Side Chain Hydroxylation of Aromatic Compounds by Fungi. Part 4.[1] Influence of the *para* Substituent on Kinetic Isotope Effects During Benzylic Hydroxylation by *Mortierella isabellina*

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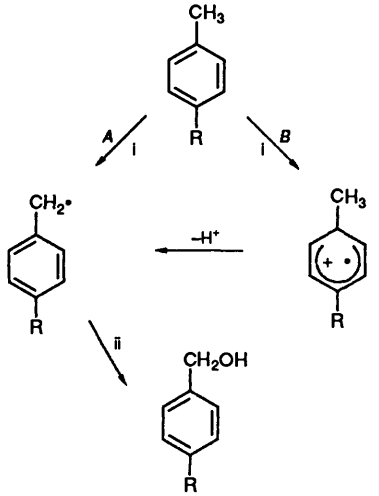
The benzylic hydroxylation of a series of *para*-substituted toluenes by the fungus *Mortierella isabellina* has been studied by using CD_3, CHD_2, and CH_2D methyl labelled substrates. Inter- and intramolecular primary and secondary deuterium kinetic isotope effect ratios have been determined: the intermolecular primary effects are maximal with strongly electron-withdrawing *para* substituents (R = CN and CF_3), while the intramolecular primary effects are minimal for R = H but increase in instances where R is electron donating or withdrawing. These results are interpreted in terms of a dependence of the hydroxylation mechanism on the nature of the *para* substituent.

In a series of papers on the side chain hydroxylation of aromatic compounds by fungi,[1-3] we have identified micro-organisms capable of oxidizing a methyl group on an aromatic ring to hydroxymethyl. It has been known for many years that this enzymic reaction occurs in mammals,[4] but only recently have bacteria,[5] fungi,[1-3,6-8] and isolated enzymes from bacterial[9] and mammalian[10,11] sources been used to study the process.

For those instances in which the nature of the enzyme performing the reaction has been investigated, the enzyme has been identified as a cytochrome P-450 dependent mono-oxygenase which functions with the stoichieometry of equation (1). The mechanism by which this enzyme performs benzylic

$$H^+ + NADPH + O_2 \longrightarrow NADP^+ + ROH + H_2O \quad (1)$$

hydroxylation has been the subject of intensive study: current proposals may be summarized as outlined in Scheme 1, in which



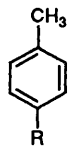
Scheme 1. Proposed routes for benzylic hydroxylation. i Fe-O•; ii Fe-OH.

an active site species of the enzyme (Fe–OH) can produce the product by reaction with a benzylic radical; the latter may be produced either directly by abstraction of a hydrogen atom from the benzylic carbon (route *A*), or indirectly by loss of a proton from a radical cation generated by a one-electron oxidation of the aromatic ring (route *B*).

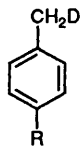
Radical cations have been produced from aromatic systems by cytochrome P-450 models,[12] and are thought to be intermediates in heteroatom dealkylations catalysed by cytochrome P-450 dependent mono-oxygenases.[13] We have proposed, on the basis of kinetic isotope effect studies, that benzylic hydroxylation of toluene by the fungus *Mortierella isabellina* proceeds by formation of an intermediate radical cation (route *B* of Scheme 1),[2] and in support of this proposal have shown that both *m*- and *p*-fluorotoluenes are hydroxylated at the same rate, a rate which is lower than that for hydroxylation of toluene itself.[1] This observation is compatible with hydroxylation by route *B* of Scheme 1 in which electron abstraction from the aromatic ring is wholly or partly rate limiting, but incompatible with hydroxylation *via* rate-limiting benzylic hydrogen radical abstraction, in which the influence of a *para* substituent on the rate would differ from that of an identical group in the *meta* position.

