Maurice C.R. Franssen

Potential Application of Haloperoxidases in Organic Chemistry

Abstract

Haloperoxidases are enzymes which are able to chlorinate, brominate or iodinate organic compounds at the expense of the corresponding halide ion and hydrogen peroxide. All haloperoxidases isolated thus far produce hypohalous acid (HOX), which is the actual halogenating agent. Therefore, no regio- or stereoselective halogenation reactions have been observed with these enzymes. The nonheme haloperoxidases are extremely stable towards hydrogen peroxide, HOX, elevated temperatures and organic solvents. The heme enzymes are much more labile, but show significant selectivity in the halide-independent oxidation of sulphides to sulphoxides, and alkenes to epoxides. As soon as enzymes are isolated or developed which combine the selectivity of heme-containing peroxidases with the stability of nonheme haloperoxidases, large-scale applications will follow very soon.

Introduction

The use of enzymes as catalysts in organic reactions has become common practice in chemical laboratories nowadays and is also gaining increasing industrial interest (Poppe and Novak, 1992; Faber and Franssen, 1993; Faber, 1995). In order to be useful to the organic chemist, enzymes have to meet the following criteria:

1. They have to be regio- and/or stereoselective;
2. They should be stable for a prolonged period under storage conditions and under turnover conditions, even at elevated temperatures or in the presence of organic solvents;
3. They should display high turnover (fast reactions);
4. They should be easily available, either commercially, or through a microorganism that is easy to cultivate.

Many hydrolases, like proteases, lipases and esterases fulfil these requirements and are therefore the enzymes which are applied most frequently at the moment.

For example, the kinetic resolution of the C3-isomers of methyl 2-methoxytetrahydrofuran-3-carboxylate (**1**, see Scheme 1) has been performed on a preparative scale using the commercially available lipase of *Candida rugosa*. The enzyme accepts only the 3S-isomers of the mixture and the product is formed with an e.e. of more than 98% (Franssen *et al.*, 1996). The reaction was run in octane as a solvent. Since a very crude lipase preparation is used, the reaction rate per mg biocatalyst is rather low but this is compensated for by the low price of the enzyme. Furthermore, the enzyme can be used repetitively under these conditions (Franssen *et al.*, 1992). Compound **2** is a building block for some naturally occurring insect antifeedants.

Enzymes which would be able to incorporate halogen atoms in organic compounds, using halide ions as the source of F, Cl, Br and I, would definitely be of interest to the organic chemist. The reasons for this are threefold:

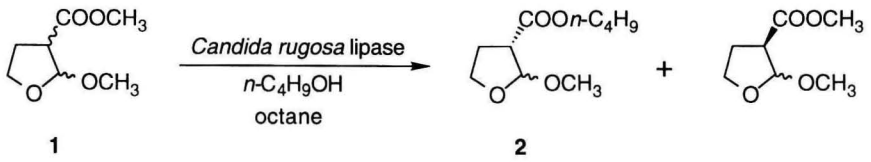
1. Halogen atoms are mostly easy to replace by nucleophiles, making halogen-containing compounds useful intermediates in synthetic routes;
2. Halogenated compounds have, in many cases, enhanced physiological activity when compared to their parent compounds;
3. The use of fluoride, chloride, bromide and iodide ions is strongly preferred above molecular fluorine, chlorine, bromine and iodine, both from a technical and from an environmental point of view.

Three kinds of halogenating enzymes have been described thus far: an S-adenosyl methionine: halide methyl transferase (Wuosmaa and Hager, 1990; Harper, these proceedings), haloperoxidases (Neidleman and Geigert, 1986; Franssen and van der Plas, 1992; Franssen, 1994) and NADH-dependent halogenating enzymes (Van Pée, these proceedings).

The first enzyme catalyses the transfer of a methyl group of SAM to chloride, bromide or iodide ions. It has been isolated from fungi and terrestial plants. The enzyme reacts according to the equation (1):

$$SAM + X^- \rightarrow SAHC + CH_3X \qquad (1)$$

This enzyme is only able to produce methyl halides and is therefore of very limited preparative value. Since the NADH-dependent halogenating biocatalysts will be dealt with elsewhere, the remainder of this paper will be devoted to haloperoxidases.



1 2

Scheme 1. Example of a lipase-mediated preparation of a chiral building block for natural products.

Haloperoxidases

Haloperoxidases are enzymes which are able to halogenate organic compounds using halide ions and hydrogen peroxide as substrates. The general reaction equation reads as follows:

$$AH + H_2O_2 + H^+ + X^- \rightarrow AX + 2H_2O \quad (2)$$

In eq. (2), AH stands for an organic substrate and AX for the halogenated product. X^- can be chloride, bromide or iodide, depending on the enzyme; fluoride is never accepted as a substrate. The enzymes are called chloro-, bromo- or iodoperoxidases, depending on the smallest halide ion they are able to oxidise.

