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A REVIEW

New frontiers in biological halogenation

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1. SUMMARY

The synthesis of halogenated compounds in biological systems is well established, yet the mechanisms by which these compounds are formed are poorly understood. Many commercially important compounds, such as pharmaceuticals and agrochemicals, contain halogens; indeed some halogenated natural products, such as the antibiotic vancomycin, are themselves valuable. Furthermore, several environmentally significant organohalogens can be formed naturally, for example it is likely that a significant proportion of the atmospheric bromomethane is produced by higher plants (Gan *et al.* 1998).

While chemical synthesis of organohalogens can be difficult, the biological production of these compounds occurs under relatively mild conditions and often with a greater degree of specificity. Therefore an understanding of the biosynthesis of halometabolites, and in particular, the enzymology of carbon–halogen bond formation, may provide convenient biotechnological methods for the halogenation of organic compounds. For over 30 years haloperoxidases were the only halogenating enzymes that had been identified and it was largely accepted that these enzymes were responsible for almost all biological halogenation reactions. However, in recent years evidence has accumulated pointing to the existence of other halogenases and now the nature of these enzymes is being revealed. This review concentrates on the occurrence, mechanism and biocatalytic potential of the halogenating enzymes that are currently known.

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2. HALOMETABOLITES

The first report of a halogen-containing natural product (halometabolite) was that of the iodinated amino acid diiodotyrosine (Fig. 1) from the coral *Gorgonia cavolii* in the late nineteenth century (Drechsel 1896). For many years such compounds were considered rare and of little biological significance and there is still a perception that organohalogens present in the environment are of anthropogenic origin only. However, even well-known pollutants such as polychlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDD/PCDF), appear to be also formed naturally (Hoekstra *et al.* 1999). Currently there are over 3600 halogenated natural products known, from bacteria, fungi, algae, higher plants and animals. Examples of halometabolites are shown in Fig. 1, but for a comprehensive review of the structural diversity of these compounds the reader is directed to Gribble (1998). Chlorometabolites and bromometabolites predominate; iodinated and fluorinated natural products are much less common. The functions of halometabolites are varied and they can have distinct physiological or biochemical roles, for example the lone star tick uses 2,6-dichlorophenol as a sex-pheromone (Berger 1972), while 4-chloroindolyl-3-acetic acid is a plant growth hormone (Marumo *et al.* 1968). Several halometabolites, particularly those of marine origin, appear to have a defensive role (Gribble 1999) and a number of halometabolites isolated from bacteria and fungi have antibiotic activity, for example chloramphenicol and chlortetracycline. The assumed role of many bacterial halometabolites conferring an advantage on the producer by inhibiting the growth of competing organisms has been questioned, as these compounds are usually produced in extremely small quantities in nature (van Pée 1996). Nevertheless, their biosynthesis

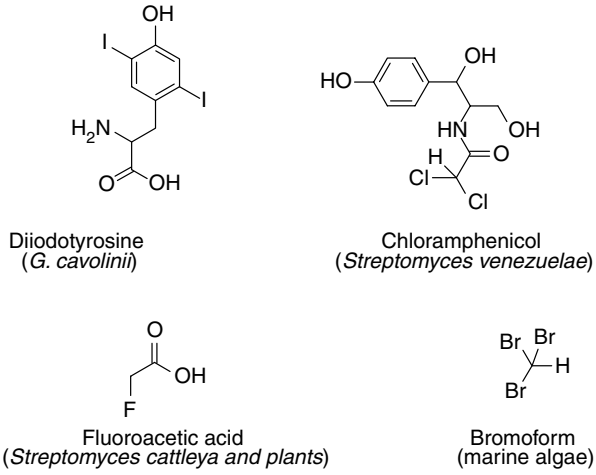

Fig. 1 Examples of naturally occurring organohalogen compounds

represents a considerable metabolic investment, thus it is reasonable to assume they are in some way useful to the producing organism.

