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Side Chain Hydroxylation of Aromatic Compounds by Fungi

3. Direct Observation of Fungal Biotransformation by NMR

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The enzymatic hydroxylations by intact fungal cells at the benzylic positions of toluene-d_8, *meta*-fluorotoluene-α,α,α-d_3, *para*-fluorotoluene-α,α,α-d_3, ethylbenzene-α,α-d_2, *meta*-fluoroethylbenzene-α,α-d_2, and *para*-fluoroethylbenzene-α,α-d_2 can be observed directly in an NMR tube by using ^{2}H NMR as a probe. This technique provides a new method for the rapid screening of microorganisms for biotransformative capability. In all cases, the rate of product accumulation was uniform over a period of at least 4 days. The fluoro-substituted products were formed at a rate independent of the position of substitution, but slower than the corresponding unsubstituted substrates.

As part of our continuing program of research involving fungal biotransformation of organic compounds, we have identified fungi which are capable of introducing the hydroxy group into the benzylic position of simple substituted aromatic hydrocarbons such as ethylbenzene and toluene (*1*, *2*). We now report that this process can be monitored directly by conducting the incubation in an NMR tube, using ^{2}H NMR as a probe to study the hydroxylation of deuterium-labeled substrates. Deuterium NMR offers the advantage that when deuterium-depleted water is used as a solvent, the level of background signals from fungal constituents or solvent is so low as to cause no interference with signals from substrate or product.

Although the use of ^{2}H NMR in biosynthetic studies using deuterium-labeled substrates is now routine (*3*), and ^{2}H NMR analysis of the products of metabolism of deuterium-labeled substrates by isolated enzymes in solution has been reported (*4*), we believe that our technique is the first reported instance of the direct observation by ^{2}H NMR of the metabolism of an exogenous substrate by an intact microorganism in a heterogeneous living system.

Thus when toluene-d_8 **(1)** was introduced into a 10-mm NMR tube containing an aqueous suspension of resting mycelial growth of *Mortierella isabellina*, a ^{2}H NMR spectrum showed only signals at δ 2.1 (3D) and 7.2 ppm (5D). After 24 h, a new signal at δ 4.7 had appeared, corresponding to the benzylic deuteria of benzyl alcohol-d_7 **(2)**. Using the signals of the deuteria on the aromatic ring as an internal standard, the course of the reaction could be followed by integration of product signals at various time intervals. The results, expressed as percentage conversions of substrate to product, are presented on the first line of Table 1. The values

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TABLE 1

Percentage Hydroxylation of Deuterated Substrates by *M. isabellina* Determined by ^2H NMR

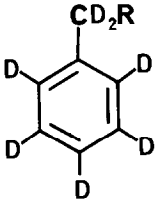
Substrate	Product	Percentage conversion[a]			
		1 day	2 days	3 days	4 days
1	**2**	8.5	17	24	34
3	**5**	3	7.5	10.5	13
4	**6**	4	6	8	10
11	**14**	11.5	21.5	28	35.3
12	**15**	5	14	20	20
13	**16**	7.5	12	20	20

[a] ±2%.

obtained were independent of the concentration of fungus over a range varying from one-half to twice that normally used and were reproducible from batch to batch of fungal growth.

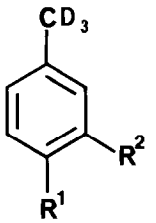
Using ethylbenzene-α,α-d_2 **(11)** as substrate, we were also able to monitor conversion to 1-phenylethanol-1-d_1 **(14)** in a similar manner, using ^2H NMR signals at δ 2.6 ($PhCD_2CH_3$) and 5.1 ppm ($PhCDOHCH_3$) to estimate the ratio of substrate to product at given time intervals. These data are also presented in Table 1. To further demonstrate the utility of the technique, it was used to investigate the biotransformation by *M. isabellina* of the fluoro-substituted aromatic compounds **3, 4, 12,** and **13,** whose hydroxylation will form the basis of a future report on the mechanistic aspects of this reaction. The toluenes with deuterium label at the benzylic position (**3** and **4**) were synthesized by lithium aluminium deuteride reduction of the corresponding methyl benzoates, followed by conversion to the substituted benzyl chlorides and subsequent treatment with lithium triethylborodeuteride. The corresponding ethylbenzenes **(11–13)** were similarly prepared starting from the appropriate acetophenones. In all cases, deuteration was essentially complete (>99% by NMR and mass spectral analysis) and homogeneous.

