The potential role of *cis*-dihydrodiol intermediates in bacterial aromatic hydroxylation and the NIH Shift

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Specifically deuteriated samples of toluene, anisole, chlorobenzene, α,α,α-trifluoromethylbenzene, naphthalene and quinoline have been synthesised and used as substrates for dioxygenase-catalysed asymmetric dihydroxylation studies to yield the corresponding *cis*-dihydrodiols as major bioproducts. Phenols were also detected as minor metabolites in some cases. Dehydration of the deuterium-labelled *cis*-dihydrodiol metabolites, under thermal conditions, in all cases, resulted in phenol formation accompanied by the NIH Shift. A comparison of NIH Shift results, obtained when phenols are produced by aromatisation of chemically synthesised deuteriated arene *cis*- and *trans*-dihydrodiols (dehydration) and arene oxides (isomerisation), suggests that this phenomenon may be associated with both monooxygenase- and dioxygenase-catalysed aromatic hydroxylations.

Introduction

Aromatic hydroxylation is a very common metabolic pathway which occurs across a wide range of compounds and organisms including plants, animals, fungi and bacteria. Early studies of the *para*-hydroxylation of phenylalanine using either very substrate-specific enzymes, *e.g.* phenylalanine hydroxylase, or much less selective enzymes, *e.g.* cytochrome P-450 (monooxygenase) isozymes, were found to be accompanied by the migration of a hydrogen (deuterium or tritium) atom to an adjacent carbon atom and partial retention of the transferred atom. This migration and retention process associated with aromatic hydroxylation was first observed at the National Institutes of Health, Bethesda, USA, and has been described as the NIH Shift.[1–3] The ubiquitous nature of the NIH Shift is evident from the large number (>100) of examples of enzyme-catalysed aromatic hydroxylations involving migration and retention of either atoms (hydrogen, deuterium, tritium, halogen) or groups (methyl, methoxycarbonyl), in both eucaryotic (animal, plant, fungal) and procaryotic (bacterial) systems, which have been reported in the literature up to 1985.[4] The phenomenon of the NIH Shift has continued to appear in the literature over the past decade[5–13] and the concept is now well established.

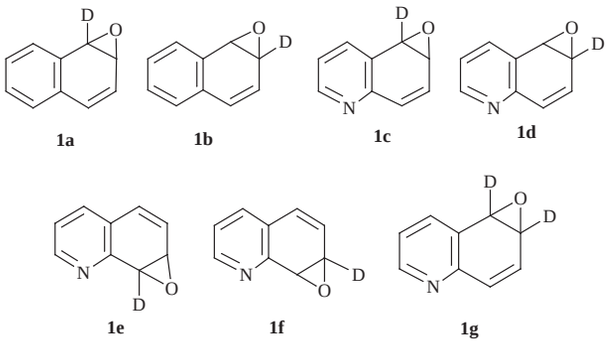
The majority of the early examples of the NIH Shift occurring during aromatic hydroxylation reactions were associated with eucaryotes and were assumed to involve transient arene oxide intermediates.[2–4] Aromatic hydroxylation has also been associated with procaryotes.[7,10,12,14–17] Thus bacterial dioxygenase-catalysed oxidation of arenes may indeed result from the initial formation of transient *cis*-dihydrodiol metabolites, followed by spontaneous dehydration. Kinetic studies on the dehydration of isolable *cis*-dihydrodiol metabolites of monosubstituted benzenes to yield phenols have shown that when electron donating groups, *e.g.* amino, alkoxy and thioalkoxy substituents are present, the rate of aromatisation under acidic conditions is much faster.[18,19] Some carbocyclic *cis*-dihydrodiol metabolites of heterocyclic arenes, *e.g.* benzofuran [6,7-$(OH)_2$],[20,21] benzothiophene [4,5-$(OH)_2$],[21,22] and isoquinoline [5,6-$(OH)_2$],[23] have also been found to aromatise at ambient temperature under the conditions of biotransformation or work-up. Aromatic hydroxylation of azaarenes, *e.g.* pyridine, quinoline, isoquinoline, quinoxaline and quinazoline again appears to be a very common biotransformation pathway in bacterial systems.[23] *cis*-Dihydrodiol metabolites of a pyridine ring system have not yet been isolated and may be too unstable to be isolated. A recent attempt to isolate the dihydrodiol metabolites resulting from *cis*-dihydroxylation at the 3,4-bond of 2-substituted quinolines yielded a *cis*-3,4-dihydroxyquinolone.[24]

Since the NIH Shift was initially associated with the isomerisation of arene oxides,[25–27] its observation during monooxygenase-catalysed aromatic hydroxylation became accepted as evidence of arene oxide involvement. This interpretation has subsequently been shown to be too restrictive and alternative mechanisms for enzyme-catalysed aromatic hydroxylation involving the NIH Shift, without involvement of arene oxide intermediates, have also been proposed.[6,12,28–30] It has been shown that the ability to form carbocation and/or oxo diene intermediates was important for the NIH Shift to occur during aromatic hydroxylation. A major objective of the present (and preliminary[6]) study was thus to demonstrate, using deuterium labelled compounds, that phenols can be produced *via* isomerisation of arene oxides or dehydration of *cis*- or *trans*-dihydrodiols and that any of these aromatisations can show evidence of the NIH Shift.

Results and discussion

The NIH Shift of a deuterium atom has been observed during the isomerisation of labelled toluene[25,27] and naphthalene oxides[26] to yield the corresponding phenols. The initial phase of the current study involved the synthesis of deuterium labelled arene oxides of naphthalenes (**1a**–**b**) and quinolines (**1c**–**g**) using synthetic methods similar to those reported for naphthalene oxides (Scheme 1).[26] Arene oxides **1a**–**g** were synthesised from 1,2-dihydro[4-^{2}H]naphthalene (**12**, X = Y = CH), 1,2-dihydro[3-^{2}H]naphthalene (**8**, X = Y = CH), 7,8-dihydro-[5-^{2}H]quinoline (**12**, X = N, Y = CH), 7,8-dihydro[6-^{2}H]-quinoline-(**8**, X = N, Y = CH), 7,8-dihydro[5,6-$^2H_2$]quinoline, 5,6-dihydro[8-^{2}H]quinoline (**12**, X = CH, Y = N), and 5,6-dihydro[7-^{2}H]quinoline (**8**, X = CH, Y = N) respectively by the literature methods.[26,31]

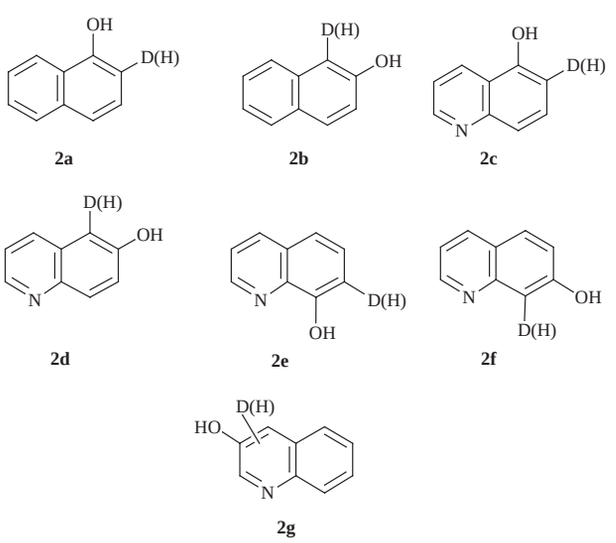
Earlier kinetic studies[32] had shown that marked differences