3. HALOGENATING ENZYMES

3.1 Haloperoxidases

Hager and co-workers made the first discovery of a halogenating enzyme while investigating the biosynthesis of the chlorinated metabolite caldariomycin (Fig. 2a) by the fungus *Caldariomyces fumago* (Shaw and Hager 1959). The enzyme that they identified catalysed the chlorination of late intermediates on the caldariomycin biosynthetic pathway in the presence of hydrogen peroxide and chloride ions, thus was termed 'chloroperoxidase' (Shaw and Hager 1961). It was also observed that the enzyme could utilize bromide and iodide, but not fluoride, in halogenating reactions (Hager *et al.* 1966). The enzyme assay used for the initial characterization of chloroperoxidase employed a synthetic substrate, monochlorodimedone (Fig. 2b), which is structurally similar to 2-chloro-1,3-cyclopentanedione (Fig. 2c), a late intermediate in caldariomycin biosynthesis. The chloroperoxidase-catalysed chlorination of monochlorodimedone to dichlorodimedone can be monitored spectrophotometrically and this convenient assay has been used to identify haloperoxidases from a range of prokaryotic and eukaryotic organisms (Neidleman and Geigert 1986). These enzymes can be further classified according to which halide ions they are capable of utilizing in the halogenating reaction. Chloroperoxidases can use chloride, bromide and iodide; bromoperoxidases can use bromide and iodide; and iodoperoxidases can only use iodide.

The chloroperoxidase from *C. fumago* is the most widely studied halogenating enzyme. It is a glycoprotein with a molecular weight of approx. 42 000 and has a haem

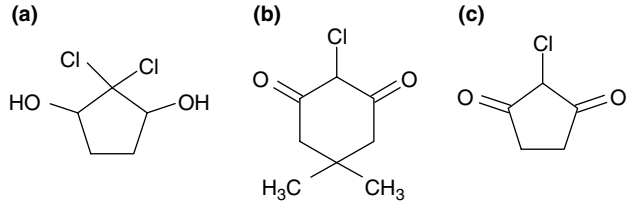

Fig. 2 Structures of caldariomycin (a), monochlorodimedone (b) and 2-chloro-1,3-cyclopentanedione (c)

prosthetic group, in common with other peroxidases (Morris and Hager 1966). However, not all haloperoxidases have haem as a prosthetic group; vanadium-dependent haloperoxidases have been isolated from fungi and algae (Vilter 1995) and halogenating enzymes that require hydrogen peroxide, but contain no haem or metal cofactor have been identified in bacteria (Wiesner *et al.* 1986; Burd *et al.* 1995). The latter enzymes were initially classified as haloperoxidases, but further studies demonstrated that they were in fact hydrolases that generate peracids from short chain carboxylic acids and hydrogen peroxide; the peracids then oxidize the halide ions, generating hypohalous acid (Hecht *et al.* 1994; Picard *et al.* 1997).

There has been much debate in the literature on the mechanism of haloperoxidases and in particular the nature of the halogenating reagent. Reduction of hydrogen peroxide by the enzyme generates a redox potential sufficient to oxidize halide ion, yielding an electrophilic halogenating reagent, which is generally believed to be hypohalous acid (van Pée 2001). Recently, crystallographic studies on *C. fumago* chloroperoxidase have shed more light on the structure of the active site and the likely mechanism of chlorination (Sundaramoorthy *et al.* 1998). Based on these observations the reaction is believed to proceed by heterolytic cleavage of the O–O peroxide bond facilitated by a glutamate residue in the active site (glu 183) acting as an acid–base catalyst, which first deprotonates hydrogen peroxide, then reprotonates the haem-bound ionized peroxide, releasing water and leaving an oxyferryl centre (compound I). This reacts readily with Cl^-, generating a Fe–OCl adduct, and as the chlorination reaction proceeds optimally at pH 3, this is most likely protonated to Fe–HOCl. The release of HOCl returns the enzyme to its native state and the hypohalous acid reacts with the organic substrate outside the active site. Franssen and van der Plas (1987) were unable to determine a K_m value for monochlorodimedone in a chloroperoxidase-catalysed reaction with substrate concentrations ranging from 2 to 100 μM, which is consistent with the suggestion that the halogenation reaction occurs outside the active site. However, the crystallographic studies revealed a possible binding site for hydrophobic substrates, and when careful measurement of chlorination rates were made using

monochlorodimedone as a substrate at concentrations between 0·5 and 10 μM, it was observed that as the substrate concentration was lowered, the chlorination rate decreased (Murphy and White, unpublished data). Therefore, a Michaelis–Menten complex between the organic substrate and chloroperoxidase may occur. It has also been reported that hypobromous acid is not released from the bromoperoxidase of *Ascophyllum nodosum* when 2-methylindole is included in the assay mixture, suggesting that in the presence of an organic substrate the halogenating reagent remains enzyme bound (Tschirret-Guth and Butler 1994). Therefore, the exact nature of the interaction between haloperoxidases and organic substrates has yet to be fully resolved.

