Biosynthesis of Chloromethane in *Phellinus pomaceus*

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The effects of L- and D-methionine on production of the gaseous secondary metabolite chloromethane (CH₃Cl) by the white-rot fungus *Phellinus pomaceus* were investigated. Although D-methionine stimulated CH₃Cl biosynthesis, the L-isomer either failed to affect or depressed production depending on the concentration. Nevertheless, very high levels of incorporation of label (~80%) from L-[methyl-²H₃]methionine into CH₃Cl could be achieved demonstrating that the amino acid is a highly effective precursor of CH₃Cl. Experiments using labelled D-methionine showed substantial but lower incorporation. Patterns of incorporation of label from both isomers into methyl benzoate, another important volatile fungal metabolite, were very similar to those observed with CH₃Cl. This behaviour is only explicable in terms of either (a) the utilization of the same enzyme system requiring an identical methyl donor for methylation of both chloride and benzoate ions or (b) the presence of a transmethylation system. The incorporation of label from L-[methyl-²H₃]methionine into methyl 2-furoate was similar to that of methyl benzoate implying that the same methylating system was used for esterification of both 2-furoic and benzoic acids. However, the incorporation pattern for methyl salicylate was different and suggests that methyl salicylate is probably formed by *o*-hydroxylation of methyl benzoate.

INTRODUCTION

Chloromethane (CH₃Cl) is a gaseous secondary metabolite produced in high yield by many species in the Hymenochaetaceae, a widespread family of white-rot fungi (Cowan *et al.*, 1973; Harper, 1985; Harper & Kennedy, 1986; Harper *et al.*, 1988). Biosynthesis of this compound occurs in the majority of species in the genera *Phellinus* and *Inonotus*, both of which are characterized by bracket-like perennial fruiting bodies found on angiosperm and gymnosperm wood in temperate and tropical regions. CH₃Cl of fungal origin may provide an important contribution to the 5 Mt per year of CH₃Cl estimated to be released to the atmosphere from natural sources (Harper and Kennedy, 1986; Harper *et al.*, 1988).

Biochemically, the enzymic incorporation of halide ion into a 1-carbon compound poses some very interesting questions. Although a large number of organohalogen compounds have been isolated from natural sources the only biological route so far identified for their biosynthesis has been the haloperoxidase-catalysed incorporation of halide ion in the presence of hydrogen peroxide (Neidleman & Geigert, 1986). Clearly, such a mechanism is not feasible if halogen is directly incorporated into a 1-carbon compound. The formation of CH₃Cl if haloperoxidase were responsible must necessarily involve a 2- or more carbon intermediate which either spontaneously decomposes or is enzymically converted to CH₃Cl. This mechanism has been invoked to explain the formation of di- and tribromomethane by an algal bromoperoxidase

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acting on 3-oxooctanoic acid in the presence of hydrogen peroxide and bromide (Theiler *et al.*, 1978). However, no indication of the biosynthesis of monohalogenated compounds by this route has yet been reported.

Alternatively, a novel enzyme system catalysing the direct methylation of halide ion by a methyl donor such as *S*-adenosylmethionine, *N*⁵-methyltetrahydrofolate or methylcobalamin may be present in CH₃Cl-producing fungi. Investigations by White (1982) using labelled L-methionine suggested that the amino acid could act as a source of the methyl group in CH₃Cl produced by *Phellinus pomaceus*. Unfortunately, significant incorporation was only obtained at high concentrations of methionine (i.e. between 2.5 and 10 g l⁻¹) likely to disrupt the normal nitrogen and carbon metabolism of the fungus. Harper & Kennedy (1986) found no evidence of stimulation of CH₃Cl production by methionine at concentrations of 100 and 200 mg l⁻¹ and concluded that the rate of synthesis of CH₃Cl was not limited by the endogenous concentration of the amino acid. An apparent stimulation of CH₃Cl production by folic acid was reported by White (1982), although again the concentration (0.23 mM) used was greatly in excess of normal physiological requirements. Harper & Kennedy (1986), using a more accurate assay system, showed no enhancement of CH₃Cl production by folic acid at concentrations between 0.023 and 230 µM. Thus the evidence for the involvement of *S*-adenosylmethionine or *N*⁵-methyltetrahydrofolate as methyl donor in the biosynthesis of CH₃Cl as postulated by White (1982) is equivocal. Further studies are clearly warranted to establish that methionine can act as precursor of CH₃Cl under normal conditions of growth and to identify the actual methyl donor.