Haloperoxidases are ubiquitous enzymes: they have been found in many different classes of organisms, as can be concluded from the brief overview given in Table 1 (Franssen, 1994).

The wide occurrence of haloperoxidases in Nature indicates that these enzymes are very important, if not essential, for life on Earth. However, the exact role of many haloperoxidases is unknown. Most probably, these enzymes are involved in the defence mechanism of their owners, since a large number of living species produce halogenated compounds (so-called halometabolites) which are more or less toxic. Alternatively, the mammalian chloroperoxidases called myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are involved in the production of the antimicrobial agents hypohalous acid (acc. to eq. (3)) and singlet oxygen (acc. to eq. (4)):

Table 1. Some sources of haloperoxidases.

name	source		prosthetic group
chloroperoxidases			
chloroperoxidase	*Caldariomyces fumago*	(mold)	heme[1]
"	*Curvularia inaequalis*	(mold)	vanadium
"	*Pseudomonas pyrrocinia*	(bacterium)	none[2]
myeloperoxidase	white blood cells	(vertebrates)	heme
bromoperoxidases			
bromoperoxidase	*Streptomyces aureofaciens*	(bacterium)	none[2]
"	*Penicillus capitatus*	(green alga)	heme
"	*Corallina pilulifera*	(red alga)	vanadium
"	*Ascophyllum nodosum*	(brown alga)	vanadium
"	*Xanthoria parietina*	(lichen)	vanadium
lactoperoxidase	milk, saliva, tears	(mammals)	heme
iodoperoxidases			
horseradish peroxidase	horseradish roots	(higher plant)	heme
thyroid peroxidase	thyroid glands	(vertebrates)	heme

[1]heme = ferriprotoporphyrin IX; [2]the enzyme contains a catalytic triad

$$H_2O_2 + X^- + H^+ \xrightarrow{\text{MPO, EPO}} HOX + H_2O \quad (3)$$

$$HOX + H_2O_2 \xrightarrow{\text{spontaneous}} {}^1O_2 + H_2O + H^+ + X^- \quad (4)$$

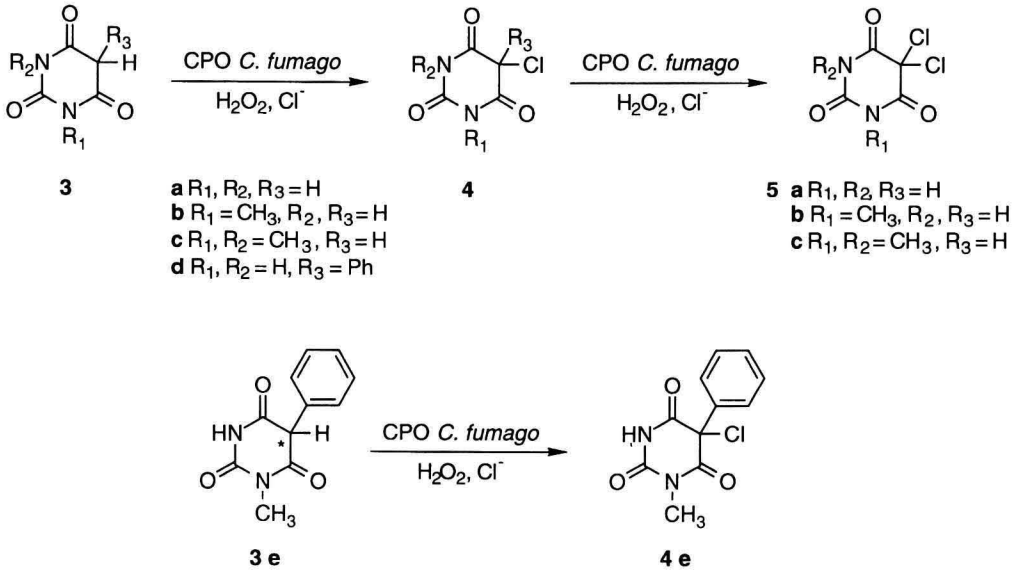
Haloperoxidases may also have other physiological roles, e.g. thyroid peroxidase is involved in the biosynthesis of the iodinated hormone thyroxin.

Of course, the lack of knowledge of the natural function of most haloperoxidases does not preclude their application in organic synthesis, for many enzymes in industrial or university laboratories are being used for the conversion of non-natural compounds. In the following sections, the state of affairs concerning the application of haloperoxidases as catalysts in organic synthesis will be presented. The examples are clustered by the prosthetic group of the enzymes.