The use of haloperoxidases as halogenating biocatalysts is limited because haloperoxidase-catalysed halogenations lack substrate specificity and regioselectivity, which is consistent with the premise that hypohalous acid is the actual halogenating reagent. Nevertheless, the halogenating activity of haloperoxidases may have some biocatalytic potential; the biotransformation of indene to 1*S*,2*R*-indene oxide, which is required in the synthesis of the HIV-1 protease inhibitor Crixivan® (Merck), can be catalysed by a crude enzyme extract of the fungus *Curvularia protuberata* (Zhang *et al.* 1999). It was found that the extract contained a bromoperoxidase, which converted indene to racemic *trans*-bromoindanols, and a dehydrogenase that stereoselectively oxidized the 2*R*,1*R*-bromoindanol to bromoindenol, leaving the 2*S*,1*S*-bromoindanol which yielded the epoxide upon the addition of base (Fig. 3). Also, the chlorinating activity of *C. fumago* chloroperoxidase has been shown to remove nickel and vanadium from the asphaltene fraction of crude oil (Mogollon *et al.* 1998) and regioselective chloroperoxidase-catalysed bromohydroxylation of alkenes has been achieved by adsorbing the enzyme on talc (Aoun and Baboulene 1998). Interestingly, chloroperoxidase exhibits peroxidase, catalase and cytochrome P450-like activities, and significantly the products of some chloroperoxidase-catalysed epoxidations are highly enantiomerically enriched (Zaks and Dodds 1995). Thus, chloroperoxidase may be useful as a chiral catalyst for certain oxidative reactions.

Until quite recently, it was assumed that the physiological function of haloperoxidases was in the biosynthesis of halometabolites. There is some evidence supporting the involvement of haloperoxidases in the production of some natural organohalogens, for example bromoform biosynthesis in the alga *Penicillus capitatus* appears to involve the action of a bromoperoxidase on 3-oxooctanoic acid (Beissner *et al.* 1981) and thyroid peroxidase has been shown to catalyse the iodination and coupling of tyrosine to yield the hormone thyroxine (Nunez 1984). However, investigations of haloperoxidases from various sources have relied mainly on the synthetic substrate monochlorodimedone for measuring enzyme activity, without demonstrating halogenation of the natural substrates by the isolated enzymes. The lack of substrate and regio-specificity of haloperoxidases is also inconsistent with the apparent specific halogenations required for the biosynthesis of many halometabolites. Genetic investigations on the biosynthesis of chlortetracycline also add support to the contention that haloperoxidases are not involved in many biological halogenation reactions. When the gene coding for the chlorination reaction in chlortetracycline biosynthesis in *Streptomyces aureofaciens* was cloned and sequenced, no similarity was found between this sequence and the sequences of known haloperoxidases (Dairi *et al.* 1995). Furthermore, the biosynthesis of the leucine-derived natural product, barbamide (Fig. 4), produced by the cyanobacterium *Lyngbya majuscula,* does not appear to involve a chloroperoxidase (Sitachitta *et al.* 1998). The methyl group of leucine that eventually becomes C-9 of barbamide requires activation before it could undergo electrophilic chlorination via a haloperoxidase. This activation would probably involve desaturation of the bond between C-2 and C-3 and subsequent carboxylation of C-9. However, feeding experiments with deuterium-labelled leucine demonstrated that no protons are lost from C-3 or C-4 of leucine as it is incorporated into barbamide,

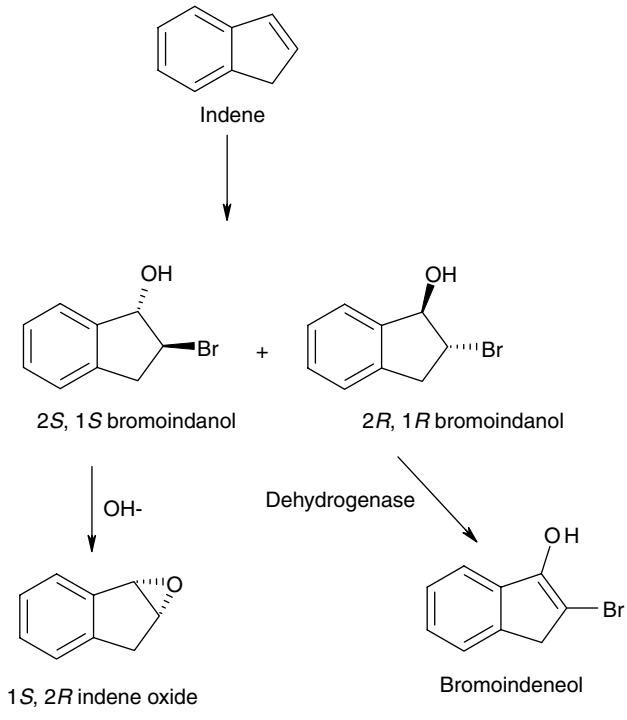

Fig. 3 Generation of 1*S*,2*R*-indene oxide by the action of a bromoperoxidase and a dehydrogenase from cell-free extracts of *Curvualria protuberata* on indene