The rates of hydroxylation of the fluorotoluenes **3** and **4** were identical within experimental error (see (Table 1), both being hydroxylated more slowly than



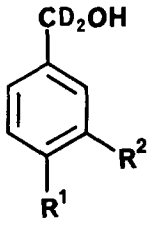
1 R = D

2 R = OH



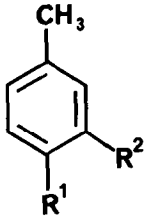
3 R^1 = F, R^2 = H

4 R^1 = H, R^2 = F



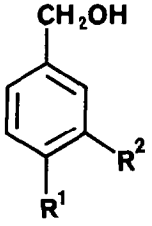
5 R^1 = F, R^2 = H

6 R^1 = H, R^2 = F



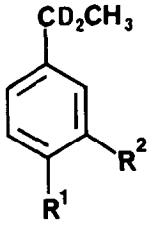
7 R^1 = F, R^2 = H

8 R^1 = H, R^2 = F



9 R^1 = F, R^2 = H

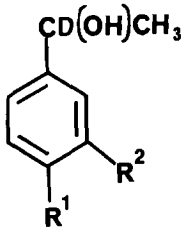
10 R^1 = H, R^2 = F



11 R^1 = R^2 = H

12 R^1 = F, R^2 = H

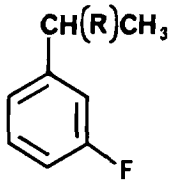
13 R^1 = H, R^2 = F



14 R^1 = R^2 = H

15 R^1 = F, R^2 = H

16 R^1 = H, R^2 = F



17 R = H

18 R = OH

toluene itself. A similar result was obtained for the fluoroethylbenzenes **12** and **13**, compared with ethylbenzene **11.** Of the above fluoro-substituted substrates, only *p*-fluoroethylbenzene **(12)** had previously been transformed on a preparative scale by *M. isabellina,* giving the alcohol **15** (*1*). To confirm validity of the interpretation of the ^{2}H NMR experiments, unlabeled *m*-fluoroethylbenzene **(17)** and the *para*- and *meta*-fluorotoluenes (**7** and **8,** respectively) were subsequently converted on a

preparative scale to the benzylic alcohols (**18, 9,** and **10,** respectively) as the only products of biotransformation by *M. isabellina.*

Our results therefore demonstrate that the ^2H NMR monitoring method can be used to establish the existence of biotransformative capability for a given substrate/microorganism combination. We envisage that this technique will prove valuable in the screening of microorganisms for their ability to metabolize exogenous organic compounds, as it requires only small amounts of deuterium-labeled substrate and completely eliminates the tedious extraction and analysis normally associated with screening procedures.

EXPERIMENTAL

Apparatus, Materials, and Methods

The general techniques used were those previously described (*1, 2*). Fungus was grown as usual (*1*), harvested, pressed dry, and then resuspended in deuterium-depleted water (Merck) at the usual concentration. Three milliliters of this fungal suspension was then placed in a 10-mm NMR tube, and substrate was (2 mg in 40 μl ethanol) added directly to the tube, which was then capped and maintained at 27°C on a rotary shaker. The ^2H NMR spectra were obtained with a Bruker AC 200 spectrometer operating at 30.722 MHz, using a sweep width of 10 ppm, 1K acquisition and 2K data transform giving a resolution of 0.293 H/pt. Spectra were run unlocked and referenced externally to $CDCl_3$. The number of scans were typically 2000 and line broadening was set at 2.0 Hz. Integration of substrate and product signals were performed between preset chemical shift limits, and percentage conversions were determined directly from the integral values. In duplicate runs, reproducibility was ±2% conversion. With the exception of those listed below, the substrates used were commercial samples.