Methyl esters of aromatic acids are major components of the volatile fraction of *P. pomaceus* (Harper & Kennedy, 1986). In the light of this observation and the finding that the pseudohalide ion thiocyanate can act as a powerful inhibitor of both halomethane biosynthesis and methyl benzoate formation, Harper & Kennedy (1986) suggested that methylation of halide ion and benzoate was closely linked, perhaps even mediated by the same biochemical system. Further support for this hypothesis was provided by the results of a survey (Harper *et al.*, 1988) which indicated that whilst ester biosynthesis occurred in 52% of CH₃Cl-producing species of *Phellinus* examined, esters were not synthesized by any *Phellinus* species lacking the CH₃Cl-biosynthesis trait. Ester biosynthesis in evolutionary terms may therefore represent the consequence of an extension of the substrate range of the methylating system involved in CH₃Cl production.

In this paper we describe experiments involving media supplementation with possible precursor compounds which were designed to determine the metabolic source of the methyl group in CH₃Cl and the methyl esters of aromatic acids produced by *P. pomaceus*. We measured the incorporation of C²H₃ from C²H₃-labelled L- and D-methionine into both CH₃Cl and the methyl esters of benzoic, salicylic and 2-furoic acids at various stages of fungal growth and attempted to ascertain the metabolic relationship between the compounds.

METHODS

Organisms and maintenance media. *Phellinus pomaceus* (Pers.) Maire (NCWRF-FPRL 33A) was acquired from the National Collection of Wood Rotting Fungi, Princes Risborough Laboratory, Building Research Establishment, Aylesbury, Bucks., UK. The fungus was maintained on slants of 5% (w/v) malt extract agar (Oxoid) containing chloramphenicol (25 µg ml⁻¹). Cultures used for inoculation were grown at 25 °C for 3 weeks in 2 ml vials with loosely fitting screw caps.

Chemicals. L-[methyl-¹³C]Methionine (99 atom% ¹³C), L-[methyl-²H₃]methionine (99.4 atom% ²H) and D-[methyl-²H₃]methionine (97 atom% ²H) were obtained from MSD Isotopes, Montreal, Canada. Chloromethane, bromomethane, methyl benzoate, methyl salicylate and agarose '10' were purchased from BDH; methyl 2-furoate was obtained from Aldrich.

Methionine analysis. The methionine content of mycological peptone was estimated using an LKB model 4400 Amino Acid Analyser as described by Dirar *et al.* (1985).

Culture media. For investigation of the percentage conversion of chloride ion to CH₃Cl when the fungus was grown in the presence of supplemental L-, D-, or DL-methionine, the basic media used was glucose/mycological peptone. The medium contained (g l⁻¹) glucose (30), mycological peptone (5), agarose '10' (10) and NaCl to give a final chloride concentration of 9.5 mM. Where required L-, D- or DL-methionine was added to the medium at 0.2 or 0.4 g l⁻¹. The pH was adjusted to 6.5 prior to autoclaving.

For incorporation studies the fungus was grown on a cellulose-based medium in 40 ml screw-capped septum

vials sealed with Teflon-lined silicone discs. Into each vial filter paper (3.5g; Whatman 40) was placed as sheets which were wrapped around approximately 80% of the circumference of the vial. For the preparation of cellulose/mycological peptone medium, a solution (6 ml) of mycological peptone (5 g l⁻¹) was added to the filter paper in each vial. To prepare cellulose/amino acids medium, a synthetic amino acids and mineral salts medium (6 ml) was added to the filter paper in each vial. The composition of this solution was as follows (g l⁻¹): KH_2PO_4 (7.0), Na_2HPO_4 (2.4), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1.0), $(\text{NH}_4)_2\text{SO}_4$ (2.5), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (0.02) and the L-amino acids, alanine (0.258), arginine free base (0.223), aspartic acid (0.340), cysteine (0.034), glutamic acid (0.607), glycine (0.229), histidine free base (0.091), isoleucine (0.142), leucine (0.302), lysine free base (0.306), phenylalanine (0.306), proline (0.230), serine (0.037), threonine (0.094), tryptophan (0.036), tyrosine (0.100) and valine (0.218). The pH of the solution was adjusted to 6.5 and thiamin mononitrate (1.2 mg l⁻¹) added together with a trace element solution (10 ml l⁻¹) of the following composition (mg l⁻¹): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (4), $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ (20), $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ (40), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (40) and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (40).