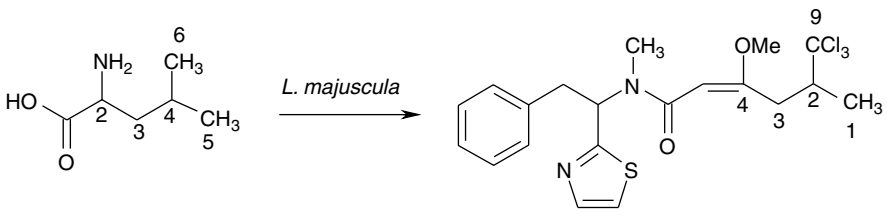

Fig. 4 Biosynthesis of the chlorinated metabolite barbamide from leucine by *Lyngbya majuscula*. The C-1 to C-4 and C-9 carbons of barbamide are derived from C-2 to C-6 of leucine

indicating that no double bond is formed between C-2 and C-3 of barbamide, hence C-9 is not activated prior to chlorination (Sitachitta *et al.* 2000). A halogenating mechanism involving radicals has been proposed (Hartung 1999), although no experiments have yet been reported that investigate this possibility.

The role of haloperoxidases in many organisms is therefore poorly understood.

3.2 $FADH_2$-dependent halogenase

The most convincing example of the non-involvement of haloperoxidases in biological halogenations is in the biosynthesis of the antifungal compound pyrrolnitrin by a number of *Pseudomonas* species, in which a new type of halogenating enzyme has been discovered. Four genes encode the biosynthetic pathway to pyrrolnitrin; *prnABCD* (Fig. 5) and the functions of the genes have been determined by identifying the intermediates that accumulated in cultures of *prn* deletion mutants (Hammer *et al.* 1997; Kirner *et al.* 1998). The gene product of *prnA*, the enzyme that catalyses the regiospecific chlorination of tryptophan, has been isolated from cell-free extracts of a *Pseudomonas fluorescens* recombinant mutant harbouring the prnA gene on a plasmid (Keller *et al.* 2000). The enzyme requires $FADH_2$ and O_2 for activity, suggesting a monooxygenase-type of mechanism, where flavin hydroperoxide activates the substrate via an epoxide, which is attacked by chloride ion, generating a halohydrin from which water is removed to yield 7-chlorotryptophan (Fig. 6). Several tryptophan and indole derivatives are also chlorinated by the halogenase, although chlorination of these substrates is at C-2 or C-3 of the indole ring, not C-7 (Hölzer *et al.* 2001).

Halogenase genes have also been found in the biosynthetic gene clusters of balhimycin, pyoluterin and chloroeremomycin (van Wageningen *et al.* 1998; Nowak-Thompson *et al.* 1999; Puk *et al.* 2002), and most recently a halogenase gene fragment was cloned from *Streptomyces venezuelae*, which produces chloramphenicol (Piraee and Vining 2002). Thus, this class of enzyme appears to play a central role in biological chlorination reactions. The sequence of the *prnC* gene, which codes for the second halogenating enzyme in pyrrolnitrin biosynthesis, does not have any homology with

