ^{2}H-NMR spectra using a 400 MHz Bruker WP400 instrument. Optical rotation ($[\alpha]_D$) measurements were carried out with a Perkin-Elmer 214 polarimeter at ambient temperature (*ca.* 20 °C) and are given in units of 10^{-1} deg cm^2 g^{-1}. Enantiopurity was determined by CSPH-PLC using specified Chiralcel columns and hexane–2-propanol (9 : 1) as eluent at a flow rate of 0.5 cm^3 min^{-1}. CD spectra were recorded using a Jasco J-720 instrument and acetonitrile as solvent.

High resolution MS were recorded at 70 eV on a VG Autospec mass spectrometer using a heated inlet system. Accurate MW values were determined by the peak matching method with perfluorokerosene as standard. GC-MS analysis of the concentrated crude EtOAc extract, obtained from metabolism of chromane **30$_D$** using *P. putida* 9816/11, was carried out using a Hewlett Packard 6890 gas chromatograph directly attached to a Hewlett Packard model 5973 Mass

Selective Detector. The GC oven was fitted with a wall-coated open-tubular capillary column (12 m × 0.2 mm) with 100% dimethyl polysiloxane (0.33 μm) as the bonded phase. The injector port was held at 250 °C and the oven was programmed at 30 °C for 1 min and then ramped to 300 °C at 10 °C min^{-1}. The products obtained using chromane **30$_D$** as the substrate for the purified TDO and NDO enzymes were analysed by GC-MS using a Hewlett Packard model 5890 gas chromatograph equipped with an HP Ultra-1 capillary column (25 m × 0.2 mm, 0.33 μm film thickness). The column temperature was programmed from 70–240 °C at 10 °C min^{-1} with a helium flow of 25 cm^3 s^{-1}. Temperatures of the injection port and transfer line were 220 and 280 °C, respectively. Samples (1 μl) were injected at a split ratio of 50 : 1 and MS were obtained using an HP model 5970 Mass Selective Detector with electron impact ionization (70 eV).

Substrates were metabolized (18 h unless otherwise mentioned) using whole cell cultures of the mutant strain *Pseudomonas putida* UV4[15] and *Pseudomonas putida* 9816/11[29] under conditions reported earlier. The bioproducts were generally harvested by repeated solvent extraction (EtOAc) of the sodium chloride-saturated aqueous solution containing the biotransformed material, followed by concentration of the combined organic extracts under reduced pressure. ^{1}H NMR spectra of the crude mixture of bioproducts obtained from each biotransformation were routinely examined prior to any purification.

Indan-1-one **15**, chroman-4-ol **31** and 5-bromoindan-1-one **20** were available commercially. Samples of chroman-2-ol **37** and *cis*-3,4-dihydroxychromane **40** were available from earlier studies.[33] (−)-(3*R*,4*S*)- and (+)-(3*S*,4*R*)-3,4-Epoxychromane **34** were prepared from the corresponding bromo-MTPA diastereoisomers (3*S*,4*S*)- and (3*R*,4*R*)-*trans*-3-bromo-4-[methoxy(phenyl)trifluoromethylacetoxy]chromane **33** by the reported procedure.[33]

Synthesis of indene substrates 8, 16 and 17

[3-^{2}H]-indene 8. Sodium borodeuteride reduction of indan-1-one **15** to yield [1-^{2}H]-indan-1-ol followed by acid-catalysed dehydration yielded [3-^{2}H]-indene **8** (45% overall yield) using the literature method.[6] ^{1}H-NMR and MS analysis showed that [3-^{2}H]-indene **8** had a deuterium atom incorporated (> 98% ^{2}H) only at the C-3 position.

6-Bromoindan-1-one 23. 6-Bromoindan-1-one **23** (4.7 g, 44%) was obtained by the literature procedure,[36] mp 106–107 °C (hexane) (lit.,[36] 108–109 °C); δ_H(300 MHz, $CDCl_3$) 2.72 (2 H, t, $J_{2,3}$ 5.8, 2-H), 3.10 (2 H, t, $J_{3,2}$ 5.8, 3-H), 7.37 (1 H, d, $J_{4,5}$ 8.1, 4-H), 7.68 (1 H, d, $J_{5,4}$ 8.1, $J_{5,7}$ 1.7, 5-H), 7.87 (1 H, d, $J_{7,5}$ 1.7, 7-H).

Synthesis of 5-bromoindan-1-ol and 6-bromoindan-1-ol

Sodium borohydride (50 mmol) was added portion-wise (0.5 h) to a solution of indanone (**20** or **23**, 25 mmol) in MeOH (50 cm^3) at 0 °C. The solution was stirred at room temperature for a further 2 h and the solvent removed under reduced pressure. The residue was extracted into CH_2Cl_2 (3 × 50 cm^3), the extract dried ($MgSO_4$) and the solvent evaporated off to yield the crude indanol.