Depending on the experiment the basic cellulose/mycological peptone or cellulose/amino acids medium was supplemented with NaCl or NaBr (0.5 g l⁻¹) and L-[methyl-¹³C]methionine, L-[methyl-²H₃]methionine or D-[methyl-²H₃]methionine at levels of 200 or 400 mg l⁻¹. Choline (25 mg l⁻¹) and benzoic acid (50 mg l⁻¹) were added in some experiments.

Cultural conditions. For assessment of total CH_3Cl generated during the growth of the fungus, cultures were grown at 25 °C in 250 ml conical flasks containing 20 ml of medium. The inoculum, which was spread uniformly over the surface of the medium, was a mycelial suspension (1 ml) prepared by homogenizing mycelia (60 mg wet wt) in sterile distilled water (20 ml) for 30 s with an Ultra Turrax homogenizer. Flasks were fitted with outlets prepared as described by Harper & Kennedy (1986) permitting a limited exchange of gases between the internal and external atmospheres.

CH_3Cl assay. Samples of headspace (2 ml) were withdrawn from culture flasks through the sampling port and injected into a Pye Unicam 104 (model 74) gas chromatograph fitted with a flame ionization detector and equipped with a glass column (1.5 m × 2 mm i.d.) packed with Tenax GC operated at a nitrogen gas flow rate of 20 ml min⁻¹. The oven temperature was programmed from 60 to 120 °C at 24 °C min⁻¹.

Calculation of total CH_3Cl generated during growth. The total CH_3Cl generated by a fungal culture was calculated by means of a computer integrating program as described by Harper & Kennedy (1986) from measurements of CH_3Cl concentrations in the headspace of the flask at regular intervals during incubation and a knowledge of the half-life of CH_3Cl in the flask obtained from a previous calibration procedure. Each experimental treatment of the fungus was repeated in triplicate and the mean CH_3Cl yield expressed as the percentage of chloride ion originally present in the medium converted to CH_3Cl . CH_3Cl yields for replicates differed by an average of 6%.

Isotopic incorporation studies. Preliminary experiments involved the measurement of the degree of isotopic labelling in CH_3Cl after growth of the fungus on cellulose/amino acids or cellulose/mycological peptone medium with various supplements and isotopically labelled L- or D-methionine. Vials (eight for each treatment) with filter paper substrate were inoculated with a mycelial suspension (1 ml) prepared as described under 'Cultural conditions'. The inoculum was spread as uniformly as possible over the surface of the filter paper and the screw cap of the vial replaced but tightened only sufficiently to maintain sterility and not to prevent access of air. After incubating the vial for 9 d at 25 °C the caps were screwed down and incubation was continued for a further 8 d. The culture was then frozen and stored at -15 °C pending measurement of the isotopic labelling of CH_3Cl by gas chromatography/mass spectrometry.

Subsequent incorporation experiments required measurement of the degree of isotopic labelling not only in CH_3Cl but also in methyl esters of benzoic, salicylic and furoic acids at various stages during the growth of the fungus on cellulose/mycological peptone medium with isotopically labelled L- and D-methionine in the presence and absence of benzoic acid. Vials (18 for each treatment) were inoculated as described above and the screw caps replaced loosely as in the previous experiments. After 5 d, three vials from each treatment were tightly closed. This procedure was repeated each day up to and including day 11. Using this protocol no appreciable CH_3Cl formed biologically was lost; previous experiments had demonstrated no significant synthesis of CH_3Cl before day 11 in the system. After closure each set of vials was incubated for a further 7 d at 25 °C and then frozen at -15 °C pending measurement of isotopic ratios.