Halogenation reactions

Heme enzymes

Halogenation reactions with heme-containing haloperoxidases have mainly been carried out using the CPO[1] from the mould *Caldariomyces fumago*, although

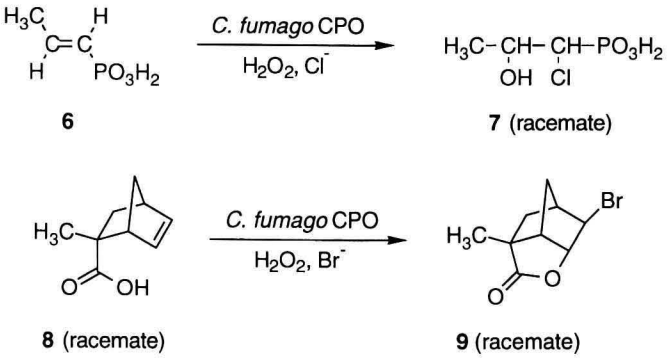


Scheme 2. Conversions of barbituric acid derivatives by the chloroperoxidase from *C. fumago* (Franssen and van der Plas, 1987).

[1] CPO = chloroperoxidase, BPO = bromoperoxidase, HRP = horseradish peroxidase

recently some interesting work using lactoperoxidase, a mammalian bromoperoxidase, has been published (Ishihara *et al.*, 1995). The *C. fumago* CPO is commercially available, though expensive. However, *C. fumago* is easy to cultivate, it produces the enzyme in copious amounts and a reliable prescription for the isolation of enzyme has been published (van Deurzen *et al.*, 1994).

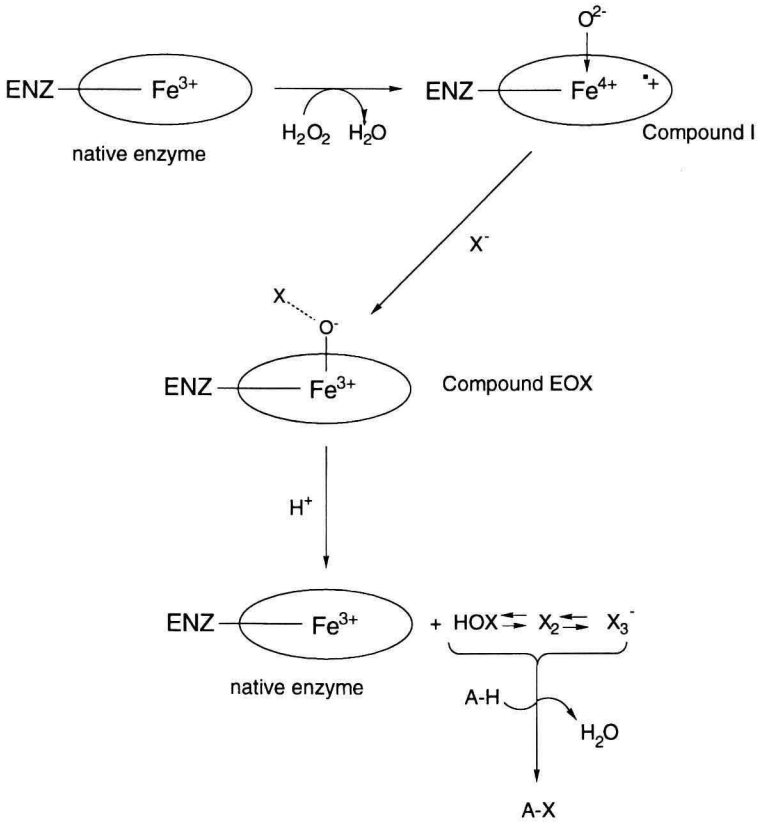
A large number of halogenation reactions using the *C. fumago* CPO have been published (see for reviews: Neidleman and Geigert, 1986; Franssen and van der Plas, 1992; Franssen, 1994). During the 1980s, we have been working on the CPO-mediated chlorination of barbituric acid and its derivatives (**3**, see Scheme 2). These compounds are smoothly converted into their monochloro derivatives (**4**) and, in case of **4a–c**, into their dichloro derivatives **5a–c**, in high yields. In order to investigate the stereochemical course of this reaction, racemic **3e** was subjected to CPO-mediated chlorination. However, the product **4e** did not show any optical rotation, indicating that the enzymatic reaction was not stereoselective (Franssen and van der Plas, 1987). The same conclusion was reached by Kollonitsch *et al.* (1970) for the formation of the chlorohydrin of propenylphosphonic acid (**6**, see Scheme 3) and by Ramakrishnan *et al.* (1983) for the bromolactonisation of the bicyclic acid **8**.