1a **1b** **1c** **1d**

1e **1f** **1g**

were found in the stability of individual arene oxides toward isomerisation under aqueous conditions. Thus, quinoline oxides **1c**–**f** were *ca*. 100–1000 fold less reactive than naphthalene oxides **1a** or **1b**. A small sample of each arene oxide (**1a**–**f**, *ca*. 0.005 g) was sealed under vacuum in a glass capillary tube and thermal isomerisation was carried out under controlled conditions to yield the corresponding phenols **2a**–**f** respectively as major products (85–95% yield of total phenolic products, Table 1). The location and incorporation of deuterium atoms were determined by ^{1}H-NMR spectroscopy (arene oxide reactant, **1a**–**f**) and GC-MS analysis of the trimethylsilyl ether derivatives (phenol products, **2a**–**f**). The value of the NIH Shift (±4% deuterium), determined from the proportion of migration and retention of deuterium label in phenol **2a** (73–81% deuterium), was similar to that reported[26] (80–81% deuterium) for 1-naphthol **2a** or 2-naphthol **2b** formation from naphthalene 1,2-oxide **1a** or **1b**. The results obtained were in accord with the mechanism outlined in Scheme 2 where evidence of only a minor contribution from the direct loss pathway was found.

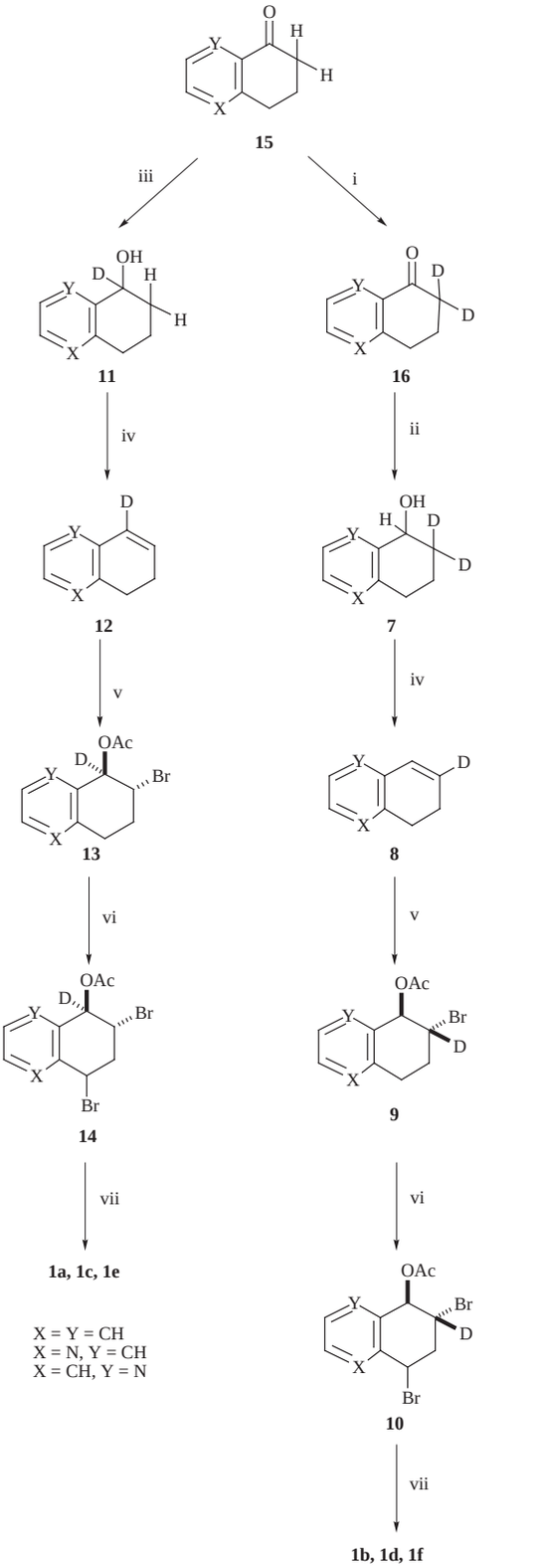


2a **2b** **2c**

2d **2e** **2f**

2g

The magnitude of the NIH Shift observed for isomerisation of the 5,6- (**1c**,**d**, 43–69% deuterium) and the 7,8- (**1e**,**f**, 42-71% deuterium) arene oxides of quinoline was generally lower and showed a greater variation between experiments. These results confirmed that the NIH Shift could be observed for phenol formation *via* isomerisation of benzene oxides fused to both carbocyclic and heterocyclic rings. It also raised the question of whether or not a proportion of the original deuterium label could be lost by alternative mechanisms including the direct loss pathway or an exchange process, particularly where fused azaarene rings are present. The observation that the NIH Shift values were slightly higher for isomerisation of arene oxides **1d** and **1f** to phenols **2c** (69% deuterium) and **2f** (56–71% deuterium), compared with arene oxides **1c** and **1e** to phenols **2c** (43–50% deuterium) and **2e** (42–46% deuterium), could be due to a small contribution from the direct loss pathway (Scheme 2).



15

11 **16**

12 **7**

13 **8**

14 **9**

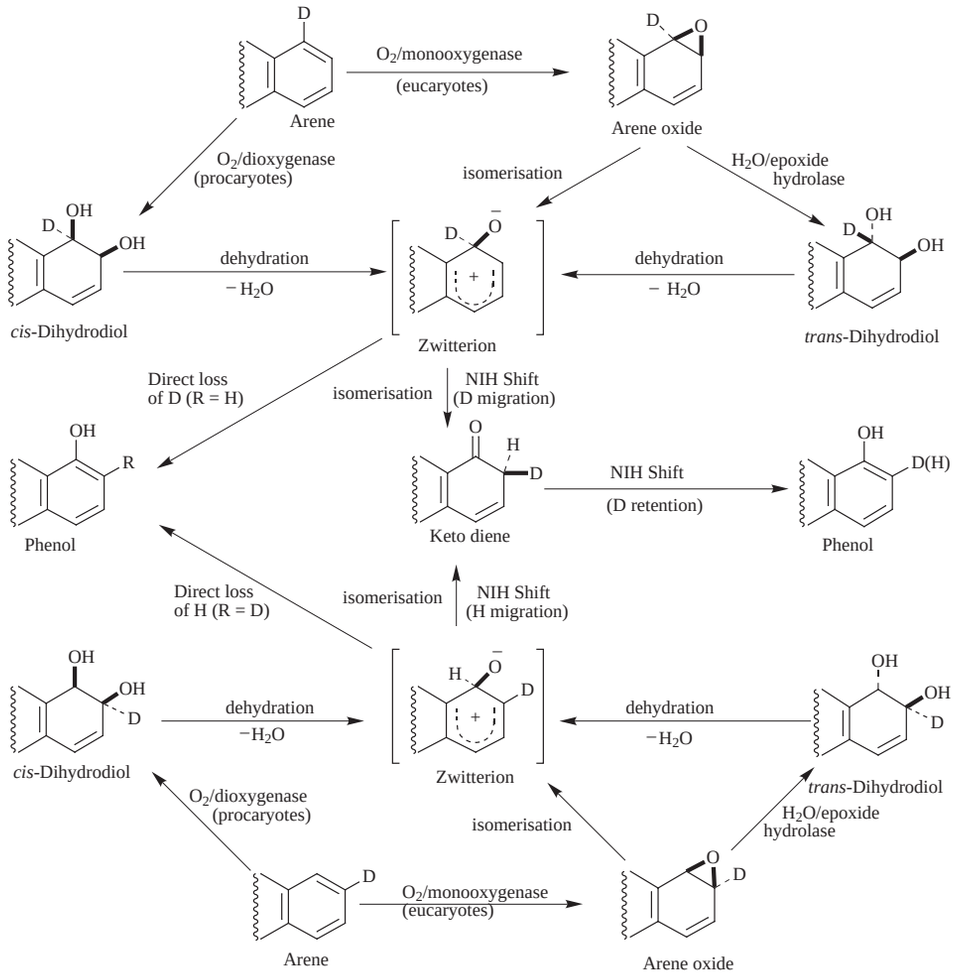
1a, 1c, 1e **10**

1b, 1d, 1f

X = Y = CH
X = N, Y = CH
X = CH, Y = N

Scheme 1 *Reagents and conditions*: i, KO^{t}Bu, D_2O; ii, $NaBH_4$; iii, $NaBD_4$; iv, PPA; v, NBA, LiOAc, $MeCO_2H$; vi, NBS, CCl_4; vii, NaOMe, THF.