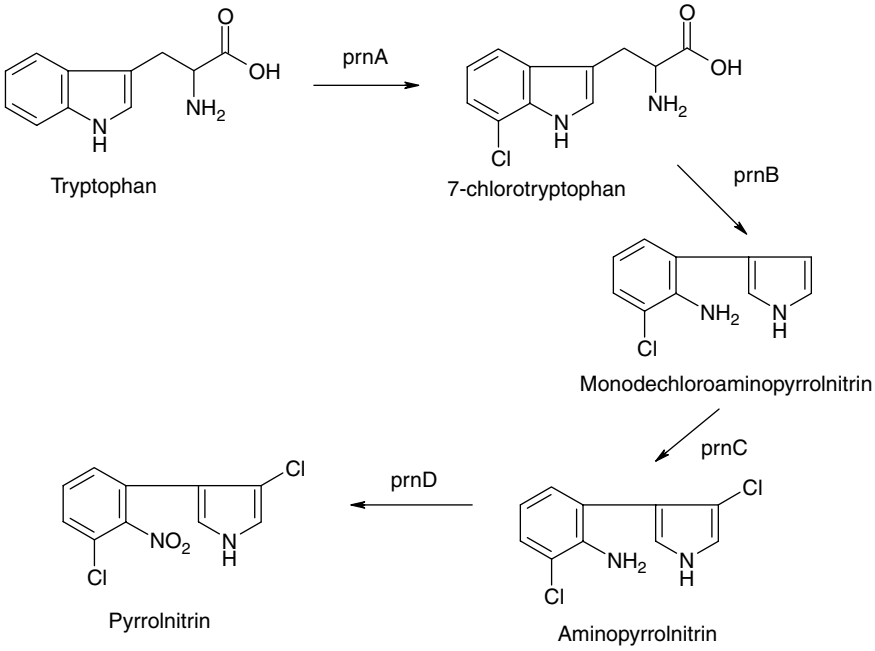

Fig. 5 Biosynthetic steps to pyrrolnitrin in *Pseudomonas fluorescens* (Kirner *et al.* 1998)

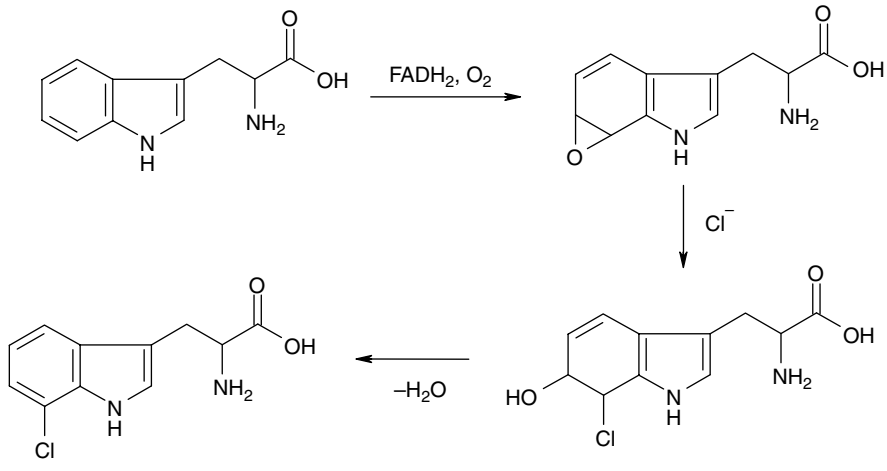

Fig. 6 Regiospecific chlorination of tryptophan by a $FADH_2$-dependent halogenase

prnA, but does have homology with the gene coding for the chlorinating enzyme in chlortetracycline biosynthesis. However, no other cell-free halogenase activity has yet been reported.

3.3 Methyl transferases

Halomethanes, in particular chloromethane, are known to be produced by fungi, algae and higher plants. Investigations of cell-free extracts have led to the identification of methyl transferase enzymes (Wuosmaa and Hager 1990; Saxena *et al.* 1998), which transfer a methyl group from *S*-adenosylmethionine (SAM) to a chloride, bromide or iodide ion (Fig. 7). Some of these enzymes are quite labile, making purification and characterization difficult, but kinetic measurements indicate that the preference of halides is $I^- > Br^- > Cl^-$, although the concentration of halide ions in the environment probably determines the proportions of the halomethanes eventually produced by the organism. It is thought that the biosynthesis of halomethanes may regulate the concentrations of halide ions in algae (Itoh *et al.* 1997) or contribute to halotolerance adaptations in plants (Ni and Hager 1998). In the wood rotting fungus *Phellinus pomaceus* it has been demonstrated that chloromethane is used biosynthetically in the methylation of aromatic acids, thus the biosynthesis of chloromethane in this organism from SAM is somewhat counter-intuitive (Harper *et al.* 1989).