Scheme 3. Examples of (unselective) halogenation reactions, catalysed by the chloroperoxidase from *C. fumago*.

The background of this lack of selectivity was unraveled by Libby *et al.* (1989, 1992), who showed by careful kinetic studies that the *C. fumago* CPO produces HOX/X_2 as the ultimate product. The complete reaction scheme for the halogenation of organic compounds by this enzyme (and other heme-containing haloperoxidases) is shown in Scheme 4.

Despite the lack of selectivity, heme-containing haloperoxidases can still be useful to the organic chemist. These enzymes produce the very reactive species HOX in a relatively slow manner, in a homogeneous, diluted solution. Therefore, yields obtained in haloperoxidase-mediated halogenation reactions are often higher than halogenation reactions where diluted HOX is added to the substrate. Compare, for example, the reaction of *C. fumago* CPO with pyrazole and two derivatives thereof (**10**) to the chlorination of these compounds by

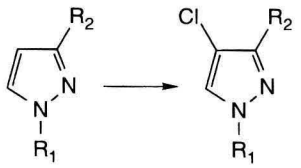


Scheme 4. Halogenation mechanism of heme-containing haloperoxidases. The protein part of the enzyme is represented by ENZ, the heme group is depicted as Fe^{x+} (x = 3 or 4) in the center of an ellipse, for reasons of clarity. Compound I contains an Fe^{4+} species and a radical cation which is situated in the heme moiety. A-H = organic substrate, A-X = halogenated product, X = Cl, Br or I.

adding diluted HOCl (Franssen *et al.*, 1987). As can be concluded from the data in Table 2, the yields of the CPO-mediated reactions are significantly higher.

It should be stressed here that the *only* way to find whether a haloperoxidase-mediated reaction is regio- or stereoselective, is to compare the product outcome to the products obtained by very slow addition of 1-2 eq. of HOX to the substrate *under the same reaction conditions*, i.e. in the same buffer, using the same concentration of substrates (including H_2O_2), and at the same temperature. The yield of the product(s) might differ from the enzymatic reaction, as shown above. However, *the structure of the product(s) in the enzymatic reaction should be different from that of the HOX-mediated reaction in order to make a claim for a selective haloperoxidase-mediated reaction justified.* Comparing the enzymatic reaction to reactions of HOX or other halogenating agents in pure water, organic solvents or mixtures thereof (Coughlin *et al.*, 1993) does not make sense,

Table 2. Yields of the chlorination reaction of pyrazole (**10a**) and its 1- and 3-methyl derivatives (**10b,c**), mediated by *C. fumago*

CPO or by HOCl[a].	substrate	yield (%) CPO, H_2O_2, Cl^-	HOCl
 **10** **11**	**10a** (R_1, R_2 = H)	68	20
	10b (R_1 = CH_3, R_2 = H)	83	68
	10c (R_1 = H, R_2 = CH_3)	91	67

[a]Taken from Franssen *et al.*, 1987.

since the reaction pattern of HOX is strongly dependent on the solvent (M.C.R. Franssen, unpublished results).

A disadvantage of heme-containing haloperoxidases is their limited stability towards elevated temperatures, towards their substrate hydrogen peroxide and product HOX, and towards organic solvents. Especially the vulnerability towards H_2O_2 and HOX poses serious problems; e.g., in case of *C. fumago* CPO the concentration of H_2O_2 should be kept below ± 2 mM in order to prevent inactivation of the enzyme due to bleaching of the heme group (Itoh *et al.*, 1987; Liu *et al.*, 1987). Care should be taken that there is always enough organic substrate present to react with the HOX as soon as it is formed. The optimum pH for this enzyme is 2.7, which might be a problem for the conversion of acid-labile substrates. Water-miscible organic cosolvents can be used without problems only up to 10% v/v in case of methanol and DMSO (Cooney and Hueter, 1974), although halide-independent oxidation reactions can be run in 30% *t*-butyl alcohol (van Deurzen *et al.*, 1994).