5-Bromoindan-1-ol. White crystals (4.8 g, 90%); mp 77–79 °C (from hexane) (Found: C, 50.7; H, 4.1. C_9H_9OBr requires C, 50.7; H, 4.3%); δ_H(500 MHz, $CDCl_3$) 1.94 (1 H, m, 2′-H), 2.48 (1 H, m, 2-H), 2.80 (1 H, m, 3-H), 3.03 (1 H, m, 3′-H), 5.18 (1 H, m, 1-H), 7.26 (1 H, m, Ar-H), 7.36 (2 H, m, Ar-H).

6-Bromoindan-1-ol. White crystals (4.7 g, 88%); mp 85–86 °C (hexane) (lit.,[37] 84–86 °C); δ_H(300 MHz, $CDCl_3$) 1.94 (1 H, m, 2-H), 2.49 (1 H, m, 2′-H), 2.75 (1 H, m, 3-H), 2.98 (1 H, m, 3′-H), 5.20 (1 H, d, $J_{1,2}$ 5.2, 1-H), 7.11 (1 H, d, $J_{4,5}$ 8.0, 4-H), 7.35 (1 H, dd, $J_{5,4}$ 8.0, $J_{5,7}$ 1.8, 5-H), 7.52 (1 H, d, $J_{7,5}$ 1.8, 7-H).

Synthesis of 5-bromoindene 16 and 6-bromoindene 17

A solution of 5-bromoindan-1-ol or 6-bromoindan-1-ol (4.3 g, 20 mmol) and *p*-toluenesulfonic acid (*ca.* 0.075 g) in benzene (150 cm^3) was refluxed (2 h) and the resulting water continuously removed from the reaction mixture with a Dean–Stark trap. The cooled benzene solution was washed with water, dried ($MgSO_4$) and concentrated under reduced pressure; purification of the residue by flash chromatography (hexane) yielded pure 5-bromoindene **16** or 6-bromoindene **17**.

5-Bromoindene 16. Colourless crystals (3.43 g, 87% yield), mp 39–40 °C (hexane) (lit.,[38] mp 41 °C); δ_H(500 MHz, $CDCl_3$) 3.35 (2 H, dd, $J_{1,2}$ 2.0, $J_{1,3}$ 1.9, 1-H), 6.59 (1 H, dt, $J_{2,1}$ 2.0, $J_{2,3}$ 5.6, 2-H), 6.81(1 H, dt, $J_{3,2}$ 5.6, $J_{3,1}$ 1.9, 3-H), 7.29–7.33 (2 H, m, Ar-H), 7.52 (1 H, d, $J_{4,5}$ 1.4, Ar-H).

6-Bromoindene 17. An oil (3.62 g, 92% yield), bp 75 °C/1 mm Hg (lit.,[39] bp 50 °C/0.1 mm Hg) (Found: M^+, 193.9733, C_9H_7Br requires M^+ 193.9731); δ_H(300 MHz, $CDCl_3$) 3.38 (2 H, d, $J_{1,2}$ 2.0, 1-H), 6.54 (1 H, dt, $J_{1,2}$ 2.0, $J_{2,3}$ 5.5, 2-H), 6.83 (1 H, m, 3-H), 7.26 (1 H, d, $J_{4,5}$ 8.1, 4-H), 7.41 (1 H, d, $J_{5,4}$ 8.1, 5-H), 7.61 (1 H, s, 7-H).

Synthesis of chromane 30

p-Toluenesulfonic acid (*ca.* 0.030 g) and hydroquinone (*ca.* 0.05 g) were added to a solution of racemic chroman-4-ol **31** (8.0 g, 53 mmol) in benzene (150 cm^3). The reaction mixture was heated under reflux using a Dean–Stark trap (2 h), washed with water, dried (Na_2SO_4) and concentrated under reduced pressure to give chrom-3-ene (5.1 g, 74%), bp 30 °C/0.2 mm Hg (lit.,[40] 51–53 °C/1.2 mm Hg); δ_H(300 MHz, $CDCl_3$) 4.81 (2 H, m, 2-H), 5.76 (1 H, m, 3-H), 6.41 (1 H, d, $J_{4,3}$ 9.6, 4-H), 6.76 (1 H, d, $J_{8,7}$ 8.0, 8-H), 6.85 (1 H, dd, $J_{6,7}$ 8.6, $J_{6,5}$ 7.4, 6-H), 6.95 (1 H, dd, $J_{5,6}$ 7.4, $J_{5,7}$ 1.5, 5-H), 7.09 (1 H, m, 7-H).

A solution of chrom-3-ene (1.06 g, 8.03 mmol) in $CHCl_3$ (35 cm^3) was stirred (12 h) in the presence of Pd/C (10%, 0.10 g) under an atmosphere of hydrogen at ambient temperature and pressure. The catalyst was filtered off and the filtrate concentrated to give the crude hydrogenated product **30**. Distillation under reduced pressure yielded chromane **30** (0.70 g, 67%), bp 50–51 °C/0.1 mm Hg (lit.,[41] 97–98 °C/19 mm Hg); δ_H (300 MHz, $CDCl_3$) 1.95 (2 H, m, 3-H), 2.74 (2 H, t, $J_{4,3}$ 6.6, 4-H), 4.13 (2 H, t, $J_{2,3}$ 5.1, 2-H), 6.76 (2 H, m, 6-H and 8-H), 7.00 (2 H, m, 5-H and 7-H).