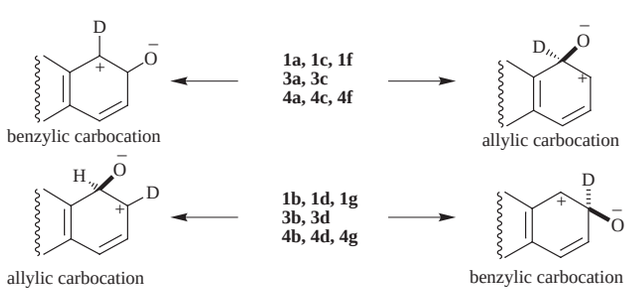
In order to examine the possibility that the values of the NIH Shift from isomerisation of arene oxides **1c**–**f** were also lower than expected due to an exchange process, a doubly labelled arene oxide of quinoline (**1g**) was synthesised, using a combination of the labelling methods (Scheme 1), and aromatised thermally. The results (Table 1) indicate that the deuterium content (58–65% deuterium/42–35% hydrogen), is consistent with a significant degree of deuterium exchange and loss. The deuterium content of the 5-hydroxyquinoline product (**2c**) was found



Scheme 2

to decrease slightly with time (130 °C, 5 min 65% deuterium, 45 min 58% deuterium). A possible mechanism for the loss of deuterium label could involve base-catalysed equilibration between the keto (keto diene) and enol (phenol) tautomers. Evidence for this mechanism was obtained when unlabelled 5-hydroxyquinoline was heated in a sealed tube (180 °C, 60 min) with a trace of D_2O to yield product **2c** with a deuterium incorporation of *ca*. 75% exclusively at C-6. A parallel experiment with 1-naphthol under identical conditions gave product **2a** with slightly lower deuterium incorporation (*ca*. 50%) at C-2. These observations suggest that loss of deuterium label from the carbon atom adjacent to the carbon bearing a phenolic hydroxy group can occur by an exchange process. Thus, deuterium retention results obtained by direct GC-MS analysis of phenolic products, particularly at the higher temperatures required for dehydration of *cis*- and *trans*-dihydrodiols or when basic nitrogen atoms are present, may be lower than expected.

The preceding results have highlighted the fact that a low value of the NIH Shift during phenol formation may be due to deuterium loss by exchange, and/or a direct loss pathway. Such low values may thus still be consistent with an arene oxide intermediate (Scheme 2). Isomerisation of arene oxides **1a**, **1e**, and **1f** under acidic conditions again showed evidence of the NIH Shift (49–79% deuterium). However, due to the possibility of protonation of the nitrogen atom in azaarene oxides in the presence of acids, the majority of isomerisation studies were carried out thermally.[31] 1-Naphthol **2a** (95% yield), 5-hydroxyquinoline **2c** (86% yield) and 8-hydroxyquinoline **2e** (95% yield) were found to be the major phenols obtained by thermal isomerisation of the corresponding arene oxides (**1a**–**g**) *via* ring opening to an allylic carbocation. 2-Naphthol **2b** (5% yield), 6-hydroxyquinoline **2d** (14% yield) and 7-hydroxyquinoline **2f** (5% yield) were assumed to result from benzylic carbocations (zwitterions) (Scheme 3). In view of the low yields



Scheme 3

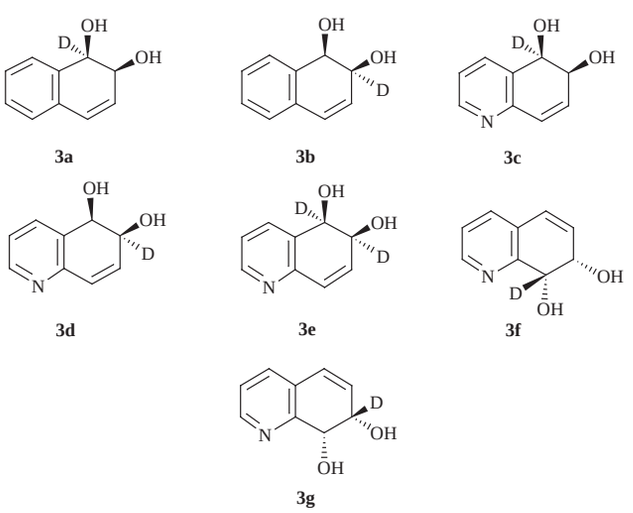
of the latter phenols, reliable values for the deuterium migration and retention (NIH Shift) were not obtained.

Quinoline had earlier been shown to be a substrate for the UV4 mutant strain of the soil bacterium *Pseudomonas putida* yielding mainly *cis*-dihydrodiols **3c** and **3d** but also minor amounts of *cis*-dihydrodiols **3f** and **3g**. Additional quinoline metabolites included 3-hydroxyquinoline, **2g** and anthranilic acid.[23] During the study, deuteriated samples of *cis*-dihydrodiols **3c** and **3d** were isolated as metabolites of 5-deuterio- and 6-deuterio-quinoline respectively under similar conditions. The metabolism of naphthalene to produce the *cis*-dihydrodiol derivative **3a** or **3b** by *P. putida* UV4[33] was not considered useful due to the presence of four equivalent arene double bonds in the naphthalene substrate. Samples of *cis*-dihydrodiols specifically labelled at C-1 (**3a**) and C-2 (**3b**) were chemically synthesised using [1-^{2}H]- (**12**, Y = X = CH, $R^1 = {}^2H$, R^2 = H) and

Table 1 Migration and retention of D-atoms (NIH Shift) during isomerisation of deuteriated arene oxides and dehydration of deuteriated dihydrodiols to yield phenols