In summary, heme-containing haloperoxidases possess the following features:

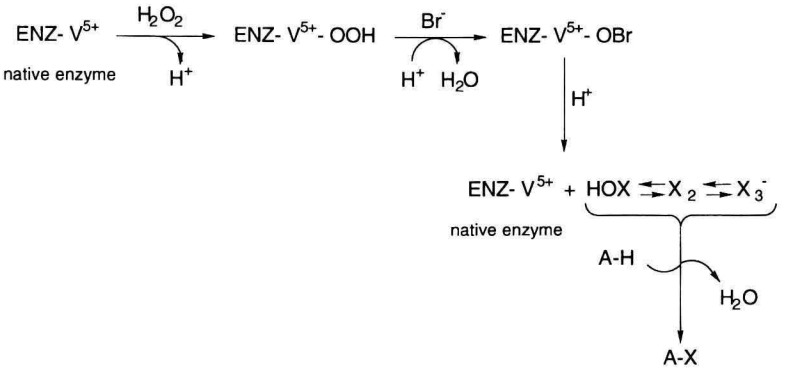
1. They catalyse the smooth, yet unselective halogenation of organic compounds, producing hypohalous acid (HOX) as the active halogenating species;
2. Their rate of reaction is moderate, except for the CPO from *C. fumago*, which is an extremely active enzyme ($k_{cat} = \pm 1000\ s^{-1}$);
3. They are not stable against ambient concentrations of their substrate (H_2O_2) and their product (HOX), as well against elevated temperatures and higher concentrations of organic solvents;

4. Several enzymes are commercially available, although they are rather expensive. However, *C. fumago* is easy to cultivate and its CPO is easy to isolate.

Vanadium enzymes

As pointed out in Table 1, many haloperoxidases are known to date which possess vanadium as the prosthetic group. The BPO from the brown alga *Ascophyllum nodosum* was the first vanadium-containing haloperoxidase which was purified to homogeneity (De Boer *et al.*, 1986). This enzyme has been tested with the barbituric acids which were already listed in Scheme 2. Just as with the CPO from *C. fumago*, 1-methyl-5-phenylbarbituric acid (**3e**) was not brominated in a stereoselective way (Franssen *et al.*, 1988). From this work and the extensive kinetic investigations in the group of Wever (De Boer and Wever, 1988; van Schijndel *et al.*, 1994) it became clear that vanadium haloperoxidases also produce HOX as the active halogenating agent, which precludes any regio- or stereoselective reaction of these enzymes. The simplified reaction sequence is depicted in Scheme 5.

Although the vanadium enzymes are just as unselective in halogenation reactions as the heme haloperoxidases, they have one distinct advantage: they are, without exception, extraordinary stable. E.g., incubation of the CPO from the fungus *Curvularia inaequalis* with 0.5 mM HOCl for 2 min still gave 90% residual activity, whereas the *C. fumago* CPO was completely inactivated in that period under the same conditions. The *Curv. inaequalis* enzyme does not loose any activity when treated with 200 mM H_2O_2 for 25 h; the *C. fumago* CPO is already completely inactive after 2 min. under those conditions (Liu *et al.*, 1987). Incubation of this *Curv. inaequalis* vanadium CPO at 80°C resulted in an initial decrease in activity of about 20% after which the enzyme remains stable for 6.5 h. Enzyme activity is not inhibited by guanidine/HCl until 2 M, and the



Scheme 5. Halogenation mechanism of vanadium-containing haloperoxidases. The protein part of the enzyme is represented by ENZ, for reasons of clarity. A-H = organic substrate, A-X = halogenated product, X = Cl, Br or I.

enzyme activity also remains constant when organic cosolvents like methanol are added until 40% v/v (Van Schijndel *et al.*, 1994). The same phenomena were observed with the vanadium-containing CPO from the red seaweed *Corallina officinalis* (Sheffield *et al.*, 1992; Rush *et al.*, 1995). The turnover of these enzymes is quite acceptable.

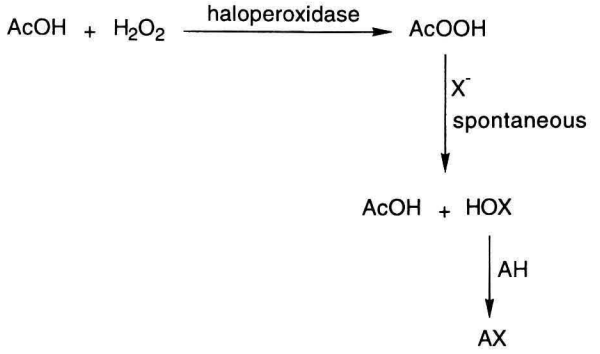
Unfortunately, only one of these interesting enzymes is commercially available (for an outrageous price), namely the above-mentioned CPO from *Corallina officinalis*. Vanadium haloperoxidases are not difficult to isolate, but definitely not so easy as the *C. fumago* CPO.

Summarising, the characteristics of vanadium-containing haloperoxidases are:

1. They catalyse the smooth, yet unselective halogenation of organic compounds, *via* enzymatically produced HOX;
2. Their turnover is rather high, k_{cat} ranging from 10–50 s^{-1};
3. They possess excellent stability towards H_2O_2, HOX, organic solvents and high temperatures;
4. Only one vanadium-containing haloperoxidase is commercially available, and very expensive; isolation of these enzymes from their natural sources is not difficult.