Synthesis of Substrates

p-Fluorotoluene-α,α,α-d_3 (***3***). *p*-Fluorobenzoic acid (10 g) was converted to the methyl ester in the standard manner, and the latter (10 g) treated with lithium aluminium deuteride (2 g) in dry ether (200 ml). The reaction mixture was refluxed for 24 h and gave, following the usual workup, *p*-fluorobenzyl alcohol-α,α-d_2 **(5)** (8.5 g). The latter compound was dissolved in dry ether and treated with thionyl chloride (10 ml), and the chloride, obtained by washing ($NaHCO_3$, H_2O) and evaporation of the reaction mixture, was treated directly with lithium triethylborodeuteride by the procedure described (*2*). The resulting *p*-fluorotoluene-α,α,α-d_3 **(3)** exhibited spectral properties (^1H NMR, ^2H NMR, MS, and ir) consistent with its structure and contained >99% d_3 species by MS analysis.

m-Fluorotoluene-α,α,α-d_3 (***4***). This was prepared from *m*-fluorobenzoic acid by a route analogous to that described above for the preparation of the *para*-isomer **3** and was obtained in an overall yield of 36%. Spectral properties (^1H NMR, ^2H NMR, MS, ir) confirmed the structure, and deuterium content by MS analysis was >99% d_3.

m-Fluoroethylbenzene (**17**). This was prepared from *m*-fluoroacetophenone, via reduction ($LiAlH_4$) to *m*-fluoro-1-phenylethanol, (**18**), conversion to the chloride ($SOCl_2$), and then treatment of the latter with lithium triethylborohydride ("Super Hydride"), in the manner previously described for the conversion of acetophenone and substituted derivatives to ethylbenzenes (*2*), in an overall yield of 40%. The title compound had physical and spectral properties which accorded with those reported (*5*).

m-Fluoroethylbenzene-α,α-d_2 (**13**). This was prepared from *m*-fluoroacetophenone by the route described above, using $LiAlD_4$ and Super Deuteride as reagents, in an overall yield of 33%. Mass spectral and NMR analyses indicated the presence of >99% d_2 species.

p-Fluoroethylbenzene-α,α-d_2 (**12**). This was prepared as described for compound **13**, but starting with *p*-fluoroacetophenone, in an overall yield of 45%. Mass spectral and NMR analysis showed >99% d_2 labeling.

Preparative Scale Biotransformations

p-Fluorotoluene (**7**). Incubation of **7** (1 g) with *M. isabellina* for 96 h followed by extraction of the culture medium with methylene chloride gave an extract (0.2 g) which afforded, following chromatography on silica gel, *p*-fluorobenzyl alcohol (**9**, 50 mg) identical in all respects to an authentic (commercial) sample.

m-Fluorotoluene (**8**). Incubation of **8** (1 g) with *M. isabellina* by the products outlined above gave the alcohol **10** (65 mg), identified by comparison with an authentic sample.

m-Fluoroethylbenzene (**17**). Incubation of **17** (1 g) with *M. isabellina* for 72 h, followed by extraction of the culture medium with methylene chloride, gave an extract (0.2 g) which, upon chromatography, afforded 3′-fluoro-1-phenylethanol (**18**, 0.11 g), whose spectral and analytical properties accorded with those reported (*6*). Compound **18** had $[\alpha]_D^{20}$ + 2.2° and an enantiomeric excess of 18% determined from the ^1H NMR of the benzylic proton in the presence of Tris(3-heptafluoropropylhydroxymethylene-(+)-camphorato)-europium(III). Based on similar compounds (*1*) **18** has been assigned the *R* configuration.

^2H NMR data acquisition. For toluene-d_8 (**1**), in which the signal from the ring-substituted deuterium nuclei could be used as an internal standard of five deuteria, the relative product and substrate concentrations were determined directly from the integral values at δ 4.7 and 2.1 ppm, respectively. For the remaining substrates in this study, the extent of diminution of the substrate $-CD_2-$ or $-CD_3$ peak and the growth of the product $-CD(OH)-$ or $-CD_2OH$ peak gave the relative proportions of substrate and product at given times, these values being converted to a percentage conversion for expression in Table 1.

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