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Isolation of a stable benzene oxide from a fungal biotransformation and evidence for an 'NIH shift' of the carbomethoxy group during hydroxylation of methyl benzoates

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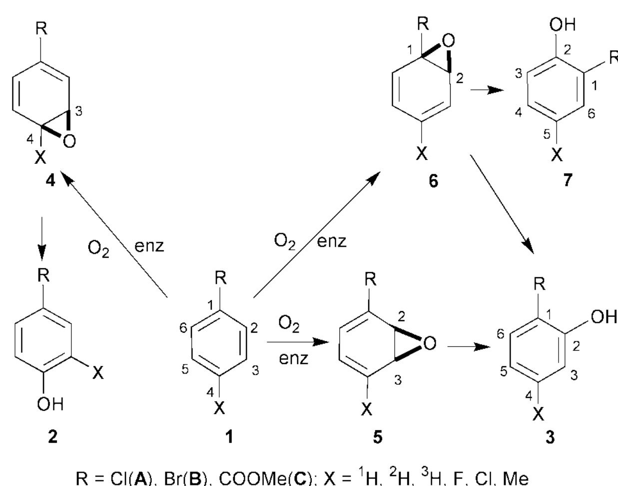
Substituted methyl benzoates are biotransformed in the fungus *Phellinus ribis* by enzyme-catalysed epoxidation to yield an isolable benzene oxide and transient benzene oxides, whose presence was inferred from isomerisation to the corresponding methyl salicylates with concomitant migration of the carbomethoxy group.

Aromatic hydroxylation in eucaryotes is widely assumed to involve the monooxygenase-catalysed epoxidation of the benzene ring.[1,2] Although benzene oxides have often been proposed as intermediates in the literature,[1–4] to date none have been isolated as a metabolite. Their role as transient intermediates during aromatic hydroxylation has been inferred on the basis of hydrogen atom migration from the hydroxylation site and its retention at an adjacent carbon atom ('NIH shift').[1,2]

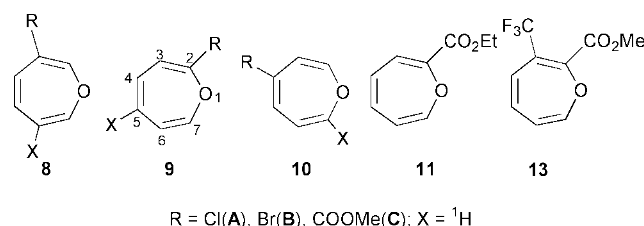
The 'NIH shift' was first observed during the *para*-hydroxylation of phenylalanine to yield tyrosine[5,6] **2** (Scheme 1, R = $CH_2CH(NH_2)CO_2H$, X = ^3H) and more than 100 examples have since been reported with ^1H, ^2H or ^3H atoms as the migrating species X. The detection of arene oxides of naphthalene[7] and quinoline[8] during liver microsomal hydroxylation of these arenes provided direct evidence of bicyclic arene epoxidation. By contrast, no unequivocal evidence of enzyme-catalysed benzene oxide formation from monocyclic arene substrates has been available and recently the role of transient arene oxide intermediates in the 'NIH shift' mechanism in biological systems has been questioned.[9–11] In earlier studies[12] we demonstrated that hydroxylation of ^2H-labelled halobenzenes **1A** (X = ^2H) and **1B** (X = ^2H) in fungi mainly occurs at the *ortho* position to yield phenols **3A** and **3B** (X = ^2H, Scheme 1) and proceeds *via* the 2,3- (**5A**, **5B**, X = ^2H) rather than the isomeric 1,2-benzene oxide (**6A**, **6B**, X = ^2H)



Scheme 1

which would yield phenols **7A** and **7B** (X = ^2H) after halogen migration.