Nonheme, nonvanadium enzymes

Several bacterial haloperoxidases have been isolated, which do not possess heme or vanadium as an essential constituent. Very recently, it was shown that the BPOs from *Streptomyces aureofaciens* strains ATCC10762 and Tü24, as well the CPOs from *S. lividans*, *Pseudomonas pyrrocinia* and *P. fluorescens* possess a catalytic triad (Pelletier *et al.*, 1995). For all these enzymes, acetate is essential for their halogenating activity, which brought the authors to the tentative reaction mechanism as depicted in Scheme 6. The catalytic triad in the enzyme active



Scheme 6. Tentative reaction mechanism for the halogenation of organic compounds as catalysed by the nonheme, nonvanadium haloperoxidases. A-H = organic substrate, A-X = halogenated product, X = Cl or Br.

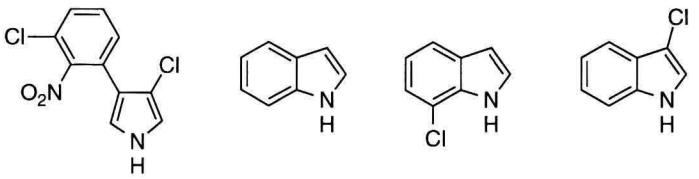
site is responsible for the reaction of acetic acid with hydrogen peroxide, forming peracetic acid. This reaction is well known for lipases (Björkling *et al.*, 1990). The peracetic acid on its turn reacts with halide ions, presumably in a spontaneous reaction, outside the enzyme active site. This would, again, yield HOX as the active halogenating agent.

These findings shed new light on the papers which have appeared on the chlorination of indole by the CPO from the bacterium *Pseudomonas pyrrocinia.* This bacterium produces pyrrolnitrin (**12**, see Scheme 7), and it was thought that this CPO was involved in its biosynthesis. According to two papers from the group of van Pée (Wiesner *et al.*, 1986; van Pée, 1990), the *P. pyrrocinia* CPO converts indole (**13**) into 7-chloroindole (**14**) upon incubation with H_2O_2 and chloride ions. This would be a regioselective reaction, since the pyrrole ring in indole is much more reactive than the benzene ring. However, recent investigations have shown that not 7-chloro-, but *3*-chloroindole (**15**) is the actual reaction product (K.-H. van Pée, personal communication), which is in line with the production of HOCl by this CPO as described above. The actual enzyme which is involved in the conversion of tryptophane into 7-chloro-tryptophane, the first step in the biosynthesis of pyrrolnitrin (**12**), appears not to be a haloperoxidase (K.-H. van Pée, these proceedings).

Nonheme nonvanadium enzymes are just as stable as their vanadium counterparts, but their activity is low (1–5 s^{-1} for standard substrates, $\pm$ 0.2 s^{-1} for indole). None of these enzymes is yet commercially available, but that will change in the near future (K.-H. van Pée, personal communication).

In summary, the following can be said about the nonheme, nonvanadium enzymes:

1. Most likely, they produce HOX as the active halogenating species, just like all the other haloperoxidases. However, the mechanism by which they produce HOX is most extraordinary;
2. Their reaction rates are lower than those of other haloperoxidases;
3. Their stability is excellent, comparable to the vanadium enzymes;
4. None of these enzymes is commercially available yet.



12 **13** **14** **15**

Scheme 7. The chemical structures of pyrrolnitrin (**12**), indole (**13**), 7-chloroindole (**14**) and 3-chloroindole (**15**).