Results and Discussion

In order to examine the mechanism of benzylic hydroxylation by *M. isabellina* more closely, we have extended our study of kinetic isotope effects to a series of *para*-substituted toluenes, **(1)**–**(29)**, members of which carry from zero to three deuterium atoms in the methyl group. In view of the severe problems associated with the isolation of mono-oxygenases of fungal origin, we have employed the standard techniques of fungal biotransformation in this study, namely the use of whole cell methodology. Our earlier data[2,3] suggest that an enzyme of *M. isabellina*, readily accessible to the aqueous growth medium, is responsible for the benzylic hydroxylation of a range of alkyl-



(1) R = H
(2) R = CH_3
(3) R = OCH_3
(4) R = F
(5) R = CF_3
(6) R = Cl
(7) R = CN



(8) R = H
(9) R = CH_3
(10) R = OCH_3
(11) R = F
(12) R = CF_3
(13) R = Br
(14) R = CN

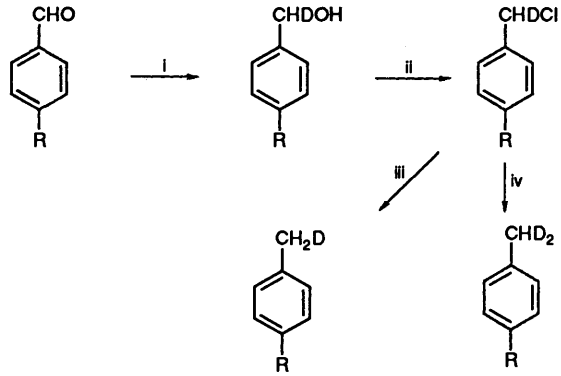
(15) R = H
(16) R = CH_3
(17) R = OCH_3
(18) R = F
(19) R = CF_3
(20) R = Br
(21) R = CN

(22) R = H
(23) R = CH_3
(24) R = CD_3
(25) R = OCH_3
(26) R = F
(27) R = CF_3
(28) R = Br
(29) R = CN

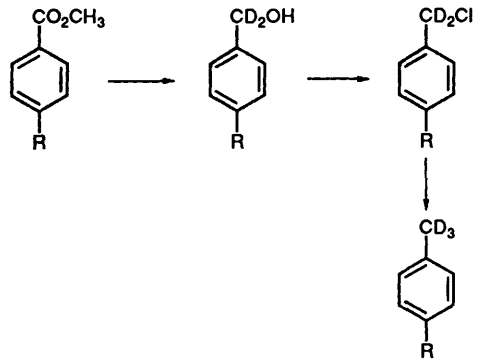
substituted aromatic compounds, but in the absence of purified enzyme preparations and genetic information we cannot state definitively that a single enzyme is responsible for the hydroxylation of all of the substrates of this study.

However, in the instance of competition experiments between molecules or sites of molecules which differ only in isotopic substitution, problems such as transport differences or metabolism by different enzymes do not arise unless isotopically sensitive metabolic branching is observed. There is no evidence for this phenomenon in the present study, and we have been able to obtain intramolecular primary and secondary k_H/k_D ratios, and combined primary and secondary intermolecular deuterium isotope effects for the range of substrates used.

The majority of substrates were prepared by the routes outlined in Schemes 2 and 3, with the introduction of deuterium from

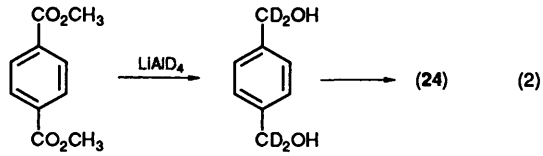


Scheme 2. i, $LiAlD_4$; ii, $SOCl_2$; iii $LiEt_3BH$; iv, $LiEt_3BD$.

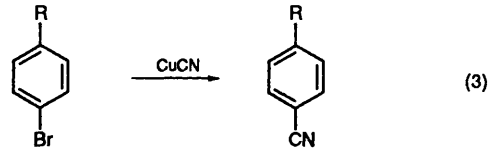


Scheme 3. i, $LiAlD_4$; ii, $SOCl_2$; iii, $LiEt_3BD$.

lithium aluminium deuteride or lithium triethylborodeuteride at the appropriate stage. Of the substrates not prepared in this way, [$^2H_6$]*p*-xylene was prepared from dimethyl terephthalate, equation (2), followed by the reactions of Scheme 3, and the *para*-cyano series was prepared from the corresponding *p*-bromo compounds, equation (3), by reaction with CuCN.