Some substituted benzene oxides are unstable, *e.g.* the 1,2-oxide of chlorobenzene **6A** (X = ^1H) could not be isolated after synthesis,[12] while the 2,3-oxide **5A** and 3,4-oxide **4A** (X = ^1H) were found to be stable.[13] Benzene oxides have been assumed to equilibrate spontaneously with the corresponding oxepine valence tautomer by a disrotatory electrocyclic rearrangement mechanism.[1,2] However, in practice, dependent on substituent position, monosubstituted benzene oxides have been found to exist almost exclusively as either the epoxide, *e.g.* 2,3-benzene oxides **5A–C** rather than oxepines **8A–C**, or the oxepine valence tautomers *e.g.* **9C** and **10A–C** rather than 1,2-benzene oxides **6C** or 3,4-benzene oxides **4A–C**.[14] The preferred benzene oxide valence tautomers containing an electron withdrawing carboalkoxy group, *e.g.* **5C** (X = ^1H), and the preferred oxepine tautomers, *e.g.* **9C** and **10C** are among the most stable known compounds in this arene oxide–oxepine class.[15–17] Thus oxepine tautomer **9C** could be chemically synthesized and was isolated as a fungal metabolite.[18,19]



The biosynthetic origin of the secondary metabolites methyl benzoate **1C** (X = ^1H) and methyl salicylate **3C**/**7C** (X = ^1H) in *Phellinus*, a widespread genus of white rot fungi, has been the subject of ongoing investigations in these laboratories. The genus is unusual in utilising CH_3Cl as a methyl donor in the biosynthesis of methyl benzoate and methyl 2-furoate.[20–23] However, the time course study of incorporation of C^2H_3 from labelled methionine (the immediate metabolic precursor of CH_3Cl) into methyl salicylate **3C**/**7C** (X = ^1H) by *Phellinus pomaceus* was quite different from that of methyl benzoate **1C** (X = ^1H). This suggests that the compound is formed by *ortho*-hydroxylation of compound **1C** (X = ^1H) rather than methylation of salicylic acid,[20] a conclusion consistent with the observation that isolated mycelia cannot utilize CH_3Cl in the methylation of salicylic acid.[20] A more recent study has detected carbomethoxyoxepine **9C** (X = ^1H) as a natural metabolite in *Phellinus tremulae*.[18] The propensity for this oxepine to rearrange *via* the postulated,[15] but undetected, benzene oxide tautomer **6C** (X = ^1H) with migration of the carbomethoxy group has prompted speculation[18] that it may be an intermediate in methyl salicylate biosynthesis. This hypothesis is given strong support by the results of incorporation experiments with this fungus involving administration of α-

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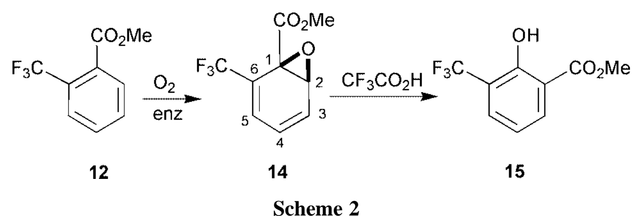
^{13}C-benzoic acid.[19] We have undertaken further biotransformation studies using *Phellinus ribis* and a range of substituted methyl benzoate substrates in the quest for the first evidence of carbomethoxy group migration *in vivo*.

P. ribis cultures were grown on 0.5% mycological peptone in shake flasks at 25 °C and the culture medium monitored for methyl esters of aromatic acids, oxepines and arene oxides by HPLC and GC/MS. As in the earlier investigations of Ayer and Cruz,[18,19] using *P. tremulae*, oxepine **9C** was detected in the medium after 12 days in addition to methyl benzoate and methyl salicylate as natural products of this fungus.[23] When cultures were supplemented after 12 days growth with 0.5 mM methyl 2,6-$^2H_2$-benzoate and examined after a further 2 days incubation, substantial incorporation of label into oxepine, 3,7-$^2H_2$-**9C** (*ca.* 70% incorporation), and methyl salicylate, 3,6-$^2H_2$-**3C** and 3-^2H-**7C** (*in toto* 40% incorporation) was found. GC/MS and NMR analyses of the metabolites showed that the major metabolic route involved migration of the carbomethoxy group to yield methyl salicylate **7C** (*ca.* 75% relative yield, 3-^2H, X = ^1H). A minor pathway (*ca.* 25% relative yield) involved migration and partial retention of a deuterium atom yielding methyl 3,6-$^2H_2$-salicylate **3C** (X = ^1H).