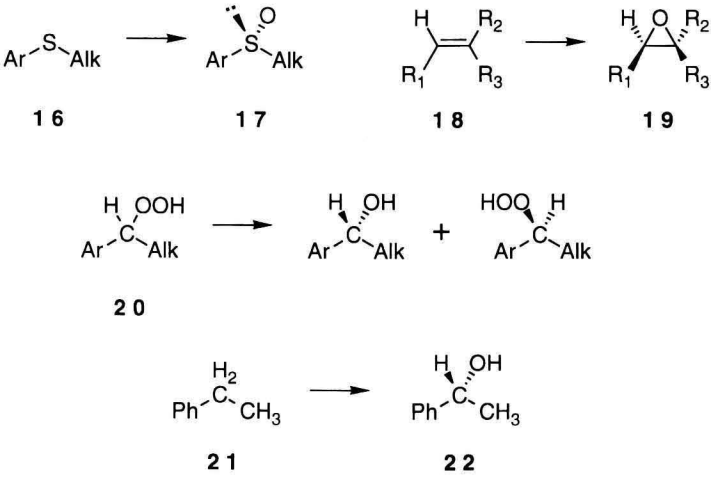
Oxidation reactions

One might get the impression that haloperoxidases are completely unselective enzymes and therefore of no commercial use. This is not true, as it has been pointed out above that they can be beneficial as mild alternatives of molecular halogens (X_2) or HOX. Besides this, there are some very selective *oxidative* reactions known of these enzymes. These reactions are examples of the so-called "classical" oxidation reactions (oxidation of X^- to X^+-species being nonclassical oxidation), which are restricted to the heme-containing haloperoxidases like horseradish peroxidase (HRP) and, in particular, the *C. fumago* CPO. Recently, a truncated cytochrome c known as microperoxidase-11, was also shown to be able to carry out stereoselective oxidation reactions (Colonna *et al.*, 1994).

These oxidation reactions comprise the direct, stereoselective transfer of the ferryl-oxygen atom in Compound I (see Scheme 4) to the organic substrate. Very high enantiomeric excesses have been obtained in the conversion of aryl alkyl sulphides (**16**, see Scheme 8) to chiral sulphoxides (**17**) (Colonna *et al.*, 1992; van Deurzen *et al.*, 1994), the epoxidation of alkenes (**18** → **19**) (Allain *et al.*, 1993; Dexter *et al.*, 1995; Lakner and Hager, 1996), the kinetic resolution of hydroperoxides (**20**) (Fu *et al.*, 1992; Hoeft *et al.*, 1995) and the stereoselective hydroxylation of alkylbenzenes (**21** → **22**) (Zaks and Dodds, 1995).

In general, the heme-containing haloperoxidases possess the following features when used as *oxidation* catalysts:

1. They are highly selective catalysts for the preparation of homochiral sulphoxides, epoxides, hydroperoxides and benzylic alcohols, the CPO from *C. fumago* being the best;
2. The reactions are catalysed in a rather slow manner (k_{cat} is ± 0.5–$1\ s^{-1}$);



Scheme 8. Examples of stereoselective oxidation reactions displayed by heme peroxidases.

3. Enzyme stability is low, as pointed out before. This is especially a problem in these oxidation reactions, since high substrate concentrations are needed in order to achieve acceptable reaction rates. Irreversible inactivation by H_2O_2 is frequently encountered;

4. The enzymes are expensive, although the *C. fumago* CPO is easy to obtain from its culture broth, as mentioned earlier.

General conclusions and outlook

We have seen in this review that there are two different combinations of properties in haloperoxidase-catalysed reactions:

1. There are *very stable* enzymes (vanadium haloperoxidases) which carry out the smooth, yet *unselective halogenation* of a large variety of organic substrates;
2. There are relatively *unstable* enzymes (heme peroxidases) which perform the *very selective oxidation* of organic substrates.

The question now arises: how can we combine the stability of the nonheme enzymes with the selectivity of the heme enzymes, in other words: how can we obtain stable enzymes which catalyse selective halogenation reactions?

In my opinion, there are three options to do this.

1. Increase the selectivity of the stable, active, vanadium haloperoxidases;
2. Increase the stability of heme-containing haloperoxidases;
3. Continue the screening for novel halogenating enzymes which are both stable and selective.

These options are discussed below.

1. Increase the selectivity

Increasing the selectivity of haloperoxidases implies, in practice, either completely changing the catalytic mechanism, or preventing the release of free HOX into the reaction medium. The goal of both strategies is to keep the active, electron-deficient halogen species inside the active site, preferably bound to an amino acid residue, because it is only in this chiral environment that stereoselectivity is to be expected.

There is some evidence for binding of organic substrates to the active site of *Ascophyllum nodosum* BPO (Tschirret-Guth and Butler, 1994). When this binding occurs before or during the formation of the active halogenating agent, some selectivity might be expected (Butler, these proceedings).

Another option is changing the structure of the enzyme. Making HRP more selective in sulphoxidation and epoxidation reactions by site-directed mutagenesis has been shown to be fruitful. The phenylalanine residue at position 41

blocks the active site in HRP but is not essential for activity. Substitution of phe-41 by an alanine makes the heme more accessible. As a result thereof, the mutant F41A is able to epoxidise styrene, which native HRP cannot do (Newmyer and Ortiz de Montellano, 1995). F41L increases the enantioselectivity of sulphoxidation (Ozaki and Ortiz de Montellano, 1994) and broadens the scope of the epoxidation reactions (Ozaki and Ortiz de Montellano, 1995).

Although these site-directed mutagenesis studies seem to be very useful to increase the scope and selectivity of peroxidase-catalysed oxidation reactions, it should be stressed that this approach might be less succesful for the halogenation reactions. One could consider to decrease the dimensions of the active site in order to slow down the release of free HOX, but on the other hand the active site should remain accessible to the organic substrate as well.