(2)



(3)

(13), (20), or (28) (14), (21), or (29)

R = CH_2D, CHD_2, CD_3

Incubations with *M. isabellina* of [$^2H_1$]- and [$^2H_2$]-labelled substrates alone, and equimolar amounts of [$^2H_0$]- and [$^2H_3$]-substrates in combination, were carried out as described previously.[2] In addition to these experiments, we were able to perform an intramolecular [$^2H_0$]/[$^2H_3$] competitive incubation in the *p*-xylene series [substrate (**23**)]. In all instances, benzylic hydroxylation occurred to give the corresponding alcohol as the only isolable biotransformation product, and the benzylic hydrogen and deuterium contents were determined by NMR analysis. The results of these experiments are summarized in the Table.

The kinetic isotope effect ratios associated with benzylic hydroxylation of these substrates can then be calculated as outlined in Scheme 4 for the *para*-substituted toluenes, and in Scheme 5 for the *p*-xylenes. Kinetic isotope effects are referred to as p (intramolecular primary k_H/k_D), s (secondary k_H/k_D), and p* (intermolecular primary k_H/k_D). The method of analysis presented in Scheme 4 assumes that primary and secondary effects are expressed independently, and is that developed by Hanzlik[10] and used previously by us[2] to determine the kinetic isotope effects for the hydroxylation of methyl-labelled toluenes by *M. isabellina*. The analysis of the effects from the [$^2H_1$] and [$^2H_2$] xylenes (**9**) and (**16**) requires a knowledge of both benzylic hydrogen and benzylic deuterium contents, as described in Scheme 5.

The results obtained from the analyses performed as outlined above are presented in the Table. The relatively high errors associated with the higher p/s values arise from a $\pm 2\%$ error in NMR integration, and are a function of the method of analysis which involves a reciprocal relationship of principal numbers and the small differences between them: high error limits are inherent in such determinations and have been reported by others.[14] It is apparent from the data presented in the Table that the magnitude of the primary isotope effects associated with benzylic hydroxylation by *M. isabellina* are dependent upon the nature of the *para* substituent, values of p/s being minimal when that group is hydrogen, and increasing for both electron donating (R = CH_3 or OCH_3) and electron withdrawing (R = F, CF_3, or CN) groups. The values obtained by intermolecular competition (p^*s^2) are low for electron-donating groups and for hydrogen, but substantial for highly electron-withdrawing groups (R = CF_3 or CN).

Intermolecular primary kinetic isotope effects are often close to unity for cytochrome P-450 dependent carbon hydroxylation reactions,[15] a phenomenon attributed to the fact that steps other than those involving loss of hydrogen or deuterium from the substrate can be rate limiting.[16,17] The values of p^*s^2 for *para*-CF_3 and -CN may, therefore, suggest a difference in the kinetic parameters of the reactions of these substrates when compared with those of the other substrates used in this study.

Intramolecular primary kinetic isotope effects, which arise

Table. Hydroxylation of substituted toluenes by *M. isabellina*.

Substrate compound	*p*-R-C_6H_4-R′ R	R′	Yield (%)	Benzylic H content	p/s[a]	p*s^2[b]	ps^2[c]
(14)	CN	CH_2D	15–20	1.04	12 ± 5		
(21)	CN	CHD_2		0.17	10 ± 3		
(7) }	CN	CH_3		1.74		6.7 ± 1.5	
(29)[f] }	CN	CD_3					
(12)	CF_3	CH_2D	10–12	1.05	9.5 ± 3		
(19)	CF_3	CHD_2		0.15	11.5 ± 4		
(5) }	CF_3	CH_3		1.69		5.7 ± 1.0	
(27)[f] }	CF_3	CD_3					
(11)	F	CH_2D	2–5	1.19	2.1 ± 0.3		
(18)	F	CHD_2		0.42	2.7 ± 0.4		
(4) }	F	CH_3		1.25		1.7 ± 0.1	
(26)[f] }	F	CD_3					
(8)[d]	H	CH_2D	4–10	1.40	0.75 ± 0.05		
(15)[d]	H	CHD_2		0.73	0.74 ± 0.05		
(1) }	H	CH_3		1.42[g]		1.9 ± 0.1	
(22)[d,f] }	H	CD_3					
(9)	CH_3	CH_2D	1–2	1.60	4.0 ± 0.5		
(16)	CH_3	CHD_2		1.30	4.8 ± 1.0		
(2) }	CH_3	CH_3		1.28		1.8 ± 0.1	
(24)[f] }	CH_3	CD_3					
							6 ± 1[e]
(23)	CH_3	CD_3					4.5 ± 1[f]
(10)	OCH_3	CH_2D	2–5	1.08	5.8 ± 1.0		
(17)	OCH_3	CHD_2		0.21	7.5 ± 2.5		
(3) }	OCH_3	CH_3		1.31		1.8 ± 0.1	
(25)[f] }	OCH_3	CD_3					