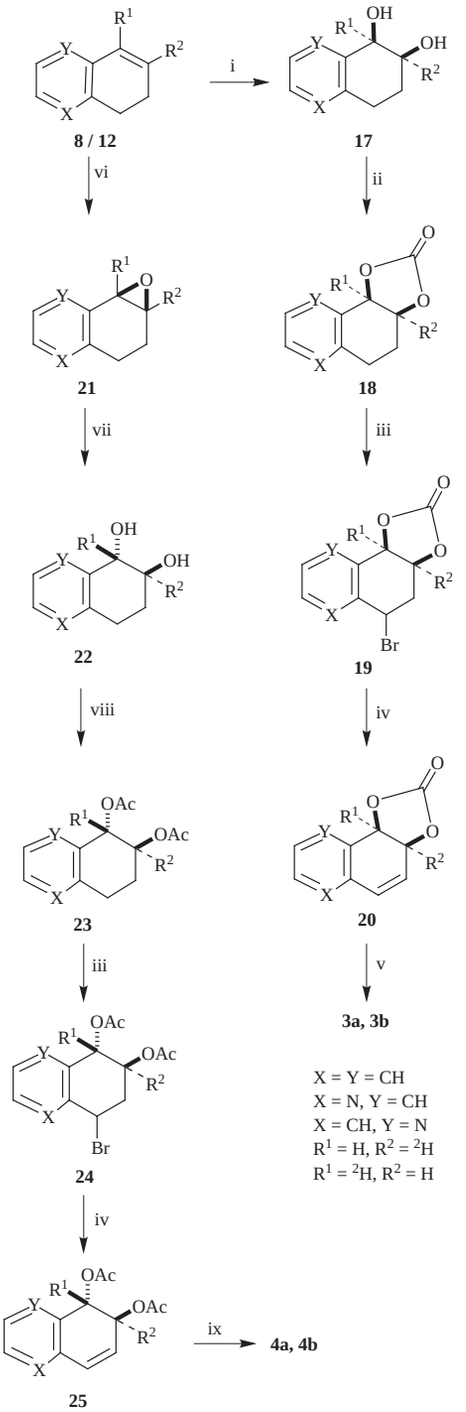
Substrate	Phenols	Conditions	Deuterium retention (%)
Arene oxides			
1a[a]	**2a**	Δ (H^+)	73,[c] 80,[d] (49)[e]
1b[a]	**2a**	Δ	81[d]
1c[a]	**2c**	Δ	43,[f] 50[g]
1d[a]	**2c**	Δ	69[f]
1e[a]	**2e**	Δ (H^+)	42,[f] 46,[h] (53)[i]
1f[a]	**2e**	Δ (H^+)	56,[f] 71,[g] (79)[i]
1g[a]	**2c**	Δ	58–65[h]
cis- and *trans*-Dihydrodiols			
3a[a]	**2a**	Δ (H^+)	73,[c] (45)[e]
3b[a]	**2a**	Δ	53[j]
3c[b]	**2d**	Δ	12[k]
3d[b]	**2d**	Δ	15[k]
3e[a]	**2d**	Δ	45[k]
4a[a]	**2a**	Δ (H^+)	70,[k] (47)[d]
4b[a]	**2a**	Δ	72[k]
4c[a]	**2d**	Δ	32[k]
4d[a]	**2d**	Δ	41[k]
4e[a]	**2d**	Δ	60[k]
4f[a]	**2e**	Δ	51[k]
4g[a]	**2e**	Δ	49[k]
5a[b]	**6a**	Δ	26[l]
5b[b]	**6a**	Δ	24[l]
5c[b]	**6b**	Δ	31[l]
5d[b]	**6b**	Δ	32[l]
5e[b]	**6c**	Δ	30[l]
5f[b]	**6c**	Δ	27[l]
5g[b]	**6d**	Δ	34[l]
5h[b]	**6d**	Δ	19[l]

[a] Non-enzymatic synthesis. [b] Dioxygenase-catalysed synthesis. Aromatisation conditions. [c] 150 °C, 5 min. [d] pH 7 buffer. [e] pH 1.8 buffer. [f] 130 °C, 10 min. [g] 120 °C, 15 min. [h] 130 °C, 5–45 min. [i] CF_3CO_2H. [j] 150 °C, 15 min. [k] 180 °C, 60 min. [l] 110 °C, 60 min.



[2-^{2}H]- (**8**, Y = X = H, R^1 = H, R^2 = ^{2}H) 3,4-dihydronaphthalene (Scheme 4). The synthetic sequence used for *cis*-dihydrodiols **3a** and **3b** involves the formation of the tetrahydroarene carbonate (**18**, X = Y = CH), bromocarbonate (**19**, X = Y = CH), dihydroarene carbonate (**20**, X = Y = CH) intermediates and provides a more convenient route to labelled *cis*-dihydrodiols **3a** and **3b** compared with the literature method (see Scheme 4).[34] This alternative approach to the synthesis of *cis*-dihydrodiols using cyclic carbonate intermediates has also been applied to the formation of *cis*-1,2-dihydroxy-1,2-dihydrochrysene and *cis*-3,4-dihydroxy-3,4-dihydrochrysene.[35]

The *cis*-dihydrodiols **3a**–**g** were generally found to be more stable to aromatisation (dehydration) than the corresponding

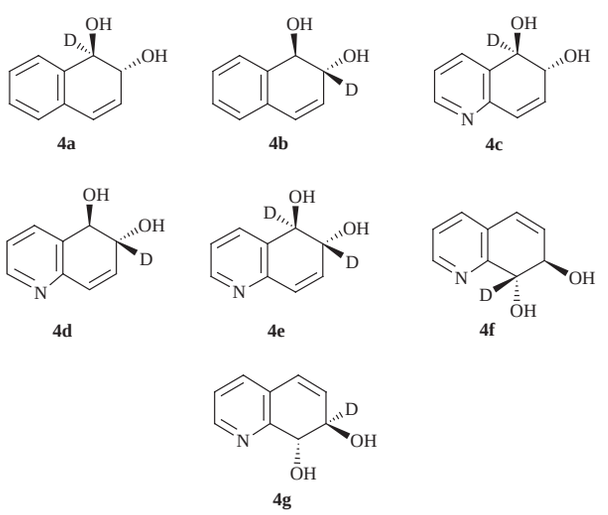


Scheme 4 *Reagents and conditions*: i, OsO_4, NMNO; ii, 1,1′-carbonyldiimidazole, C_6H_6; iii, NBS, CCl_4; iv, DBN, THF; v, Et_3N, aq. MeOH; vi, MCPBA, CH_2Cl_2; vii, aq. KOH, tBuOH; viii, Ac_2O, pyridine; ix, NaOMe, MeOH.

arene oxides **1a**–**g** (isomerisation) at elevated temperatures and under acidic conditions. NIH Shift studies could not be carried out on the very minor and relatively unstable *cis*-dihydrodiol metabolites **3f** and **3g** of quinoline. Thermal dehydration of *cis*-dihydrodiols **3a**–**e** was carried out under conditions similar to those used for isomerisation of arene oxides **1a**–**g** except that the samples were generally maintained at slightly higher temperatures for a longer period of time. An identical phenolic product **2a** (*ca*. 95% yield) and similar relatively large values for the NIH shift (*ca*. 53–81% deuterium) were observed for aromatisation of arene oxides **1a** and **1b** and *cis*-dihydrodiols **3a** and **3b**, a result which is consistent with preferential formation of an allylic carbocation intermediate (Scheme 3). The formation of phenol **2c** as a major product (86% yield) from arene