Determination of ¹³C and ²H incorporation. For measurement of isotopic ratios in CH_3Cl and CH_3Br the following procedure was adopted. The vial containing the fungal culture was attached to a Hewlett Packard 7675A Purge and Trap sampler and purged at 25 °C with helium at a flow rate of 20 ml min⁻¹ for between 30 s and 2 min depending on the concentration of halomethane in the sample. The volatile components were adsorbed on a Tenax trap. The Purge and Trap sampler was linked to a Carlo Erba Mega Series gas chromatograph equipped with a fused silica WCOT capillary column (10 m × 0.32 mm i.d.) coated with Poraplot Q porous polymer. The entrapped volatiles were thermally desorbed using a split ratio of 10:1 on to the first 10 cm of the capillary column by cooling the latter in liquid N₂. On complete desorption of the volatiles the oven was programmed from 30 to 150 °C at 10 °C min⁻¹ with helium as carrier gas at a flow rate of 2 ml min⁻¹. The gas chromatograph was linked

via a direct inlet to a Kratos MS25 RFA mass spectrometer coupled to a Data General DG30 data system. The mass spectrometer was operated in the electron impact mode at an ionizing voltage of 4 kV. The total ion current between m/e 30 and 200 was monitored by scanning at 1 s decade⁻¹.

The relative isotopic abundance of ¹³C in atom% in CH₃Cl was determined as the ratio of ion currents $\frac{m/e\ 53}{m/e\ 52 + m/e\ 53} \times 100$ at the normal retention time of CH₃Cl. The percentage ¹³C incorporation was calculated from this value by subtraction of the natural ¹³C abundance in CH₃Cl. The relative abundance of ¹³C in atom% in CH₃Br was determined as the ratio of ion currents $\frac{m/e\ 97}{m/e\ 96 + m/e\ 97} \times 100$ at the normal retention time of CH₃Br. The percentage ¹³C incorporation was calculated as before, after correction for the natural ¹³C abundance in CH₃Br. The percentage incorporation of C²H₃ into CH₃Cl was measured as the ratio of ion currents $\frac{m/e\ 55}{m/e\ 52 + m/e\ 55} \times 100$ at the normal retention time of CH₃Cl.

For the measurement of isotopic ratios in methyl esters of benzoic, salicylic and 2-furoic acids the following procedure was used. After determination of halomethane isotopic ratios the vial was attached to the Purge and Trap sampler and purged at 80 °C with helium at a flow rate of 20 ml min⁻¹ for between 2 and 8 min depending on the concentration of esters in the sample. The trapped volatiles were desorbed as described above on to a fused silica WCOT capillary column (25 m × 0.32 mm i.d.) with BP10 as bonded phase. On complete desorption the chromatograph oven was programmed from 30 to 250 °C at 10 °C min⁻¹ with helium as carrier gas at a flow rate of 2 ml min⁻¹. The mass spectrometer was operated as described above except that the total ion current between m/e 30 and 300 was recorded. The percentage incorporation of C²H₃ into methyl benzoate was determined as the ratio of ion currents $\frac{m/e\ 139}{m/e\ 136 + m/e\ 139} \times 100$ at the normal retention time of methyl benzoate. Similarly the percentage incorporation of C²H₃ into methyl 2-furoate and methyl salicylate was determined as the ratio of ion currents $\frac{m/e\ 155}{m/e\ 152 + m/e\ 155} \times 100$ and $\frac{m/e\ 129}{m/e\ 126 + m/e\ 129} \times 100$ respectively at the expected retention times of the compounds.

RESULTS AND DISCUSSION

Comparison of effect of L- and D-methionine on CH₃Cl production

Harper & Kennedy (1986) failed to show any significant effect of L-methionine supplementation of the medium at 100 and 200 mg l⁻¹ on overall CH₃Cl production by *P. pomaceus* leading them to conclude that if methionine was a precursor of CH₃Cl, biosynthesis of the latter was not limited by the endogenous concentration of the amino acid. An alternative explanation may be that the exogenous methionine is not taken up when the fungus is growing on a readily utilizable carbon source such as glucose. This would also explain the failure of White (1982) to obtain appreciable labelling in CH₃Cl produced by the fungus when grown on malt extract medium in the presence of labelled L-methionine at a concentration of 400 mg l⁻¹.