(±)-[4-^{2}H]-Chroman-3-ol 35$_D$. To a solution of racemic epoxide **34** (0.75 g, 5.1 mmol) in dry Et_2O (15 cm^3) was added lithium aluminium deuteride (0.54 g, 13 mmol, 98 atom % ^{2}H) and the reaction mixture stirred (18 h) at room temperature. The reaction was quenched by the dropwise addition of water. Filtration and concentration of the dried (Na_2SO_4) ether solution yielded (±)-[4-^{2}H]-chroman-3-ol **35$_D$** (0.70 g, 91%), mp 78–79 °C (from Et_2O–hexane); δ_H (300 MHz, $CDCl_3$) 2.09 (1 H, br s, OH), 2.77 (1 H, br s, 4-H), 4.08 (2 H, m, 2-H), 4.23 (1 H, m, 3-H), 6.87 (2 H, m, 6-H and 8-H), 7.09 (2 H, m, 5-H and 7-H). The deuterium incorporation was estimated to be ≥ 98% at C-4 by ^{1}H-NMR spectral analysis.

(−)-(3*S*,4*R*)-[4-^{2}H]-Chroman-3-ol 35$_D$. Reductive ring opening of the (−)-(3*R*,4*S*)-epoxide **34** (1.34 g, 9.1 mmol, $[a]_D$ −71) with lithium aluminium deuteride (0.95 g, 22.7 mmol) yielded (−)-(3*S*,4*R*)-[4-^{2}H]-chroman-3-ol **35$_D$** (1.26 g, 92%), mp 95–96 °C (from Et_2O–hexane); $[a]_D$ −20.6 (*c* 2.0, $CHCl_3$).

(+)-(3*R*,4*S*)-[4-^{2}H]-Chroman-3-ol 35$_D$. Similar treatment of the (+)-(3*S*,4*R*)-enantiomer of epoxide **34** (1.09 g, 7.4 mmol)

gave (+)-(3*R*,4*S*)-[4-^{2}H]-chroman-3-ol **35$_D$** (1.04 g, 94%), mp 95–96 °C; $[a]_D$ +21.5 (*c* 2.1, $CHCl_3$). Enantiomers of alcohol **35$_D$** were spectrally indistinguishable from the racemic sample. The deuterium incorporation was found to be > 98% (by ^{1}H-NMR) in both the enantiomers.

(±)-[4-^{2}H]-3-(*p*-Toluenesulfonyloxy)chromane 36$_D$. *p*-Toluenesulfonyloxy chloride (1.17 g, 6.1 mmol) was added to a cooled solution of [4-^{2}H]-3-chromanol **35$_D$** (0.62 g, 4.1 mmol) in dry pyridine (4 cm^3); the reaction mixture was stirred (24 h). Pyridine was removed (reduced pressure), water (10 cm^3) was added to the residue and the reaction mixture extracted with CH_2Cl_2 (2 × 30 cm^3). The extract was dried (Na_2SO_4) and concentrated under reduced pressure to yield (±)-[4-^{2}H]-3-(*p*-toluenesulfonyloxy)chromane **36$_D$** (0.94 g, 75%), mp 91–92 °C (from Et_2O–hexane) (Found: C, 62.9; H, 5.2. $C_{16}H_{15}DO_4S$ requires C, 63.0; H, 5.6%); δ_H (500 MHz, $CDCl_3$) 2.46 (3 H, s, Me), 2.95 (1 H, br s, 4-H), 4.10 (2 H, m, 2-H), 4.95 (1 H, m, 3-H), 6.81 (1 H, d, $J_{8,7}$ 7.9, 8-H), 6.87 (1 H, m, 6-H), 6.96 (1 H, d, $J_{5,6}$ 7.1, 5-H), 7.11 (1 H, m, 7-H), 7.36 (2 H, d, *J* 8.0, Ar-H), 7.81 (2 H, d, *J* 8.0, Ar-H).

(+)-(3*S*,4*R*)-[4-^{2}H]-3-(*p*-Toluenesulfonyloxy)chromane 36$_D$. (−)-(3*S*,4*R*)-[4-^{2}H]-3-chromanol **35$_D$** (1.30 g, 8.6 mmol) when reacted with *p*-toluenesulfonyl chloride gave (+)-(3*S*,4*R*)-[4-^{2}H]-3-(*p*-toluenesulfonyloxy)chromane **36$_D$** (2.00 g, 76%), mp 94–95 °C, $[a]_D$ + 6.4 ± 0.5 (*c* 1.65, $CHCl_3$).

(−)-(3*R*,4*S*)-[4-^{2}H]-3-(*p*-Toluenesulfonyloxy)chromane 36$_D$. (+)-(3*R*,4*S*)-[4-^{2}H]-3-chromanol **35$_D$** (1.06 g, 7.0 mmol) on reaction with *p*-toluenesulfonyl chloride gave (−)-(3*R*,4*S*)-[4-^{2}H]-3-(*p*-toluenesulfonyloxy)chromane **36$_D$**, mp 94–95 °C; $[a]_D$ −6.0 ± 0.5 (*c* 1.44, $CHCl_3$).