An interesting halomethane: bisulphide/halide ion methyltransferase has been isolated from the bacterium CC495, which can use chloromethane as a sole carbon and energy source (Coulter *et al.* 1999). The enzyme has a corrinoid-bound cobalt atom and uses halomethanes as methyl donors and halide ions as methyl acceptors, thus the enzyme has transhalogenating activity. Bisulphide also acts as a methyl acceptor, but the methanethiol formed is not a methyl donor, thus *in vivo* the enzyme probably converts chloromethane to methanethiol, which can then be oxidized by methanethiol oxidase. Unlike other corrinoid-containing enzymes, the transhalogenase is not sensitive to light and the purified enzyme is quite robust, as it can be frozen and lyophilized without significant loss of activity. One possible application of this enzyme is in the measurement in biological samples of small amounts of halide ion that may not be detectable by ion-selective electrodes because of the presence of interfering compounds (Wang *et al.* 1994). For

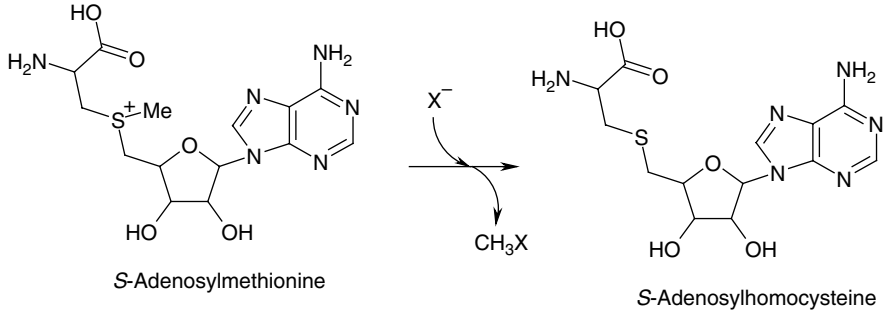

Fig. 7 Reaction catalysed by *S*-adenosylmethionine: halide ion methyl transferase

$X^- = Cl^-, Br^-$ or I^-

example, in the presence of a large excess of chloromethane the transhalogenase enzyme could volatilize bromide or iodide ions. Sensitive GC or GC/MS techniques could then determine the concentration of bromomethane or iodomethane in the headspace.

3.4 Fluorinase

Although the amount of fluorine in the earth's crust is greater than that of the other halogens, most of it is biologically unavailable because of the low solubility of fluorine-containing minerals. Additionally, the high heat of hydration of the fluoride ion means that in aqueous solution it is a poor nucleophile and the relatively high redox potential required for oxidation of fluoride ion prevents incorporation of fluorine via the haloperoxidase reaction. Consequently, fluorinated natural products are rare in nature, with only a handful of such compounds known (Fig. 8), which are produced by some higher plants growing in tropical and subtropical regions, and two streptomycetes.

Fluorine is the most electronegative element in the periodic table and has a van der Waals radius similar to hydrogen (Silvester 1993). Incorporating fluorine into an organic compound can alter its electronic properties without substantial steric effects and fluorinated compounds have different biological activities than their non-fluorinated analogues. Thus many pharmaceutical compounds contain fluorine, such as the anticancer drug fluorouracil, the serotonin uptake inhibitor fluoxetine (Prozac®, Eli Lilly) and fluoroquinolone antibiotics such as ciprofloxacin. However, specific fluorination of organic compounds with chemical methods requires corrosive reagents, such as HF, and often results in polyfluorinated products. Hence, an enzyme that catalyses the formation of C–F bonds under relatively mild conditions would be a very useful biocatalyst. Enzymatic formation of C–F bonds is possible with active-site mutants of glycosidase enzymes, where a nucleophilic glutamate residue is replaced with glycine, alanine or serine. The requirement for a nucleophile in the active site is satisfied by fluoride at high concentrations (2 M), but the glycosyl fluorides formed are transitory (Zechel *et al.* 2001).

The first report of a fluorometabolite was in the South African plant *Dichapetalum cymosum* nearly 60 years ago (Marais 1944). However, progress in understanding the biosynthesis of fluorine-containing natural products was limited for some time because of difficulties in obtaining fresh supplies of fluorometabolite-producing plants and the inconsistency of fluoroacetate biosynthesis by plant tissue cultures. The finding that the bacterium *Streptomyces cattleya* produces fluoroacetate and 4-fluorothreonine (Fig. 8) as secondary metabolites by Sanada *et al.* (1986) provided a more convenient system to study the biochemistry and enzymology of C–F bond formation. The biosynthetic pathway to the fluorometabolites in *S. cattleya* was