Data presented in Table 1 confirm the lack of effect of methionine (200 mg l⁻¹) supplementation on CH₃Cl biosynthesis and demonstrate a marked suppression of CH₃Cl production when the concentration was increased to 400 mg l⁻¹. Such inhibition may simply reflect a more general inhibition of fungal growth noted at this level of supplementation. However, D-methionine gave a significant stimulation of CH₃Cl biosynthesis at a concentration of 200 mg l⁻¹ and the stimulation was also apparent albeit to a lesser extent when the racemic compound was added. Whether this promotion of CH₃Cl production by the D-isomer indicates that it can act *per se* as a precursor of CH₃Cl or is affecting fungal metabolism in such a way as to enhance the availability of L-methionine for CH₃Cl biosynthesis in the fungal cell is not clear. Indeed, the increased CH₃Cl production induced by the presence of the stereoisomer may result from other more general effects on secondary metabolism.

Incorporation of isotopically labelled methionine into CH₃Cl

In preliminary experiments *P. pomaceus* was grown in vials for 17 d on cellulose-based medium in the presence of ¹³CH₃- and C²H₃-labelled methionine with various media supplements and the proportion of label incorporated into CH₃Cl was determined. In the

Table 1. *Effect of L- and D-methionine supplementation of medium on CH_3Cl yields in *P. pomaceus**

Supplement to glucose/mycological peptone medium	Level of supplementation (mg l^{-1})	Percentage chloride converted to CH_3Cl
None	—	59
L-Methionine	200	64
	400	37
D-Methionine	200	72
	400	65
DL-Methionine	200	68
	400	69

Table 2. *Incorporation of isotopically labelled L- and D-methionine into CH_3Cl or CH_3Br by *P. pomaceus* after 17 d growth on various media*

Supplements to medium	Level of supplementation (mg l^{-1})	Basic medium	Percentage incorporation of label*
L-[methyl- ^{13}C]Methionine NaCl	400	Cellulose/ amino acids	27.0 ± 4.6
	500		
L-[methyl- ^{13}C]Methionine NaCl	400	Cellulose/ mycological peptone	24.6 ± 3.5
	500		
L-[methyl- ^{13}C]Methionine NaBr	400	Cellulose/ amino acids	28.6 ± 3.9
	500		
L-[methyl- ^{13}C]Methionine Choline NaCl	400	Cellulose/ amino acids	26.5 ± 6.1
	25		
D-[methyl- $^2\text{H}_3$]Methionine NaCl	400	Cellulose/ amino acids	21.1 ± 5.9
	500		

*Mean values $\pm$ SD.

presence of 400 mg L-[methyl- ^{13}C]methionine l^{-1} , 27% incorporation of ^{13}C into CH_3Cl was recorded on the defined cellulose/amino acids medium. The substantial incorporation demonstrates that L-methionine can act as a precursor of CH_3Cl at relatively low levels of methionine addition and contrasts with a mere 3.4% incorporation observed by White (1982). The discrepancy can probably be ascribed, as postulated above, to the inhibition of exogenous methionine uptake in the latter study during growth on a readily utilizable carbon source (maltose) present in the malt-based medium used by White.

When the defined amino acid component of the cellulose/amino acids medium was replaced by mycological peptone very little decrease in incorporation of label was observed (Table 2) despite the fact that the mycological peptone supplement contained appreciable quantities of L-methionine (85 mg l^{-1}) which might have been expected to dilute the added labelled methionine resulting in an underestimate of the percentage incorporation of label into CH_3Cl . However, nearly all methionine in mycological peptone was found to be in a bound form, presumably as peptides and not immediately available for fungal uptake. Only 5 mg free methionine l^{-1} in the culture medium was derived from mycological peptone. This observation explains the negligible effect of substituting cellulose/mycological peptone for cellulose/amino acids medium on the level of incorporation and allowed the former medium to be used in the subsequent more detailed isotope incorporation experiments described in the next section.

When NaCl in the medium was replaced by NaBr incorporation of label into CH_3Br exhibited no significant difference from that into CH_3Cl confirming that, as anticipated, both

halomethanes are almost certainly derived by the action of the same enzyme from the same precursor pool. The finding also establishes that the observed preference of the fungal methylating system for bromide over chloride (Harper & Kennedy, 1986) does not significantly affect the rate of turnover in the pool of methyl donor. The addition of unlabelled choline to the culture medium did not alter the incorporation of $^{13}\text{CH}_3$ from L-[methyl- ^{13}C]methionine into CH_3Cl to any significant extent (Table 2) thereby appearing to eliminate any intermediary role for this compound in the biosynthesis of halomethane.