(±)-[4-^{2}H]-Chromane 30$_D$. Lithium aluminium hydride (0.60 g, 15.8 mmol) was added to a solution of racemic [4-^{2}H]-3-(*p*-toluenesulfonyloxy)chromane **36$_D$** (0.66 g, 2.2 mmol) in dry THF (10 cm^3) and the reaction mixture heated (24 h) under reflux. The cooled reaction mixture was filtered after decomposition of the complex by addition of water (1.5 cm^3). The filtrate, combined with the Et_2O washings, was dried (Na_2SO_4), concentrated under reduced pressure and the residue purified by flash column chromatography on silica gel (Et_2O–hexane) followed by distillation under reduced pressure to yield (±)-[4-^{2}H]-chromane **30$_D$** (0.21 g, 72%), bp 80 °C/0.02 mm Hg (Found: M^+, 135.0794. C_9H_9DO requires 135.0794); δ_H (300 MHz, $CDCl_3$) 2.01 (2 H, m, 3-H), 2.77 (1 H, br s, 4-H), 4.19 (2 H, m, 2-H), 6.81 (2 H, m, 6-H and 8-H), 7.05 (2 H, m, 5-H and 7-H); *m*/*z* (%) 136 ($M + 1^+$, 18), 135 (M^+, 100), 134 (23), 120 (11), 107 (18), 79 (28). The deuterium atom incorporation (≥ 98%) at C-4 was determined by ^{1}H- and ^{2}H-NMR spectral analyses.

(−)-(4*S*)-[4-^{2}H]-Chromane 30$_D$. (+)-(3*S*,4*R*)-[4-^{2}H]-3-(*p*-Toluenesulfonyloxy)chromane **36$_D$** (1.95 g, 6.4 mmol, $[a]_D$ + 6.4) was treated with excess lithium aluminium hydride to yield (4*S*)-[4-^{2}H]-chromane **30$_D$** (0.64 g, 74%), bp 80 °C/0.02 mm Hg; $[a]_D$ −1.7 (*c* 0.32, $CHCl_3$).

(+)-(4*R*)-[4-^{2}H]-Chromane 30$_D$. (−)-(3*R*,4*S*)-[4-^{2}H]-3-(*p*-Toluenesulfonyloxy)chromane **36$_D$** (1.45 g, 4.8 mmol, $[a]_D$ −6.0) afforded (4*R*)-[4-^{2}H]-chromane **30$_D$** (0.50 g, 78%), $[a]_D$ +1.0 (*c* 0.54, $CHCl_3$). (−)-(4*S*)- and (+)-(4*R*)-enantiomers of [4-^{2}H]-chromane **30$_D$** had identical ^{1}H-NMR spectra (≥ 98% ^{2}H) and mirror image CD spectra.

Biotransformation of [3-^{2}H]-indene 8$_D$

Biotransformation (*P. putida* UV4, 8 h) of [3-^{2}H]-indene **8$_D$** (0.47 g, 4.1 mmol) and extraction (EtOAc) of the centrifuged culture medium yielded three products. PLC (40% Et_2O in hexane) separation gave (−)-[1-^{2}H]-(1*S*,2*R*)-1,2-dihydroxyindane **13$_D$** (R_f 0.1), [1-^{2}H]- and [3-^{2}H]-(1*R*)-inden-1-ol **14$_D$/14′$_D$** (R_f 0.5) and 1-indanone **15**, identified by comparison with an authentic sample (0.02 g, 4%, R_f 0.8).

(−)-[1-^{2}H]-(1*S*,2*R*)-1,2-Dihydroxyindane 13$_D$. Colourless crystals (0.11 g, 18%), mp 95–96 °C (from $CHCl_3$–hexane) (lit.,[5] mp 95–96 °C); $[a]_D$ −21 (*c* 0.4, $CHCl_3$); δ_H (500 MHz; $CDCl_3$) 2.95 (1H, dd, $J_{3,2}$ 3.7, $J_{3,3'}$ 16.3, 3-H), 3.12 (1H, dd, $J_{3',2}$ 5.7, $J_{3',3}$ 16.3, 3′-H), 4.42 (1H, dd, $J_{2,3}$ 3.7, $J_{2,3'}$ 5.7, 2-H), 7.21–7.28 (3H, m, Ar-H) and 7.39–7.41 (1H, m, Ar-H); ee (*ca.* 38%) by CSPHPLC analysis (Chiralcel OD column).

[1-^{2}H]- and [3-^{2}H]-(1*R*)-Inden-1-ol 14$_D$/14′$_D$. Colourless needles (0.045 g, 8%), unstable sample, mp 83–84 °C (from ether–hexane) (Found: C, 81.5; H, 6.5. C_9H_7DO requires C 81.2; H, 6.8%); $[a]_D$ −70 (*c* 0.4, $CHCl_3$) (lit.,[24] $[a]_D$ −178 but found to increase on purification to $[a]_D$ −249); δ_H(500 MHz; $CDCl_3$) 5.21 (1H, 43% D, br s, 1-H), 6.42 (1H, dd, $J_{2,1}$ 1.8, $J_{2,3}$ 5.6, 2-H), 6.75 (1H, 57% D, d, $J_{3,2}$ 5.6, 3-H), 7.24 (3H, m, Ar-H), 7.52 (1H, d, *J* 6.9, Ar-H); ee (*ca.* 28%) by CSPHPLC analysis (Chiralcel OB column).