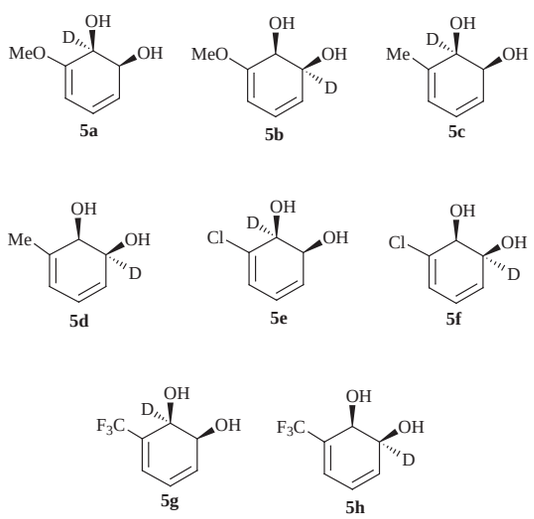
oxides **1c** and **1d** was again in accord with expectations, assuming an allylic carbocation intermediate. By contrast, phenol **2d** was isolated as the major product from *cis*-dihydrodiols **3c** and **3d** (83% yield), suggesting a preference for a benzylic carbocation intermediate (Scheme 3). It is noteworthy that the proportion of the minor phenol **2d** (14%) was higher than that observed for the corresponding phenols **2b** (5%) and **2f** (5%). Among the factors which may partially account for the formation of a higher proportion of phenol **2d** are: (i) the decreased stability of the allylic carbocation formed from *cis*-dihydrodiols **3c** and **3d** (relative to the benzylic carbocation) due to a contribution from a resonance structure having a positive charge on the nitrogen atom, (ii) the preferred formation of the benzylic carbocation from *cis*-dihydrodiols **3c** and **3d** under the higher temperature and aqueous conditions required for aromatisation compared with arene oxides **1c** and **1d** (Scheme 2). Despite the differences in product composition, the NIH Shift was again found during the formation of phenol **2d**, but with much lower values. When the doubly labelled *cis*-dihydrodiol **3e** was thermally aromatised (dehydrated), the major phenol product, **2d**, was found to have lost a significant portion of label (55% deuterium lost). This result was in accord with that obtained from aromatisation of arene oxide **1g** to yield phenol **2c**, where a marked proportion of deuterium label was also lost (30–40%). In both cases loss of deuterium label can be accounted for by an exchange process.

The mechanism for thermal dehydration (aromatisation) of *cis*-and *trans*-dihydrodiols to yield phenols was expected to be similar and thus a comparative study was undertaken. The deuterium labelled *trans*-dihydrodiols **4a**–**g** could, in principle, be

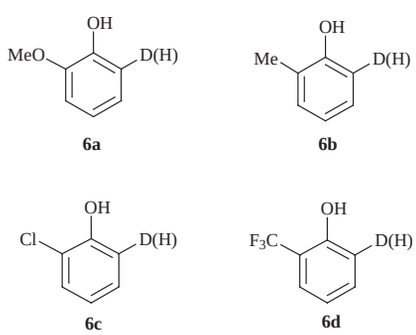


synthesised by hydrolysis of the corresponding labelled arene oxides **1a**–**g**. While this reaction occurs readily during animal liver metabolism of arenes *via* epoxide hydrolase catalysis[4] (Scheme 2) all attempts to hydrolyse the arene oxides of naphthalene **1a** and **1b** by chemical methods have to date failed. The chemical synthesis of the deuteriated *trans*-dihydrodiols, **4a** and **4b**, from precursors **8** and **12** (X = Y = CH) using the established method,[31] is outlined in Scheme 4. Treatment of the deuteriated arene oxides **1c**–**g** with potassium hydroxide in aqueous 2-methylpropan-2-ol according to the literature method[31] yielded the corresponding *trans*-dihydrodiols **4c**–**g** by exclusive attack at the allylic positions. The *trans*-dihydrodiols **4a**–**g** were found to be more stable than the corresponding *cis*-dihydrodiols **3a**–**g** and thus thermal aromatisation (dehydration) was carried out at a higher temperature (180 °C) for an extended period (60 min). The product composition of phenols obtained from the *trans*-dihydrodiols was virtually identical to that observed from aromatisation of the *cis*-dihydrodiols, *i.e.* **2a** (95% yield), **2e** (90% yield) and **2d** (83% yield). The magnitude of the NIH Shift observed was similar for 1-naphthol (**2a**, 70–72% deuterium and 53–73% deuterium) obtained respectively from *trans*-(**4a** and **4b**) and *cis*-dihydrodiols (**3a** and **3b**) of naphthalene. The lower NIH Shift values found after aromatisation of *trans*-dihydrodiols **4c** and **4d** (32–41% deuterium in **2d**), **4f** and **4g** (49–51% deuterium in **2e**), may be accounted for by the loss of label due to an exchange process. Thus, when the doubly labelled *trans*-dihydrodiol **4e** was aromatised, the resulting phenol **2d** was found to have lost 40% of the deuterium label due to exchange. The observed similarity of phenol ratios and NIH Shift values suggest that the *cis*-and *trans*-dihydrodiols both aromatise by similar mechanisms. The observation of an NIH Shift after acid-catalysed dehydration of 5,6-*trans*-dihydroxy-3-chloro[6-^{2}H]cyclohexa-1,3-diene to yield *p*-chlorophenol (23% deuterium),[36] has thus been extended to a range of *trans*-dihydrodiols **4a**–**g** (41–72% deuterium) derived from bicyclic arenes.

The most common type of bacterial dioxygenase-catalysed *cis*-dihydroxylation is found in the monocyclic series, where more than one hundred examples of *cis*-dihydrodiol derivatives of substituted benzenes have been reported,[19] and phenol formation is more likely to be encountered among this *cis*-dihydrodiol category. Having demonstrated that the NIH Shift can be associated with the aromatisation of arene oxide, *cis*-dihydrodiol and *trans*-dihydrodiol derivatives of polycyclic arenes, the final aspect of this study was focussed on the aromatisation of deuteriated *cis*-dihydrodiols (**5a**–**h**),

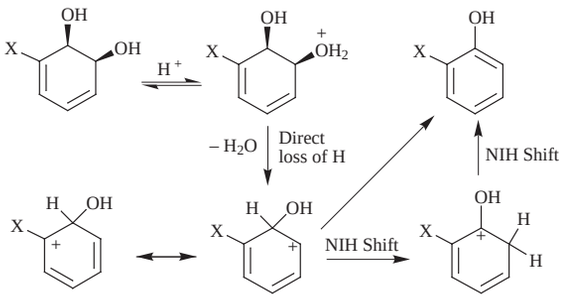


derived from methoxy-, methyl-, chloro- and α,α,α-trifluoromethyl-benzene respectively. The earlier kinetic studies on the aromatisation of the latter non-deuteriated *cis*-dihydrodiol metabolites (**5a**–**h**) showed a difference in rates of >10^6 fold.[18] The *cis*-dihydrodiols of methoxybenzene (**5a** and **5b**) proved to be the least stable; they readily aromatised under acidic ($HClO_4$) aqueous conditions to yield *o*-hydroxy(methoxy)-benzene (**6a**, 99% yield). Under similar conditions *o*-hydroxy-



toluene (**6b**, 98% yield), *o*-hydroxy(chloro)benzene (**6c**, 99% yield), and *o*-hydroxy-α,α,α-trifluoromethylbenzene (**6d**, 91% yield) were formed as major products with the *meta*-substituted

phenols as very minor components. The mechanism for the dehydration of seventeen different unlabelled *cis*-dihydrodiols under aqueous acidic conditions, based upon the rate constants obtained and the proportion of *o*- and *m*-phenols found,[18] was consistent with the NIH Shift occurring. (Scheme 5). Mono-



Scheme 5

deuterium-labelled *cis*-dihydrodiol derivatives of methoxybenzene (**5a** and **5b**), toluene (**5c** and **5d**), chlorobenzene (**5e** and **5f**) and α,α,α-trifluoromethylbenzene (**5g** and **5h**), were obtained by biotransformation of the corresponding substituted deuteriated benzene substrates in growing cultures of *P. putida* UV4. The products from acid-catalysed and thermal dehydration of the *cis*-dihydrodiols **5a**–**h** were identical. Thus thermal aromatisation yielded the *ortho*-substituted phenols as the major products (**6a**–**c**, >97% yield; **6d**, 91% yield). Since a deuterium atom was present at only one of the two *ortho*-positions in the methoxy-, methyl-, chloro- and α,α,α-trifluoromethyl-benzene substrates a suitable correction was made to allow comparisons with other NIH Shift values (Table 1). The NIH Shift observed provided confirmation of the mechanism suggested based on the kinetic studies.[18] Previous values for the NIH Shift during *o*-hydroxylation of methoxybenzene (35–67% deuterium),[37,38] methylbenzene (11–43% deuterium),[38,39] and chlorobenzene (39–52% deuterium),[40] were found to be variable. The relatively low values of the NIH Shift observed (19–32% deuterium) by the thermal isomerisation of *cis*-dihydrodiols (**5a**–**h**) may be accounted for by the conditions employed during the aromatisation which may result in a loss of label by an exchange process, leading to the generally lower values normally observed for the *ortho*-substituted phenols.