Interestingly, in view of the stimulatory effect of D-methionine on CH_3Cl production, addition of D-[methyl- $^2\text{H}_3$]methionine to the growth medium led to a substantial incorporation of C^2H_3 into CH_3Cl (Table 2), implying that either the CH_3Cl generating system was almost equally active on methyl donors derived from both isomers of methionine or that rapid enzymic racemization occurred within the fungal cell.

Comparison of incorporation of L- and D-[methyl- $^2\text{H}_3$]methionine into CH_3Cl and methyl benzoate

In order to examine the relationship between the biosynthesis of methyl benzoate and that of CH_3Cl the levels of incorporation into the two compounds from both isomers of labelled methionine after various periods of fungal growth were measured. The isotopic compositions of CH_3Cl and methyl benzoate produced by the fungus in sealed vials were determined from the point at which CH_3Cl was first present in the headspace of the vial in detectable quantities (Fig. 1). Very high ($\sim 80\%$) and almost identical levels of incorporation of the methyl group from L-methionine into both CH_3Cl and methyl benzoate were observed initially. From the 14th day of growth the levels began to fall, though at very similar rates, as the highly labelled CH_3Cl and methyl benzoate formed in the early stages of growth were diluted by the unlabelled compounds formed from endogenously synthesized L-methionine whilst at the same time the exogenous labelled methionine was depleted. By day 17 the labelling in CH_3Cl was not substantially different from that found in the preliminary experiments.

When the fungus was grown in the presence of labelled D-methionine, incorporation ($\sim 20\%$) into CH_3Cl and methyl benzoate was very much lower than that observed in the presence of L-methionine. However, the level subsequently increased to a maximum of over 50% on the 14th day of growth but declined thereafter at a rate very similar to that found with the L-isomer. The reason for this unusual incorporation pattern is not immediately apparent but can be explained in terms of the suggestions made above. If the CH_3Cl generating system can only use L-methionine as source of the methyl donor then the initial increase in incorporation may represent the induction of a racemase for the amino acid. Alternatively if the CH_3Cl generating system can utilize both L- or D-methionine as source of the methyl donor the preliminary increase may reflect the induction of an uptake system for D-methionine. Of particular note is the remarkable correspondence between the patterns of incorporation of D-methionine into CH_3Cl and methyl benzoate which did not differ from each other to a significant extent at any stage during the growth period studied (Fig. 1).

Several general conclusions regarding the origin of CH_3Cl and methyl benzoate can be drawn from the results presented in Fig. 1. The exceptionally high and initially very similar incorporations of C^2H_3 from L-[methyl- $^2\text{H}_3$]methionine into CH_3Cl and methyl benzoate leave no doubt that L-methionine is a highly effective precursor of the methyl group in both these compounds. However, it is not possible to deduce from the results obtained with D-methionine whether the methyl group of the D-isomer or a methyl donor derived from it can be used directly for methylation of chloride or benzoate or whether the amino acid must first undergo racemization. The striking similarity of the incorporation patterns into CH_3Cl and methyl benzoate when grown on both labelled L- and D-methionine can only be interpreted in terms of one of the following biochemical possibilities.

(a) The same enzyme system requiring an identical methyl donor is utilized for the methylation of both chloride and benzoate ions. The slight disparity between the incorporation patterns into the two compounds from day 14 onwards in the L-methionine experiment can be adequately explained on the basis that for a short period between days 13 and 14 the formation

Fig. 1. Incorporation of methyl- $^2\text{H}_3$ from L-[methyl- $^2\text{H}_3$]methionine (---) and D-[methyl- $^2\text{H}_3$]methionine (—) into CH_3Cl (○) and methyl benzoate (●) by *P. pomaceus*. The fungus was grown on cellulose/mycological peptone medium supplemented with NaCl (0.5 g l^{-1}) and labelled L- or D-methionine (0.4 g l^{-1}).