Biotransformation of 5-bromoindene 16

Biotransformation (*P. putida* UV4, 7 h) of 5-bromoindene **16** (0.150 g, 0.77 mmol) and extraction (EtOAc) of the centrifuged culture medium yielded two products; these on separation by PLC (60% Et_2O in pentane) gave 6-bromoindan-1-one **23** (0.007 g, 4%, R_f 0.5) and (1*R*,2*S*)-5-bromoindane-1,2-diol **18** (R_f 0.1).

6-Bromoindan-1-one 23. It was spectrally identical to a sample obtained from the biotransformation of 6-bromoindene **17**.

(1*R*,2*S*)-5-Bromo-1,2-dihydroxyindane 18. White crystals (0.07 g, 42%), mp 160–161 °C (from CH_2Cl_2); $[a]_D$ + 54.1 (*c* 0.77, MeOH) (Found: C, 46.8; H, 3.7. $C_9H_9BrO_2$ requires C 46.8; H, 3.9%); δ_H(500 MHz, $CDCl_3$) 2.30 (1 H, d, $J_{OH,1}$ 5.2, OH), 2.54 (1 H, d, $J_{OH,2}$ 7.2, OH), 2.91(1 H, dd, $J_{3,2}$ 3.1, $J_{3,3'}$ 16.4, 3-H), 3.06 (1 H, dd, $J_{3',2}$ 5.6, $J_{3',3}$ 16.4, 3′-H), 4.54 (1 H, m, 2-H), 5.02 (1 H, dd, $J_{1,2}$ 6.1, $J_{1,OH}$ 5.2, 1-H), 7.12 (1 H, d, $J_{4,5}$ 8.0, 4-H), 7.40 (1 H, m, $J_{5,4}$ 8.0, 5-H), 7.57 (1 H, s, 7-H); electronic CD data: λ 226 nm ($\Delta\varepsilon$ −2.571), 203 nm ($\Delta\varepsilon$ 13.58); ee (*ca.* 46%) by ^{1}H-NMR analysis of the MEBBA derivative.

Biotransformation of 6-bromoindene 17

Biotransformation (*P. putida* UV4, 7 h) of 6-bromoindene **17** (0.200 g, 1 mmol) and extraction (EtOAc) of the centrifuged culture medium yielded two products; separation by PLC (30% Et_2O in hexane) gave the less polar (R_f 0.45) bioproduct, 6-bromoindan-1-one **23** (0.061 g, 28%) which was identified by spectral comparison with an authentic sample. The second, more polar (R_f 0.35) product was found to be (1*S*)-6-bromoinden-1-ol **22**.

(+)-(1*S*)-6-Bromoinden-1-ol 22. Colourless crystals (0.035 g, 16%), mp 56 °C (hexane) (Found: C, 51.1; H, 3.4. C_9H_7BrO requires C, 51.2; H, 3.3%); $[a]_D$ + 185.3 (*c* 0.8, $CHCl_3$); δ_H(300 MHz, $CDCl_3$) 1.72 (1 H, d, $J_{OH,1}$ 8.9, OH), 5.16 (1 H, m, $J_{1,OH}$ 7.4, 1-H), 6.39 (1 H, dd, $J_{2,1}$ 1.6, $J_{2,3}$ 5.6, 2-H), 6.68 (1 H, d, $J_{3,2}$ 5.6, 3-H), 7.09 (1 H, d, $J_{4,5}$ 7.9, 4-H), 7.40 (1 H, dd, $J_{5,4}$ 7.9, $J_{5,7}$ 1.4, 5-H), 7.64 (1 H, d, $J_{7,5}$ 1.4, 7-H); electronic CD data: λ 270 nm ($\Delta\varepsilon$ 0.928), 230 nm ($\Delta\varepsilon$ −1.796), 204 nm ($\Delta\varepsilon$ 7.779), 198 nm ($\Delta\varepsilon$ 7.298); > 98% ee by CSPHPLC analysis (Chiralcel OB column).

Biotransformation of chromane 30

Biotransformation (*P. putida* UV4) of chromane **30** (0.150 g, 1.12 mmol) and extraction (EtOAc) of the centrifuged culture

medium yielded a mixture of two bioproducts; these on separation by PLC (50% Et_2O in pentane) afforded (+)-(4*R*)-chroman-4-ol **31** (R_f 0.28) and chroman-4-one **32** (R_f 0.5).