While the observation of the NIH Shift during aromatic hydroxylation of arenes is consistent with involvement of either arene oxide or dihydrodiol intermediates, it is difficult to provide unequivocal evidence for such intermediates. Such intermediate metabolites are rapidly converted to further metabolites (arene oxides→*trans*-diols and *cis*-diols→catechols) by the corresponding enzymes. In general terms the more stable dihydrodiol intermediates are much more likely to be detected and isolated than the corresponding arene oxides. The difference in stability is illustrated when the deuterium labelled naphthalene 1,2-oxides **1a**,**b** were added to liver microsomal incubations and to an acidic aqueous medium.[26] 1-Naphthol was produced due to isomerisation and was found to exhibit the NIH Shift with a similar magnitude to that found when produced by hydroxylation of naphthalene.[26] During the present study, addition of deuterium labelled *cis*-1,2-dihydroxydihydronaphthalenes **3a**,**b** to the bacterial culture medium showed little evidence of spontaneous dehydration to yield 1-naphthol due to the greater stability of the *cis*-dihydrodiol intermediates compared with the corresponding arene oxides **1a**,**b**. A further example of the problem of interpretation of NIH Shift values is provided by the formation of the phenol 3-hydroxyquinoline **2g**, a minor bacterial metabolite of quinoline previously isolated from cultures of *P. putida* UV4,[23] but also detected during the present study. Addition of [3-^{2}H]quinoline as a substrate under similar conditions yielded 3-hydroxyquinoline **2g** but with evidence of the NIH Shift (*ca*. 23% deuterium by GC-MS analysis of the TMS ether). While the presence of the NIH Shift is consistent with spontaneous dehydration of the *cis*-3,4-dihydroxy-3,4-dihydroquinoline intermediate (particularly since *cis*-dihydrodiols were isolated due to oxidation at the 5,6- and 7,8-positions), all attempts to find direct evidence of the elusive 3,4-*cis*-dihydrodiol metabolite of quinoline have however been unsuccessful to date.[24] Direct GC-MS evidence of the NIH Shift occurring during the formation of the *ortho*-phenols **6a** (29% deuterium) and **6b** (38% deuterium) as minor products from biotransformation of the corresponding arenes {[2-^{2}H]anisole and [2-^{2}H]toluene} was observed. This observation could result from spontaneous dehydration of the corresponding major metabolites **5a**,**b** and **5c**,**d**, which were among the least stable *cis*-dihydrodiols studied. Support for this view was obtained when samples of the deuterium-labelled *cis*-dihydrodiols **5a**,**b** and **5c**,**d** were heated in the bacterial culture medium for an extended period to yield the corresponding phenols **6a** and **6b** having identical values for the NIH Shift (29 and 38% deuterium). Despite the difficulty of providing unequivocal evidence of the intermediacy of unstable *cis*-dihydrodiols during metabolism of arenes to yield the corresponding phenols in procaryotic systems, the results presented here and elsewhere[41] appear to support this mechanism.

Conclusion

The thermal aromatisation of a series of deuterium-labelled arene oxides, *cis*-dihydrodiols and *trans*-dihydrodiols has in each case been found to produce the NIH Shift. The magnitude of the NIH Shift was diminished by a deuterium exchange process. The results found support the view that phenolic products, isolated from the enzyme-catalysed oxidation of arenes, showing the NIH Shift, can result from the initial formation of unstable *cis*-dihydrodiol intermediates followed by their spontaneous dehydration. The presence of the NIH Shift, occurring as a result of aromatisation of arene oxide or dihydrodiol metabolites, this cannot be used to distinguish between monooxygenase- and dioxygenase-catalysed routes for aromatic hydroxylation.

Experimental

^{1}H NMR spectra were recorded at 300 MHz (General Electric QE 300) or at 500 MHz (General Electric GE 500) in $CDCl_3$ solvent unless otherwise indicated. Chemical shifts (δ) are reported in ppm relative to $SiMe_4$ and coupling constants (J) are given in Hz. High resolution MS and GC-MS data were recorded at 70 eV on an AEI-MS 902 instrument updated by VG Autospec instruments using a heated inlet system.

Biotransformations of the deuteriated samples of quinoline, methoxy-, methyl-, chloro- and α,α,α- trifluoromethyl-benzenes were carried out using growing cultures of the bacterium *Pseudomonas putida* UV4 to form the corresponding deuteriated *cis*-dihydrodiols **3c**–**g** and **5a**–**h** (under conditions reported for non-deuteriated samples).[21,42] Non-deuteriated forms of the arene oxides **1a**–**g**, *cis*-dihydrodiols **3a**–**g**, **5e**–**h**, *trans*-dihydrodiols **4a**–**g** and phenols **2a**–**f**, **6a**–**d** have previously been fully characterised and reported in the literature. The deuteriated compounds were identified by NMR spectroscopy (reduction in H signal intensity when a ^{2}H atom was present) and GC-MS comparison (increase in the M + 1 peak) with authentic samples in all cases. The deuteriated samples of [3-^{2}H]-, [5-^{2}H]- and [6-^{2}H]-quinoline were synthesised from the corresponding commercially available aminoquinoline by the general method given below.

Preparation of monodeuteriated quinolines

General preparation of diazonium fluoroborate salt. The aminoquinoline (3.25 g, 22.6 mmol) was dissolved in fluoroboric acid (40 cm^3). An aqueous solution of sodium nitrite (3.4 g in 7

cm^3 H_2O) was added dropwise to the vigorously stirred solution at 0 °C. The almost pure precipitated diazonium salt was collected by filtration, washed on the filter with portions of diethyl ether and then dried (P_2O_5). This compound (5.5 g, 100%) was used immediately for the next step without further purification.

Reduction of the diazonium salt. A suspension of the salt (3.64 g, 15.0 mmol) in chloroform (120 cm^3) was stirred at ice bath temperature. Deuteriated hypophosphorous acid (Aldrich, 50 wt% soln. in D_2O; 10.0 g, 72.5 mmol) was added in one portion along with trace amounts of cuprous oxide. The reaction mixture was stirred for five minutes and then solid potassium carbonate was added to bring the mixture to pH 8; the organic layer was separated and the remaining aqueous layer was extracted with $CHCl_3$ (3 × 50 cm^3). The combined organic extracts were washed with H_2O (100 cm^3), dried ($MgSO_4$) and the solvent distilled off under reduced pressure to give the crude product. Purification of the product by distillation under reduced pressure gave the mono-deuteriated quinoline (2.2 g, 55%). The bp (50–55 °C/0.4 mmHg), was identical to the commercially available non-deuteriated sample.