A series of methyl esters of monosubstitited benzoic acids were next examined as possible substrates by supplementation of the fungal growth medium as described above. Comparison of the metabolites from the methyl esters of *para*-substituted benzoic acids **1C** (X = F, Cl, Me), with authentic standard samples of the substituted methyl salicylate metabolites **7C** (X = F, Cl, Me) by GC/MS indicated that the 'NIH shift' involving migration of the carbomethoxy group had occurred during aromatic hydroxylation. Methyl esters of the corresponding *meta*-substituted benzoic acids were also examined as substrates but only the methyl *m*-fluoro- and *m*-methyl-salicylates were detected and no carbomethoxy migration was observed. Aromatic hydroxylation did not occur with the methyl esters of *ortho*-substituted benzoic acids or methyl esters of the *o-*, *m-* and *p*-trifluoromethyl benzoic acids as substrates. The only oxepine detected, other than oxepine **9C**, was oxepine **11** when ethyl benzoate was a substrate. However, GC/MS analysis of the bioextracts from metabolism of methyl 2-trifluoromethylbenzoate **12** as substrate, revealed a major bioproduct with a molecular weight identical to that of the corresponding oxepine **13**. This metabolite, purified by PLC, was obtained (*ca.* 2 mg from 300 cm^3 of culture medium) as a stable colourless liquid. ^1H- and ^{13}C-NMR spectra (NOE, COSY, HETCOR) of the metabolite were consistent with previously synthesised arene oxides.[13,24] It was identified as benzene oxide **14** (Scheme 2). δ_H 500 MHz, $CDCl_3$: 3.85 (s, Me), 4.23 (dd, H-2), 6.59 (ddd, H-4), 6.7 (ddd, H-3), 6.98 (dd, H-5), $J_{2,3}$ 3.7, $J_{2,4}$ = $J_{5,3}$ 1.9, $J_{3,4}$ = $J_{4,5}$ 8.6; δ_C (125 MHz, $CDCl_3$): 53.09, 61.03, 65.39, 128.02, 130.24, 130.28, 132.20, 166.55. ^{19}F-NMR, UV, IR and high resolution MS were used to confirm the identification of benzene oxide **14**.

Chemically synthesised 2,3-benzene oxides (*e.g.* **5A** and **5B** X = ^1H), derived from the *cis*-2,3-dihydrodiol enantiomers, were found[24] to have totally racemized *via* the oxepine tautomer in accord with predictions.[1,2] Benzene oxide **14** was similarly found to be racemic suggesting that total racemization had occurred *via* the undetected oxepine valence tautomer **13**. Addition of trifluoroacetic acid to oxide **14** resulted in aromatization to yield product **15** (Scheme 2) whose structure was confirmed by MS and NMR analysis (^1H-, ^{13}C-, HMBC) thus providing a further example of a carbomethoxy group migration. Interestingly phenol **15** was only detected as a trace metabolite of the methyl benzoate **12** and this appears to be a consequence of the remarkable stability of benzene oxide **14** which may be due to the presence of two strongly electron withdrawing groups, in particular the bulky CF_3 group. The location of these groups at positions C-1 and C-6 in arene oxide **14** appears to bias the equilibrium almost exclusively towards the benzene oxide valence tautomer **14**. As in other eucaryotes the enzyme is presumably a monooxygenase and attempts to isolate it are in progress.



Scheme 2

The isolation of benzene oxide **14** as a metabolite and the identification of aforesaid metabolites in cultures of *P. ribis* provide compelling evidence that *o*-hydroxylation of the methyl benzoates proceeds *via* benzene oxide intermediates. These intermediates, in turn, provide a rationale for this first report of an 'NIH shift' of the carbomethoxy group in a biological system. The apparent carboxyl group migration reported earlier[25] may in fact involve an ester intermediate.

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