(+)-(4*R*)-Chroman-4-ol 31. Colourless crystals (0.022 g, 13%), mp 68–70 °C (from Et_2O–pentane) (lit.,[40] 73–75 °C); $[\alpha]_D$ + 63 (*c* 0.38, EtOH) (lit.,[41] + 67.0); δ_H (300 MHz, $CDCl_3$) 1.96–2.08 (2 H, m, 3-H), 2.18 (1 H, br s, OH), 4.22 (2 H, m, 2-H), 4.73 (1 H, m, 4-H), 6.81–6.93 (2 H, m, 6-H and 8-H), 7.16–7.29 (2 H, m, 5-H and 7-H); *m*/*z* (M^+ 150, 100), 149 (59); ee (*ca.* 95%) by CSPHPLC analysis (Chiralcel OB column).

Chroman-4-one 32

Viscous oil (0.008 g, 5%) (lit.,[40] bp 78–80 °C at 0.3 mm Hg); δ_H (300 MHz, $CDCl_3$) 2.81 (2 H, t, $J_{3,2}$, 3-H), 4.54 (2 H, t, $J_{2,3}$ 6.6, 2-H), 7.00 (2 H, m, 6-H and 8-H), 7.47 (1 H, m, 7-H), 7.90 (1 H, dd, $J_{5,6}$ 7.9, $J_{5,7}$ 1.7, 5-H); *m*/*z* (M^+ 148, 70), 120 (100), 92 (63), 63 (19).

Biotransformation of (±)-[4-^{2}H]-chromane 30_D

Biotransformation (*P. putida* UV4) of racemic deuterium-labelled chromane $\mathbf{30_D}$ (0.050 g, 0.37 mmol, ≥ 98 atom % ^{2}H) and isolation of metabolites was carried out in a similar manner as for substrate **30**; (+)-(4*R*)-chroman-4-ol $\mathbf{31/31_D}$ (0.006 g, 11%) and chroman-4-one **32** (0.004 g, 7%) were obtained. ^{1}H-NMR spectral analysis indicated that the ratio of non-deuteriated alcohol **31** to deuteriated alcohol $\mathbf{31_D}$ was 37 : 63; ee ≥ 98% by CSPHPLC analysis.

Biotransformation of (−)-(4*S*)-[4-^{2}H]-chromane 30_D

Biotransformation (*P. putida* UV4) of (−)-(4*S*)-[4-^{2}H]-chromane $\mathbf{30_D}$ (0.050 g, 0.37 mmol, ≥ 98 atom % ^{2}H) yielded [4-^{2}H]-chroman-4-ol $\mathbf{31_D}$ (0.08 g, 14%) and chroman-4-one **32** (0.005 g, 9%). Metabolite $\mathbf{31_D}$ was shown to be deuteriated at C-4 (*ca.* 98%) by ^{1}H-NMR spectral analysis.^{1}H- and ^{2}H-NMR spectral analysis confirmed that deuterium was located at C-4; ee > 98% by CSPHPLC analysis.

Biotransformation of (+)-(4*R*)-[4-^{2}H]-chromane 30_D

Biotransformation (*P. putida* UV4) of (+)-(4*R*)-[4-^{2}H]-chromane $\mathbf{30_D}$ (0.05 g, 0.37 mmol, ≥ 98 atom % ^{2}H) yielded chroman-4-ol **31** (0.008 g, 14%) and chroman-4-one **32** (0.005 g, 9%). Chroman-4-ol **31** was found to be essentially non-deuteriated (*ca.* 1% deuterium at C-4) by ^{1}H-NMR spectral analysis; ee >98% by CSPHPLC analysis.

Biotransformation of (±)-[4-^{2}H]-chromane 30_D

Biotransformation (*P. putida* 9816/11, 18 h) of the racemic deuterium-labelled chromane $\mathbf{30_D}$ (0.20 g, 1.48 mmol, ≥ 98% ^{2}H) was carried out according to the reported method.[43] EtOAc extraction (2 × 30 cm^3) of the centrifuged culture medium yielded a mixture of bioproducts. GC-MS analysis, using the earlier specified conditions, showed the following bioproducts: substrate $\mathbf{30_D}$ (8.6 min), chroman-4-one **32** (10.5 min, 53%), (−)-(4*S*)-chroman-4-ol $\mathbf{31/31_D}$ (10.8 min, 40%) and *cis*-3,4-dihydroxychromane $\mathbf{40/40_D}$ (12.6 min, 7%). Bioproducts $\mathbf{31/31_D}$ (0.03 g, 13% isolated yield) and $\mathbf{32_D}$ (0.035 g, 16% isolated yield) were separated and purified by PLC (40% Et_2O in hexane). Chromanol $\mathbf{31/31_D}$, $[\alpha]_D$ −16.0 (*c* 1.0, EtOH) was found to have an ee of *ca.* 24% by CSPHPLC analysis. The *cis*-3,4-dihydroxychromane $\mathbf{40/40_D}$ was not isolated.