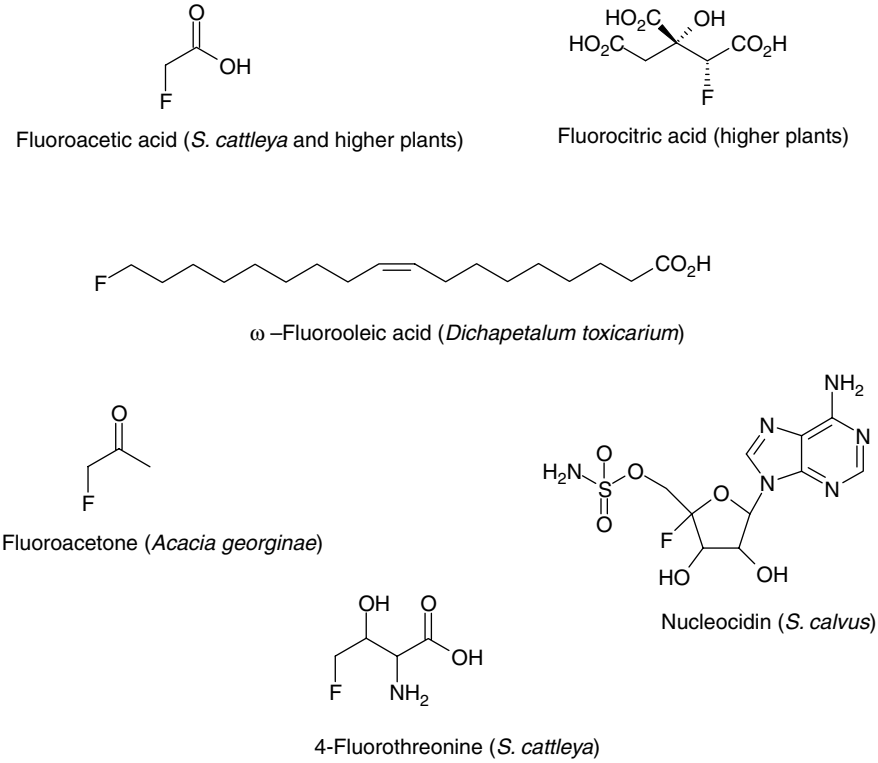

Fig. 8 The known fluorinated natural products

initially investigated by feeding radioisotope-labelled putative precursors of the fluorination reaction to resting cell cultures and measuring the specific radioactivity in fluoroacetate (Reid *et al.* 1995; Tamura *et al.* 1995), but the results of these experiments were conflicting, making it difficult to draw conclusions. A greater understanding of the biosynthetic origin of the fluorometabolites was achieved with feeding experiments using stable isotope labelled precursors (Hamilton *et al.* 1998). The isotopic enrichments of fluoroacetate and 4-fluorothreonine from almost all of the precursors examined were very similar, strongly suggesting that the C-3 and C-4 atoms of 4-fluorothreonine and both carbon atoms of fluoroacetate originated from a common precursor, which was subsequently identified as fluoroacetaldehyde (Moss *et al.* 2000). Fluoroacetaldehyde is oxidized to fluoroacetate by a specific NAD^+-dependent aldehyde dehydrogenase (Murphy, Moss and O'Hagan 2001) and 4-fluorothreonine is formed from fluoroacetaldehyde and threonine in a reaction mediated by an unusual pyridoxal phosphate-dependent transaldolase enzyme (Murphy, O'Hagan and Schaffrath 2001). Most significantly, the enzyme that catalyses the formation of the C–F bond in *S. cattleya* has very recently been identified. When cell-free extract was incubated with fluoride and SAM, an intermediate usually associated with biological methylation, organofluorine compounds were identified (O'Hagan *et al.* 2002). The initial product of fluorination was identified as 5′-fluoro-5′-deoxyadeonsine by GC/MS and ^{19}F-NMR and when this compound was prepared synthetically and added to cell-free extracts, fluoroacetate was formed. The known steps of the biosynthetic pathway to the fluorometabolites in *S. cattleya* are shown in Fig. 9. It appears that the fluorination reaction proceeds by nucleophilic attack by fluoride on the C-5′ of SAM, displacing methionine. As fluoride is such as poor nucleophile in aqueous solution, it is likely that water is excluded from the active site. It seems beyond coincidence that two of the halogenating enzymes that are currently known utilize SAM as a carbon substrate, and it will be very interesting to compare the amino acid sequences of these enzymes to evaluate their similarities. Work is currently underway to purify and characterize the fluorinase and an immediate application of the enzyme is in the generation of nucleosides labelled with the radioactive isotope of fluorine, ^{18}F, which could be used in positron emission tomography (PET) studies to assess tumour proliferation (Kim *et al.* 1996).