[a] Intramolecular primary k_H/k_D, p. [b] Intermolecular primary k_H/k_D, p*. [c] Secondary k_H/k_D, s. [d] From reference 2. [e] From ^2H NMR, δ 2.3 and 4.6 (3.0:0.33). [f] From ^1H NMR, δ 2.2 and 4.4 (3.0:9.0). [g] Substrate ratio 44% CD_3, 56% CH_3, see reference 2.

from competition within a single catalytic cycle of the enzyme, are generally regarded as a close estimate of the intrinsic isotope effect.[18] The variation of p/s with the *para* substituent noted in the Table is difficult to accommodate within a single mechanistic framework, especially as the values of p = 1.02 and s = 1.37 for toluene[2] are best interpreted in terms of a product-like transition state for the reaction in which the C–H or C–D bond breaking is nearly complete, resulting in a low primary effect, with concomitant rehydridization from sp^3 to sp^2 being responsible for the expression of a large secondary effect.[2]

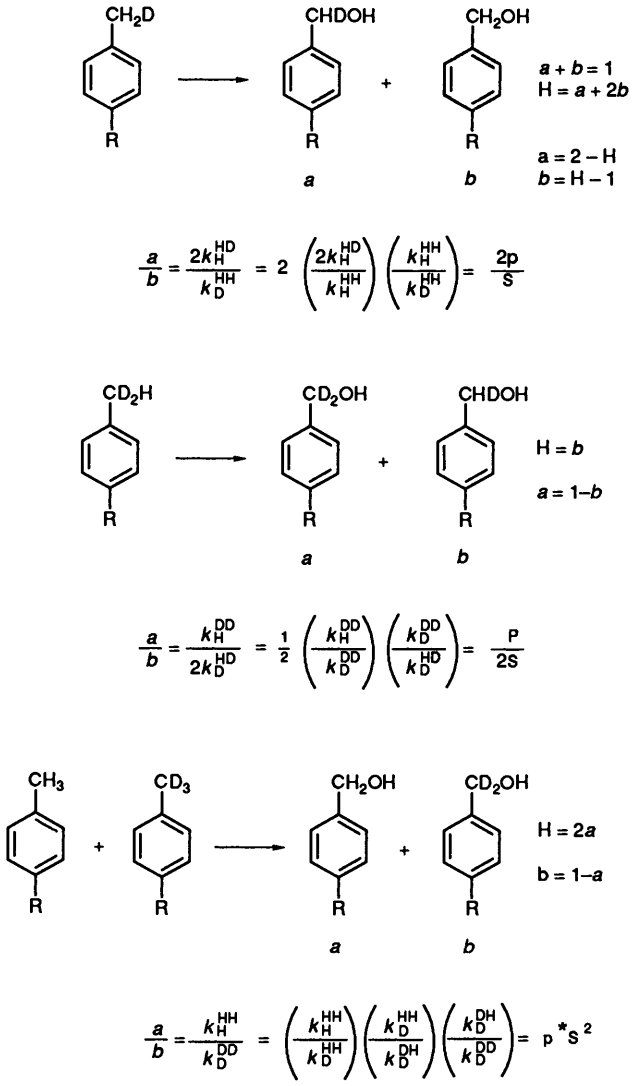
The values of the Table are, however, consistent with a situation in which there is a change in mechanism of hydroxylation as the *para* substituent becomes highly electron withdrawing. This can be accommodated by the proposals of Scheme 1, where those substrates with electron-donating substituents or hydrogen in the *para* position react *via* route *B* (electron abstraction), whereas those with highly electron withdrawing *para* substituents react by route *A* (benzylic hydrogen abstraction). A change in the rate-limiting step for the reaction is, thus, reflected in the values of p*s^2; the high values of p/s observed for *para*-CF_3 and -CN are consistent with those expected for a rate-limiting benzylic radical abstraction;[19] and the values of p/s observed for electron-donating *para* substituents may be explained by a shift towards a more symmetrical transition state, a phenomenon also observed for substrates with *ortho* alkyl substituents.[2]