Biotransformation of (−)-(4*S*)-[4-^{2}H]- and (+)-(4*R*)-[4-^{2}H]-chromane 30_D using purified TDO and NDO enzymes

Biotransformations of (−)-(4*S*)-[4-^{2}H]- and (+)-(4*R*)-[4-^{2}H]-chromane $\mathbf{30_D}$ with purified TDO and NDO enzymes were carried out by the same procedure as was used for the sulfoxidation of sulfide[14] and benzylic hydroxylation of indanone substrates.[28] Biotransformations on a medium scale were conducted in flasks (250 cm^3) which were shaken (150 rpm) in a water bath (28 °C). The reaction mixture in each flask used for TDO or NDO biotransformations contained 30 cm^3 of 2-(4-morpholino)ethanesulfonic acid (50 mmol, pH 6.8), NADH (2 mmol), ferrous ammonium sulfate (0.1 mmol), catalase (0.005 g) and the respective reductase (0.003 g), ferredoxin (0.0016 g) and ISP (0.00175 g) components of TDO and NDO. The quantities of substrates added to the pure enzyme preparations were (+)-(4*R*)-[4-^{2}H]-chromane $\mathbf{30_D}$ (0.004 g) and (−)-(4*S*)-[4-^{2}H]-chromane $\mathbf{30_D}$ (0.0067 g) respectively. The biotransformations were conducted over a 4 h period. The metabolites were recovered by extraction (EtOAc) and concentration under reduced pressure. The product mixtures were subjected to GC-MS analysis and the relative ratios of products obtained are shown in Table 1. Enantiopurity values of chroman-4-ol $\mathbf{31/31_D}$ were determined, after purification (TLC), by CSPHPLC analysis (Chiralcel OJ column).

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References

1 D. T. Gibson and R. E. Parales, *Curr. Opin. Biotechnol.*, 2000, **11**, 236.
2 D. R. Boyd, N. D. Sharma and C. C. R. Allen, *Curr. Opin. Biotechnol.*, 2001, **12**, 564.
3 T. Hudlicky, D. Gonzalez and D. T. Gibson, *Aldrichimica Acta*, 1999, **32**, 33.
4 D. R. Boyd and G. N. Sheldrake, *Nat. Prod. Rep.*, 1998, **15**, 309.
5 S. M. Resnick, K. Lee and D. T. Gibson, *J. Ind. Microbiol.*, 1996, **17**, 438.
6 L. P. Wackett, L. D. Kwart and D. T. Gibson, *Biochemistry*, 1988, **27**, 1360.
7 D. R. Boyd, N. D. Sharma, P. J. Stevenson, J. Chima, D. J. Gray and H. Dalton, *Tetrahedron Lett.*, 1991, **32**, 3887.
8 J. M. Brand, D. L. Cruden, G. J. Zylstra and D. T. Gibson, *Appl. Environ. Microbiol.*, 1992, **58**, 3407.
9 D. R. Boyd, N. D. Sharma, N. I. Bowers, P. A. Goodrich, M. R. Groocock, A. J. Blacker, D. A. Clarke, T. Howard and H. Dalton, *Tetrahedron: Asymmetry*, 1996, **7**, 1559.
10 N. I. Bowers, D. R. Boyd, N. D. Sharma, P. A. Goodrich, M. R. Groocock, A. J. Blacker, P. Goode and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 1999, 1453.
11 D. R. Boyd, N. D. Sharma, N. I. Bowers, J. Duffy, J. S. Harrison and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 2000, 1345.
12 C. C. R. Allen, D. R. Boyd, H. Dalton, N. D. Sharma, S. A. Haughey, R. A. S. McMordie, B. T. McMurray, K. Sproule and G. N. Sheldrake, *J. Chem. Soc., Chem. Commun.*, 1995, 119.
13 K. Lee, J. M. Brand and D. T. Gibson, *Biochem. Biophys. Res. Commun.*, 1995, **212**, 9.
14 D. R. Boyd, N. D. Sharma, S. A. Haughey, M. A. Kennedy, B. T. McMurray, G. N. Sheldrake, C. C. R. Allen, H. Dalton and K. Sproule, *J. Chem. Soc., Perkin Trans. 1*, 1998, 1929.
15 D. R. Boyd, N. D. Sharma, S. A. Haughey, J. F. Malone, A. King, B. T. McMurray, R. Holt and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 2001, 3288.
16 D. T. Gibson, B. Gschwendt, W. K. Yeh and V. M. Kobal, *Biochemistry*, 1973, **12**, 1520.
17 D. R. Boyd, N. D. Sharma, M. Groocock, J. F. Malone and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 1997, 1897.
18 D. R. Boyd, N. D. Sharma, V. Ljubez, B. E. Byrne, S. D. Shepherd, C. C. R. Allen, L. A. Kulakov, M. I. Larkin and H. Dalton, *Chem. Commun.*, 2002, 1914.
19 D. R. Boyd, N. D. Sharma, M. A. Kennedy, J. F. Malone, C. C. R. Allen, S. M. Resnick and D. T. Gibson, *J. Org. Chem.*, 1999, **64**, 4005.