The other known fluorometabolite from a bacterium is the antibiotic nucleocidin (Fig. 7), which was isolated from *Streptomyces calvus* (Thomas *et al.* 1957). Previously it was thought that since there was no structural similarity between fluoroacetate and nucleocidin, different fluorinating enzymes were present in the two bacteria (Harper and O'Hagan

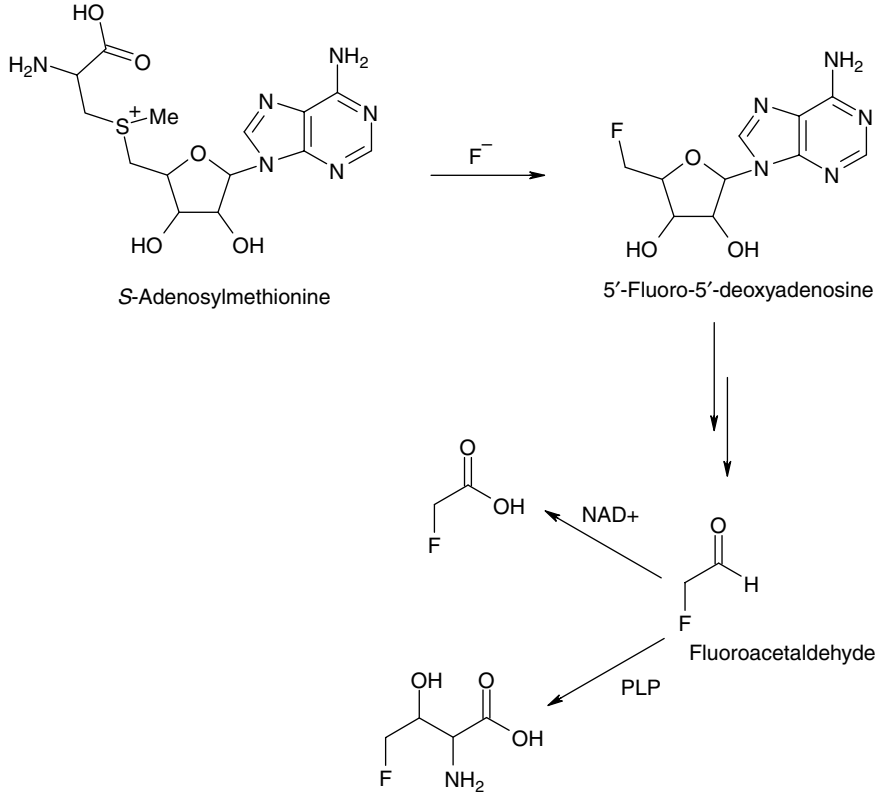

Fig. 9 The biosynthesis of fluoroacetic acid and 4-fluorothreonine in *S. cattleya*

1994). However, the structure of the initial fluorinated intermediate in *S. cattleya*, 5′-fluoro-5′-deoxyadenosine, is quite similar to that of nucleocidin, thus the fluorination reaction in *S. calvus* may be similar to the reaction in *S. cattleya*. Unfortunately, attempts to re-isolate nucleocidin from cultures of *S. calvus* have been unsuccessful (Maguire *et al.* 1993), possibly as a result of the freeze drying methods employed to preserve the culture, so further studies on the biosynthesis of this fluorometabolite may require the organism to be re-isolated from the environment.

Interestingly, the discoveries of bacteria that biosynthesize fluorinated compounds were purely fortuitous and the consequence of fluoride impurities in the culture medium. It is very likely that other microorganisms have similar biosynthetic capabilities and a screening programme for such compounds would therefore be worthwhile.

4. OUTLOOK

The perceived importance of haloperoxidase enzymes in biohalogenation has diminished in recent years and the recent findings of new types of halogenating enzymes, although significant, illustrate how little is currently known about biological halogenation reactions. Undoubtedly more such enzymes are waiting to be discovered and further work is required to characterize the enzymes that are currently known. Further research into biological halogenation in general will also provide a greater understanding of halogenated compounds in the environment and provide for more informed decisions regarding the regulation of the anthropogenic production and release of such compounds.

The use of the recently discovered halogenases in biotechnological applications has only begun to be investigated, and by employing techniques such as directed evolution it may be possible to develop biocatalysts that will regiospecifically halogenate a range of substrates in either aqueous or non-aqueous conditions. This principal has already been demonstrated with the *C. fumago* chloroperoxidase where mutant enzymes have been developed with enhanced halogenating activity in a toluene/isopropanol/water microemulsion system (Rai *et al.* 2001). Furthermore, the genes coding for halogenating enzymes may be used to generate recombinant organisms that produce halogenated derivatives of valuable natural products, for example antibiotics, with altered biological properties. The biotechnological prospects for biohalogenation would therefore appear very bright.

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