It is possible that, within this framework, the *para*-fluoro substrates represent an intermediate situation in which, in spite of the electron-withdrawing nature of the *para* substituent, the aromatic ring is still subject to one-electron oxidation. This interpretation is supported by the value of p*s^2 observed for the *para* fluoro substrates, and by the similarity in the rates of oxidation of *p*- and *m*-fluoro toluenes referred to above.[1]

Since the Hammett σ value for *para*-F is close to zero (0.06), it was considered desirable to examine substrates with σ values intermediate between this and the highly electron withdrawing CF_3. However, of such substrates, *p*-methyl acetophenone was reduced at the carbonyl group by *M. isabellina*;[3] *p*-chlorotoluene was found to be oxidized (in low yield) to *p*-chlorobenzoic acid by *M. isabellina* in addition to the normal benzylic hydroxylation reaction, thus, precluding its use as a substrate to examine the latter process; and *p*-bromotoluene was not a substrate for the hydroxylase enzyme.

Our interpretation of the benzylic hydroxylating reaction can also be used to explain the value of ps^2 obtained from substrate (**23**), α-α-α-[$^2H_3$]*p*-xylene. The hydroxylation of this substrate is represented in equation (4). The intramolecular primary effect, p, now represents the result of competition between different and distant sites of the substrate, rather than competition within a single methyl group as is the case for *p*-xylenes (**9**) and (**16**), but these two scenarios can become mechanistically identical through reaction by route *B* of Scheme 1 (R = CD_3), in which loss of a benzylic proton or deuteron to the medium is a competitive intramolecular event if not initiated by a group on the enzyme. This interpretation is supported by the magnitude of p, which indicates that hydrogen loss is wholly or partly rate limiting in the overall reaction.

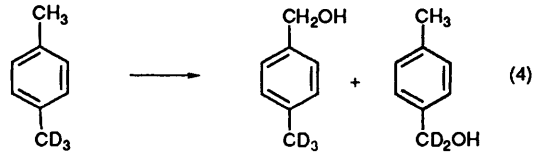
From the value of p/s = 4.4 [average of values from substrates (**9**) and (**16**)], and ps^2 = 5.25 [average of values from



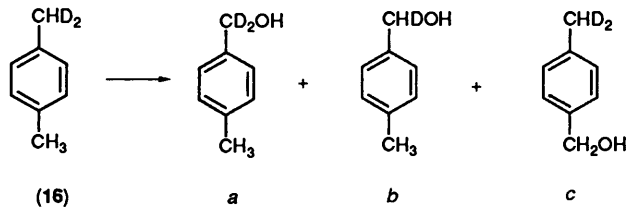
Scheme 4. Derivation of p/s and p*s^2 ratios for *para* substituted toluenes.

substrate (**23**)], it may, therefore, be concluded that p = 4.7 and s = 1.06. Any explanation of the magnitude of p based upon intramolecular competitive hydrogen abstraction is now difficult to envisage, as this would require that a single active site group of the enzyme have simultaneous access to both methyl groups in a bound substrate, a sterically unattractive proposal: the p*s^2 value of 1.8 obtained from the intermolecular competitive use of *p*-xylene/[$^2H_6$]-*p*-xylene rules out of consideration any explanation based upon differential binding of substrate or release of product.