20 D. R. Boyd, N. D. Sharma, J. G. Carroll, C. C. R. Allen, D. A. Clarke and D. T. Gibson, *Chem. Commun.*, 1999, 1345.
21 B. Kauppi, K. Lee, E. Carredano, R. E. Parales, D. T. Gibson, H. Eklund and S. Ramaswamy, *Structure*, 1998, **6**, 571.
22 E. Carredano, E. Karlsson, B. Kauppi, D. Chaudhury, R. E. Parales, J. V. Parales, K. Lee, D. T. Gibson, H. Eklund and S. Ramaswamy, *J. Mol. Biol.*, 2000, **182**, 5495.
23 K. Chen, M. Costas, J. Kim, A. K. Tipton and L. Que, *J. Am. Chem. Soc.*, 2002, **124**, 3026.
24 D. R. Boyd, N. D. Sharma, R. Boyle, B. T. McMurray, T. A. Evans, J. F. Malone, J. Chima, H. Dalton and G. N. Sheldrake, *J. Chem. Soc., Chem. Commun.*, 1993, 49.
25 D. R. Boyd, R. A. S. McMordie, N. D. Sharma, H. Dalton, P. Williams and R. O. Jenkins, *J. Chem. Soc., Chem. Commun.*, 1989, 339.
26 D. R. Boyd, N. D. Sharma, I. N. Brannigan, D. A. Clarke, H. Dalton, S. A. Haughey and J. F. Malone, *Chem. Commun.*, 1996, 2361.
27 D. R. Boyd, N. D. Sharma, S. A. Haughey, J. F. Malone, B. T. McMurray, G. N. Sheldrake, C. C. R. Allen and H. Dalton, *Chem. Commun.*, 1996, 2363.
28 S. M. Resnick, D. S. Torok and D. T. Gibson, *J. Org. Chem.*, 1995, **60**, 3546.
29 D. T. Gibson, S. M. Resnick, K. Lee, J. M. Brand, D. S. Torok, L. P. Wackett, M. J. Schocken and B. E. Haigler, *J. Bacteriol.*, 1995, **177**, 2615.
30 S. M. Resnick, D. S. Torok, K. Lee, J. M. Brand and D. T. Gibson, *Appl. Environ. Microbiol.*, 1994, **60**, 3323.
31 D. Burdi and T. P. Begley, *J. Am. Chem. Soc.*, 1991, **113**, 776.
32 D. R. Boyd, N. D. Sharma, R. Boyle, J. F. Malone, J. Chima and H. Dalton, *Tetrahedron: Asymmetry*, 1993, **4**, 1307.
33 D. R. Boyd, N. D. Sharma, R. Boyle, T. A. Evans, J. F. Malone, K. M. McCombe, H. Dalton and J. Chima, *J. Chem. Soc., Perkin Trans. 1*, 1996, 1757.
34 S. M. Resnick and D. T. Gibson, *Biodegradation*, 1993, **4**, 195.
35 A. M. Valentine, B. Wilkinson, K. E. Lieu, S. Komar-Panicucci, N. D. Priestly, P. G. Williams, H. Morimoto, H. G. Floss and S. J. Lippard, *J. Am. Chem. Soc.*, 1997, **119**, 1818.
36 L. A. M. Cornelius and D. W. Combs, *Synth. Commun.*, 1994, **24**, 2777.
37 W. J. Houlihan, J. H. Gogerty, V. A. Parrino and E. Ryan, *J. Med. Chem.*, 1983, **26**, 765.
38 M. Adamczyk, D. S. Watt and D. A. Netzel, *J. Org. Chem.*, 1984, **49**, 4226.
39 M. Quere, *Bull. Soc. Chim. Fr.*, 1971, 2983.
40 W. E. Parham and L. D. Huestis, *J. Am. Chem. Soc.*, 1962, **84**, 813.
41 J. Gripenberg and H. Tapio, *Acta Chem. Scand.*, 1966, **20**, 1561.
42 W. D. Still, Jr. and D. J. Goldsmith, *J. Org. Chem.*, 1970, **35**, 2282.
43 D. R. Boyd, N. D. Sharma, L. V. Modyanova, J. G. Carroll, J. F. Malone, C. C. R. Allen, J. T. G. Hamilton, D. T. Gibson, R. E. Parales and H. Dalton, *Can. J. Chem.*, 2002, **80**, 589.
44 D. T. Gibson, G. E. Cardini, F. C. Maseles and R. E. Kallio, *Biochemistry*, 1970, **9**, 1631.
45 M. D. Wolfe, J. V. Parales, D. T. Gibson and J. D. Lipscomb, *J. Biol. Chem.*, 2001, **276**, 1945.
46 J. T. Groves, G. A. McClusky, R. E. White and M. J. Coon, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 154.
47 C. J. Schofield and Z. Zhang, *Curr. Opin. Chem. Biol.*, 1999, **9**, 722.
48 B. Siegel, *Bio-Org. Chem.*, 1979, **8**, 2129.
49 A. D. N. Vaz, S. J. Pernecky, G. M. Raner and M. J. Coon, *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 4644.
50 A. D. N. Vaz, D. F. McGinnity and M. J. Coon, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 3555.
51 E. Carredano, A. Karlsson, B. Kauppi, D. Choudhury, R. E. Parales, J. V. Parales, K. Lee, D. T. Gibson, H. Eklund and S. Ramaswamy, *J. Mol. Biol.*, 2000, **296**, 701.
52 M. D. Wolfe and J. D. Lipscomb, *J. Biol. Chem.*, 2003, **278**, 829.
53 A. Karlsson, J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund and S. Ramaswamy, *Science*, 2003, **299**, 1039.