[5-^{2}H]-Quinoline: 88% deuteriation by ^{1}H NMR analysis.

[6-^{2}H]-Quinoline: 89% deuteriation by ^{1}H NMR analysis.

[3-^{2}H]-Quinoline: 87% deuteriation by ^{1}H NMR analysis.

Synthesis of [2-^{2}H]- and [3-^{2}H]-methoxybenzene, [2-^{2}H]- and [3-^{2}H]-methylbenzene, [2-^{2}H]- and [3-^{2}H]-chlorobenzene, [2-^{2}H]- and [3-^{2}H]-α,α,α-trifluoromethylbenzene

The *o*-deuteriated forms of methoxybenzene, methylbenzene, chlorobenzene and α,α,α-trifluoromethylbenzene were each obtained (50–70% yield) by formation of the Grignard reagents from the corresponding *o*-substituted iodobenzenes followed by quenching with D_2O using the reported method.[39] The deuterium incorporations in the *ortho*- and *meta*-monodeuteriated samples of methoxybenzene (92% *o*-^{2}H; 95% *m*-^{2}H), methylbenzene (91% *o*-^{2}H; 96% *m*-^{2}H), chlorobenzene (92% *o*-^{2}H; 95% *m*-^{2}H), α,α,α-trifluoromethylbenzene (90% *o*-^{2}H; 95% *m*-^{2}H), and in the corresponding *cis*-dihydrodiol metabolites (determined by ^{1}H NMR and MS analyses), were found to be identical. When allowance was made for the presence of a deuterium atom, the ^{1}H NMR spectra of all deuteriated *cis*-dihydrodiols were identical to those reported in the literature.

Synthesis of deuteriated arene oxides 1a–g

The deuteriated arene oxides **1a**, **1c** and **1e** were obtained by the indicated synthetic sequence (Scheme 1: **15** ⟶ **11** ⟶ **12** ⟶ **13** ⟶ **14** ⟶ **1a**, **1c** and **1e**) using reported methods.[26,31] Similarly, deuteriated arene oxides **1b**, **1d** and **1f** were obtained by the parallel sequence shown (Scheme 1: **15** ⟶ **16** ⟶ **7** ⟶ **8** ⟶ **9** ⟶ **10** ⟶ **1b**, **1d** and **1f**). Reduction of the dideuteriated intermediate **16** (X = N, Y = CH) with $NaBD_4$ yielded a trideuteriated benzylic alcohol which was converted to 5,6-epoxy-5,6-dihydro[5,6-$^2H_2$]quinoline, **1g**, using the steps shown (Scheme 1). The deuteriated arene oxides **1a**–**f** were consistently found to contain >90% deuterium ± 3% at the expected positions: **1a** (97% ^{2}H), **1b** (98% ^{2}H), **1c** (90% ^{2}H), **1d** (92% ^{2}H), **1e** (95% ^{2}H), **1f** (93% ^{2}H) and **1g** (95% $^2H_2$), based upon ^{1}H NMR and MS analyses.

Preparation of monodeuteriated *trans*-dihydrodiols 4a,b

Deuteriated 1,2-epoxy-1,2,3,4-tetrahydronaphthalene 21 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). The deuteriated alkene (**8** or **12**, X = Y = CH; 0.50 g, 3.8 mmol) was taken up in dichloromethane (75 cm^3) and phosphate buffer (pH 8, 75 cm^3) was added. *m*-Chloroperoxybenzoic acid (MCPBA, 1.30 g, 7.5 mmol) was subsequently added in portions over 10 minutes to the stirred two-phase mixture at 0 °C. After stirring the mixture for 3.5 h, another portion of MCPBA (1.30 g, 7.5 mmol) was added and the mixture stirred overnight at ambient temperature. The organic phase was separated, washed with aqueous sodium sulfite (1 M, 1 × 30 cm^3), aqueous sodium hydrogen carbonate (1 M, 1 × 30 cm^3), water (1 × 30 cm^3) and then dried (Na_2SO_4). Evaporation of the solvent gave the epoxide (**21**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H) as a yellow oil (0.50 g, 90%). Allowing for the presence of a deuterium atom at C-1 or C-2 (97 ± 2% deuterium by ^{1}H NMR analysis), the samples were spectroscopically identical with literature values.[43]

Deuteriated *trans*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene 22 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). The epoxide (**21**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H; 2.40 g, 16.3 mmol) was taken up in 2-methylpropan-2-ol (10 cm^3), aqueous potassium hydroxide (2 M, 10 cm^3, 20 mmol) was added and the mixture refluxed overnight. The reaction mixture was cooled, evaporated to dryness, the residue dispersed in chloroform (35 cm^3) and washed with water (1 × 35 cm^3). The aqueous layer was backwashed with chloroform (1 × 20 cm^3), the combined organic extracts dried (Na_2SO_4) and the solvent evaporated under reduced pressure to yield the crude product as a dark brown solid. Purification by flash chromatography (6% MeOH in $CHCl_3$) gave the title *trans*-diol (**22**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H) as a white solid (1.91 g, 71%), R_f 0.13 (2% MeOH in $CHCl_3$). Allowing for the presence of deuterium atom at C-1 or C-2 (*ca.* 97 ± 2% deuterium by ^{1}H NMR analysis) the samples were spectroscopically identical with literature values.[44]

Deuteriated *trans*-1,2-diacetoxy-1,2,3,4-tetrahydronaphthalene 23 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). The *trans*-diol (**22**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H; 0.271 g, 1.64 mmol) was dissolved in pyridine (2 cm^3) and an excess of acetic anhydride (2 cm^3) was added to it. The reaction mixture was left at room temperature overnight. The pyridine was removed under reduced pressure and the residue, after the usual work-up, was purified by PLC [40% Et_2O in light petroleum (bp 40–60 °C)] to give the title compound (**23**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H) as a viscous yellow oil (358 mg, 88%), R_f 0.32 ($CHCl_3$). Allowing for the presence of deuterium at C-1 or C-2 the sample (*ca.* 97 ± 2% deuterium by ^{1}H NMR analysis) was spectrally identical to the literature values.[45]

Deuteriated 4-bromo-*trans*-1,2-diacetoxy-1,2,3,4-tetrahydronaphthalene 24 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). To a solution of the diacetate (**23**, X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H, 0.119 g, 0.48 mmol) in carbon tetrachloride (5 cm^3), *N*-bromosuccinimide (0.094 g, 0.53 mmol) and AIBN (0.002 g) were added. The reaction mixture was stirred and heated to reflux while being irradiated with a heat lamp. After 20 minutes the solution was cooled and the precipitated succinimide by-product removed by filtration through a pad of Celite and the solvent evaporated under reduced pressure to yield the crude product **24** as a colourless oil (0.157 g, 100%). ^{1}H-NMR spectral analysis showed the presence of two diastereoisomers (1 : 1). The crude material (*ca.* 97 ± 2% deuterium by ^{1}H NMR analysis) was used without purification for the next stage.