Fig. 2. Incorporation of methyl- $^2\text{H}_3$ from L-[methyl- $^2\text{H}_3$]methionine into CH_3Cl (○) and methyl benzoate (●) by *P. pomaceus* in (a) the absence and (b) the presence of benzoic acid. The fungus was grown on cellulose/mycological peptone medium supplemented with NaCl (0.5 g l^{-1}) and labelled L-methionine (0.2 g l^{-1}) $\pm$ benzoic acid (0.05 g l^{-1}).

of methyl benzoate was limited by the endogenous concentration of benzoate whereas at no stage during growth was CH_3Cl biosynthesis constrained by chloride concentration. As the concentration of benzoate is determined by the rate of biosynthesis by the fungus whereas chloride concentration is probably governed solely by the rate of uptake from the culture medium, this assumption appears feasible. In order to test this hypothesis the incorporation of L-methionine into CH_3Cl and methyl benzoate was measured when the fungus was grown in the presence and absence of added benzoic acid in the culture medium (Fig. 2a, b). The patterns of incorporation into the compounds were almost identical in the presence of a relatively low concentration of exogenous benzoic acid suggesting that benzoic acid may, in the absence of an exogenous source of the compound, limit the rate of synthesis of methyl benzoate for a short period during fungal growth. It could be argued that the similarity of the incorporation patterns observed is explicable in terms of two distinct enzyme systems using the same methyl donor. However, for such a postulate to be tenable the possession of identical kinetic parameters by the two enzymes and their induction at identical points in the fungal growth cycle would have to be invoked. Such circumstances cannot be regarded as likely.

(b) Rapid transmethylation may occur between methyl benzoate and inorganic chloride as shown below in reaction 1, or alternatively between CH_3Cl and benzoic acid as in reaction 2. An activated derivative of benzoic acid such as benzoyl-CoA rather than the acid *per se* might conceivably be the substrate for the enzyme in reaction 2.

Fig. 3. Incorporation of methyl- $^2\text{H}_3$ from L-[methyl- $^2\text{H}_3$]methionine into methyl benzoate (●), methyl 2-furoate (---□---) and methyl salicylate (■) in *P. pomaceus*. The fungus was grown on cellulose/mycological peptone medium supplemented with NaCl (0.5 g l^{-1}) and labelled L-methionine (0.2 g l^{-1}).

The slight delay in the progressive decline over time in the labelling of methyl benzoate as compared to CH_3Cl in the L-methionine experiment illustrated in Fig. 1 would, on the basis of this hypothesis, tend to favour reaction 2. However, a third possibility cannot be ignored in which an enzyme catalyses both the forward and reverse reactions above so that the overall direction of the reaction is dependent on the relative concentrations of the reactants.

Experiments involving the incubation of fungal mycelia with isotopically labelled CH_3Cl and methyl benzoate are currently underway to distinguish between the broad specificity methylating system utilizing a common methyl donor described in (a) and the transmethylation possibilities discussed in (b).

Incorporation of L-[methyl- $^2\text{H}_3$] methionine into methyl 2-furoate and methyl salicylate

Harper & Kennedy (1986) noted that in addition to methyl benzoate, methyl esters of other aromatic acids such as 2-furoic and salicylic acids were major components of the volatile fraction of *P. pomaceus*. Such methyl esters were also associated with the presence of methyl benzoate in a number of other halomethane producing species of *Phellinus* examined by Harper *et al.* (1988). To determine whether all methyl esters present in such *Phellinus* species were formed by the same methylating system the levels of incorporation of label from L-[methyl- $^2\text{H}_3$]methionine into the various esters were compared (Fig. 3). The pattern of incorporation of label into methyl furoate is very similar to that recorded for methyl benzoate, suggesting that the methylation of furoic acid is probably mediated by the same enzyme system as that methylating benzoic acid. However, the pattern of incorporation into methyl salicylate is quite different from that into methyl benzoate. Initial incorporation into methyl salicylate is comparatively low. A low level of biosynthesis of the compound at this stage of growth presumably contributes little label to the unlabelled compound carried over in the inoculum. Maximum incorporation into methyl salicylate was not attained until the 14th day of growth by which time labelling of methyl benzoate and furoate was decreasing rapidly. This divergent behaviour implies that methyl salicylate is almost certainly not biosynthesized by direct methylation of the corresponding acid in the same manner as the other methyl esters. Instead the response is consistent with the formation of methyl salicylate by *o*-hydroxylation of methyl benzoate. Labelling of methyl salicylate will therefore tend to lag behind that of methyl benzoate.

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