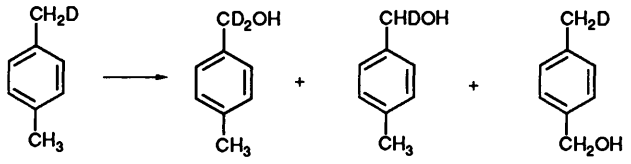
The secondary effect derived from substrate (**23**) is obtained from reactions in which hydrogen is lost only from carbons which carry hydrogen, and deuterium is likewise lost only from



(**23**)



from ^{1}H NMR	$2c + b = 1.30$	
from ^{2}H NMR	$2c/2a + b = 1.7$	
given	$a + b + c = 1$	a/b = 2p/s = 2.4,
then:	$a = 0.29, b = 0.12, c = 0.59$	p/s = 4.8



from ^{1}H NMR	$2a + b + 2c = 1.60$	
from ^{2}H NMR	$c/b = 1.35$	
given	$a + b + c = 1$	b/a = 2p/s = 8.0,
then:	$a = 0.05, b = 0.4, c = 0.55$	p/s = 4.0

Scheme 5. Derivation of p/s ratios for *p*-xylenes (**16**) and (**9**).

deuterium-substituted carbons. This effect is, thus, directly comparable with those obtained from R–CD_3 *versus* R–CH_3 intermolecular competitions, and, with the assumption that primary and secondary effects are expressed independently,[2] is the same secondary effect as that obtained from R–CH_2D and R–CHD_2 intramolecular competitions.

Conclusions

Our data, therefore, support an interpretation of the benzylic hydroxylating process as one in which an intermediate benzylic radical can be formed in two ways, either directly by hydrogen abstraction, or indirectly *via* a one-electron oxidation of the aromatic ring followed by proton loss. The former pathway is apparently followed with substrates having low electron density in the aromatic ring (electron-withdrawing substituents), while the latter may be favoured for those substrates with electron-rich aromatic rings.

At the present time we do not know if these two possibilities exist for a single enzyme or, as discussed above, whether a series of closely related isozymes is responsible for the observed differences in kinetic isotope effects. Nevertheless, the variation in isotope effects with *para* substituents clearly indicates that mechanistic conclusions based upon the study of a single substrate are not necessarily valid for other substrates, however closely related.

Experimental

Materials and Methods.—The techniques used for spectral analysis and biotransformation were those previously described.[2,3]

Synthesis of Substrates.—[$^2H_1$]- *and* [$^2H_2$]-*Labelled materials.* The preparation of the labelled toluenes (**8**) and (**15**) are described previously.[2] Compounds (**9**)–(**12**) and (**16**)–(**19**) were prepared from the corresponding aldehyde as outlined in Scheme 2. In all instances, the identity of the product was

confirmed by comparison of physical and spectral data with those of authentic unlabelled material. Deuterium content was estimated by mass spectrometry and ^{1}H NMR as >99% [$^2H_1$] or >99% [$^2H_2$] material as appropriate. A typical procedure is detailed below.

(*i*) α-[$^2H_1$]p-*Fluorobenzyl alcohol.* A solution of *p*-fluorobenzaldehyde (10 g) in dry ether (50 cm^3) was added slowly to a solution of lithium aluminium deuteride (1 g) in dry ether (50 cm^3) and the resulting mixture heated under reflux for 8 h. The mixture was then cooled, excess reagent destroyed by the addition of water (1 cm^3, dropwise), and then the mixture was treated with sodium hydroxide (1 cm^3, 15%), followed by water (3 cm^3). The resulting solid was removed by filtration, washed with ether, and the combined filtrates evaporated to give α-[$^2H_1$]*p*-fluorobenzyl alcohol, (10 g), which was used directly in the next step without further purification.

(*ii*) α-[$^2H_1$]p-*Fluorobenzyl chloride.* Thionyl chloride (12 g), followed by pyridine (2 drops) was added to α-[$^2H_1$]*p*-fluorobenzyl alcohol, (10 g), and the resulting mixture heated under reflux for 2 h. Water (200 cm^3) was then added, followed by ether (150 cm^3). The ethereal layer was removed, and the aqueous layer extracted further with ether (2 × 150 cm^3). The combined ethereal extracts were washed with 5% hydrochloric acid, dried and evaporated to give *p*-fluorobenzyl chloride (9.8 g) of sufficient purity for use in the next step.