Deuteriated *trans*-1,2-diacetoxy-1,2-dihydronaphthalene 25 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). To a stirred solution of the crude bromodiacetates **24** (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H; 0.200 g, 0.61 mmol) in dry tetrahydrofuran (7 cm^3), at 0 °C under nitrogen, was added 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) (0.25 cm^3, 2.0 mmol). The reaction mixture was stirred for 4 h at room temperature. The tetrahydrofuran was removed under reduced pressure, the residue dispersed in water and extracted with diethyl ether (3 × 10 cm^3). The organic extracts were dried ($MgSO_4$) and the solvent removed under reduced pressure to yield the crude product as an oil. Purification by PLC [40% Et_2O in light petroleum (bp 40–60 °C)] gave the title compound as a clear oil which solidified to a white

solid (0.075 g, 50%), R_f 0.41. Allowing for the presence of a deuterium atom at C-1 or C-2 (*ca.* 97 ± 2% deuterium by ^{1}H NMR analysis), the sample was spectroscopically identical to literature values.[45]

Deuteriated *trans*-dihydrodiols 4a–g. The diacetate **25** (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H; 0.036 g, 0.15 mmol) was taken up in dry methanol (5 cm^3) and sodium methoxide (0.040 g, 0.74 mmol) added to the stirred solution. All of the starting material was consumed after 30 minutes [TLC; 40% Et_2O in light petroleum (bp 40–60 °C)]. The methanol was removed under reduced pressure, the residue dispersed in water and the aqueous mixture extracted with ethyl acetate (3 × 8 cm^3). The extracts were dried ($MgSO_4$) and the solvent removed under reduced pressure to yield the crude product. Purification by PLC (6% MeOH in $CHCl_3$) gave the dihydrodiol **4a** or **4b** as a white solid (0.020 g, 84%). Allowing for the presence of a deuterium atom at C-1 or C-2 the sample was spectroscopically identical with literature values.[45]

Preparation of monodeuteriated *trans*-dihydrodiols 4c–g

Treatment of the deuteriated quinoline arene oxides, **1c**–**g**, with KOH in D_2O–*t*-BuOD according to the literature method[31] yielded the corresponding deuteriated *trans*-dihydrodiols **4c** (90% ^{2}H), **4d** (92% ^{2}H), **4e** (95% ^{2}H), **4f** (95% ^{2}H) and **4g** (93% ^{2}H).

Synthesis of deuteriated *cis*-dihydrodiol 3a or 3b

Deuteriated *cis*-1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene 17 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). To a stirred solution of the deuteriated alkene (**8** or **12**, 2.5 g, 19 mmol) in acetone (18.0 cm^3) were added *N*-methylmorpholine *N*-oxide (2.5 g, 21.4 mol), water (10 cm^3), 2-methylpropan-2-ol (6 cm^3) and a solution of osmium tetroxide (0.012 g, 0.05 mmol) in carbon tetrachloride (2.5 cm^3). After stirring the reaction mixture for 48 h at ambient temperature a saturated solution of sodium metabisulfite (5 cm^3) was added and the stirring continued for another 4 h. The mixture was diluted with water (50 cm^3), extracted with ethyl acetate (3 × 50 cm^3), the organic extracts dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to give the crude diol as a solid. Purification by flash chromatography (5% MeOH in CH_2Cl_2) gave the title *cis*-diol **17** (2.5 g, 80%), mp 98–99 °C (lit.,[46] mp 101–102 °C); (97 ± 2% deuterium at C-1 or C-2 by ^{1}H NMR analysis).

Deuteriated 3a,4,5,9b-tetrahydronaphtho[1,2-*d*][1,3]dioxol-2-one 18 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). 1,1′-Carbonyldiimidazole (0.5 g, 3.08 mmol) was added, in portions over 6 h, to a refluxing suspension of the *cis*-diol **17** (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H; 0.1 g, 0.61 mmol) in dry benzene (15 cm^3) under nitrogen; the reaction mixture was then refluxed overnight. The cooled reaction mixture was washed with water (1 × 20 cm^3) and the aqueous layer backwashed with diethyl ether (1 × 20 cm^3). The combined organic extracts were dried ($MgSO_4$) and the solvents evaporated under reduced pressure to yield the crude product as an oil (0.116 g, 100%). Recrystallization gave compound **18** (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H) as an off-white solid. Mp 82–83 °C [from Et_2O–light petroleum (bp 40–60 °C)] (Found: C, 69.6; H, 5.2. $C_{11}H_{10}O_3$ requires C, 69.5; H, 5.3%); δ_H(300 MHz, $CDCl_3$, non-deuteriated sample) 1.94–2.02 (m, 1H, H-4$_A$), 2.26–2.34 (m, 1H, H-4$_B$), 2.65–2.73 (m, 1H, H-5$_A$), 2.91–2.98 (m, 1H, H-5$_B$), 5.19 (1H, m, H-3a), 5.69 (d, *J* 7.6, 1H, H-9b), 7.11–7.42 (m, 4H, Ar-H) (97 ± 2% deuterium at C-9b or C-3a by ^{1}H NMR analysis).

Deuteriated 5-bromo-3a,4,5,9b-tetrahydronaphtho[1,2-*d*]-[1,3]dioxol-2-one 19 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). To a solution of the cyclic carbonate **18** (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H 0.350 g, 1.83 mmol) in dry carbon tetrachloride (140 cm^3), AIBN (0.03 g) and *N*-bromosuccinimide (0.380 g, 2.1 mmol) were added. The mixture was heated under nitrogen to reflux using an oil bath while also being irradiated with a heat lamp. After 15 minutes, when all the NBS was seen to have reacted, heating was continued using the heat lamp only. A short time later the succinimide by-product precipitated and the reaction mixture was allowed to cool. After the usual work up the crude title compound was obtained as a grey-white solid (0.445 g, 90%). ^{1}H-NMR analysis showed the product to be a mixture of two diastereoisomers and the crude product was immediately used for the next step.

Deuteriated 3a,9b-dihydronaphtho[1,2-*d*][1,3]dioxol-2-one 20 (X = Y = CH, R^1/R^2 = ^{2}H/H or H/^{2}H). 1,5-Diazabicyclo[4.3.0]-non-5-ene (DBN) (0.31 cm^3, 2.5 mmol) was added drop-wise to a stirred solution of the crude bromocyclic carbonate **19** (0.35 g, 1.3 mmol) in dry tetrahydrofuran (20 cm^3) at 0 °C under nitrogen and the resulting mixture was stirred for 4 h at 0 °C. Ice-cold water (20 cm^3) was added to the reaction mixture and the tetrahydrofuran removed under reduced pressure. The aqueous mixture was then extracted with ethyl acetate (3 × 25 cm^3), the combined organic extracts dried (Na_2SO_4) and the solvent evaporated under reduced pressure to give the crude product **20** as a brown solid (0.15 g, 61%). The crude compound was used as such for the next stage without purification (97 ± 2% deuterium at C-9b or C-3a by ^{1}H NMR analysis).

Deuteriated *cis*-1,2-dihydroxy-1,2-dihydronaphthalene 3a or 3b. The crude cyclic carbonate **20** (0.15 g, 0.56 mmol) was taken up in aqueous methanol (MeOH, 8 cm^3; H_2O, 2 cm^3) and triethylamine (2 cm^3) was added to the stirred solution at room temperature. The reaction was monitored by TLC. After 4 h the solvents were removed under reduced pressure and the residue was purified by PLC (6% MeOH in $CHCl_3$) to gave the title diol **3a or 3b** as an off-white solid (0.090 g, 70%). R_f 0.18 (6% MeOH in $CHCl_3$), mp 109–111 °C (from $CHCl_3$–hexane) (lit.,[34] mp 101–102 °C). (97 ± 2% deuterium at C-1 or C-2 by ^{1}H NMR analysis).

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Paper 8/05077E