(*iii*) α-[$^2H_2$]p-*Fluorotoluene.* This was prepared from the above chloride by reaction with lithium triethylborodeuteride (100 cm^3, 1 mol dm^{-3}), as described.[2] The product (3.9 g) was purified by distillation before use as a substrate.

[$^2H_3$]-*Labelled materials.* Compounds (**23**)–(**28**) were prepared from the corresponding esters as outlined in Scheme 3. A typical procedure for ester reduction, and the procedure for the preparation of (**24**) [equation (2)], are detailed below.

(*i*) α,α-[$^2H_2$]p-*Trifluoromethylbenzyl alcohol.* A solution of methyl *p*-trifluoromethylbenzoate (13 g) in dry THF (100 cm^3) was added slowly to a solution of lithium aluminium deuteride (2 g) in dry THF (100 cm^3), and the resulting mixture heated under reflux for 48 h. The mixture was then cooled and worked up by the sequential addition of water (2 cm^3), 15% sodium hydroxide (2 cm^3), and water (6 cm^3). The resulting salts were removed by filtration, washed with THF, and the combined filtrate dried and evaporated to give a product (9.09 g) of sufficient purity to use directly in the next step of the procedure.

(*ii*) α,α,α,α-[$^2H_4$]p-*Hydroxymethylbenzyl alcohol.* Dimethyl terephthalate (12 g) was placed in a Soxhlet thimble in a extractor fitted to a 1 dm^3 flask containing a solution of lithium aluminium deuteride (4 g) in THF (500 cm^3), and the mixture was heated under reflux for 48 h. The mixture was then cooled and worked up as described above following the sequential addition of water (4 cm^3), 15% sodium hydroxide (4 cm^3), and water (12 cm^3) to give the title compound (8 g) which was used directly in the next step.

(*iii*) α,α,α,α-[$^2H_4$]p-*Chloromethylbenzyl chloride.* The above alcohol was mixed with thionyl chloride (12 cm^3), and the solution heated under reflux for 2 h, cooled, diluted with ether (100 cm^3), and poured onto water (200 cm^3). The aqueous layer was extracted with further ether (3 × 100 cm^3), and the combined extracts dried and evaporated to yield the title compound (10.3 g), used directly in the preparation of α,α,α,α,α,α-[$^2H_6$]*p*-xylene, by reaction with 1 mol dm^{-3} lithium triethylborodeuteride (200 cm^3) as described.[2]

(*iv*) p-*Tolunitriles* (**14**), (**21**), *and* (**29**). These were prepared from the corresponding *p*-bromotoluenes, (**13**), (**20**), and (**28**) by reaction with CuCN as shown in equation (3) and described below for (**14**). A mixture of α-[$^2H_1$]*p*-bromotoluene (8.13 g) and CuCN (7.27 g) in anhydrous dimethylformamide (10 cm^3) was heated at reflux temperature for 7.5 h, and then poured into a hot (70 °C) solution made by dissolving anhydrous $FeCl_3$ (19.36 g) in water (28 cm^3) and hydrochloric acid (5 cm^3). The resulting mixture was stirred at 70 °C for 20 min, then cooled and extracted with toluene (4 × 50 cm^3). The combined organic extract was washed with 20% hydrochloric acid, water, and sodium hydrogencarbonate solution, and then dried and evaporated. The product was purified by Kugelrohr distillation to give 3.7 g of material, identified by comparison of spectral and analytical data with an authentic sample.

Incubations with M. isabellina.—All incubations were performed as previously described[2,3] using 72 h old cultures for a further period of 72 h. Products were isolated by continuous extraction (methylene dichloride), and purified by flash column chromatography (silica gel, benzene–ether 5% stepwise gradient). Isolated yields and hydrogen content of products are listed in the Table. In all cases, the identity of products was confirmed by spectral analysis. Hydrogen or deuterium contents were determined, following pooling of all chromatographic fractions containing product, from 200 MHz ^{1}H or 30.72 MHz ^{2}H NMR spectra. Integration of the signal due to the benzylic hydrogens, using the aromatic hydrogen signal as an internal standard of four, was used to estimate the benzylic hydrogen content. For the *p*-xylenes, integration of the aromatic methyl group signal gave the hydrogen or deuterium content at that location.

Acknowledgements

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