2. Increase the stability of heme enzymes

The most vulnerable part of the heme-containing haloperoxidases is the heme function itself. Treatment with too high concentrations of hydrogen peroxide or HOX causes complete and irreversible inactivation of the enzyme due to bleaching of the heme. Protecting the heme edge by site-directed mutagenesis could be a solution for this problem, but care should be taken that the ferric ion should remain accessible. It has recently been shown that reconstitution of the *C. fumago* apo-CPO, expressed in *E. coli*, is feasible at high pressure, albeit with low yield (Zong *et al.*, 1995). This might enable the substitution of the protoporphyrin IX of the native enzyme by a more stable heme group.

Increasing the stability of heme enzymes is absolutely needed for an industrial process (Pickard *et al.*, 1991). Maybe the three-dimensional structures of haloperoxidases can learn us which factors are involved in the (in)stability of these enzymes. The X-ray structure of the very active CPO from *C. fumago* has been solved recently (Sundaramoorthy *et al.*, 1995), as well as the structure of the stable V-CPO *Curvularia inaequalis* (Messerschmidt and Wever, 1996; Messerschmidt, these proceedings) and the stable nonheme nonvanadium CPO from *Streptomyces aureofaciens* (Hecht *et al.*, 1994).

3. Further screening

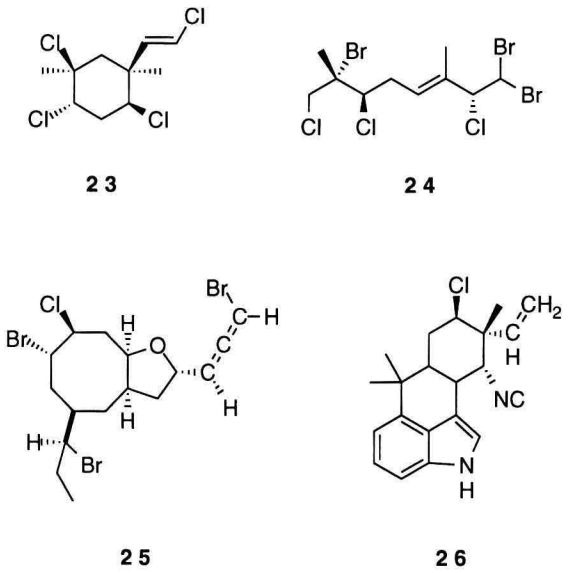
The option which is maybe the least appealing but which might be the most fruitful, is the continued screening for novel halogenating biocatalysts. The background of this option is simple: Nature contains many different halometabolites which contain chlorine or bromine atoms attached to chiral carbon atoms. For some of them, in particular the ones depicted in Scheme 9, it is very difficult to envisage a biosynthetic route *without* the involvement of stereo- and regioselective halogenating enzymes.

If such enzymes really exist, then the question arises why they have not been detected up to now. The reasons may be twofold:

1. The wrong organisms have been taken as a source for halogenating enzymes. E.g., it is known that the brown alga *Ascophyllum nodosum* produces a lot of bromoform (Wever, 1988), which can be easily formed from methyl ketones and HOBr. The formation of bromoform, of course, does not preclude the presence of regio- and/or stereoselective halogenating enzymes but it is does indicate that nonselective enzymes are abundant in this species. Considering the compounds shown in Scheme 9, screening for a halogenating enzyme in *Plocamium* might be much more interesting.

2. The correct organisms have been investigated, but the selective enzymes have not been found because the enzymes were not fed with their natural substrates. It might be that some haloperoxidases react unselectively with artificial substrates like monochlorodimedone and phenol red (De Boer *et al.*, 1987), whereas they react in a selective manner with their natural substrate because they possess a specific binding site for it.

It should be stressed that finding out which enzyme is responsible for the introduction of halogen into halometabolites is not easy, as was demonstrated by the thorough investigations of the group of van Pée on the biosynthesis of chloramphenicol (Facey *et al.*, 1996). Maybe, the selective halogenating enzymes belong to a new, yet undiscovered class of enzymes which do not need hydrogen peroxide as a substrate. An indication for this is presented elsewhere in these proceedings by van Pée.



23 24

25 26

Scheme 9. Halometabolites which contain chlorine or bromine atoms, attached to chiral carbon atoms. Compound **23** is from *Laurencia implicata* (red alga), **24** from *Plocamium sp.* (red alga), **25** from *Plocamium cartalagineum* (red alga) and hapalindole A (**26**) is from the blue-green alga *Hapalosiphon fontinalis*.

In my opinion, the key for selectivity in enzymatic halogenation resides in the chiral halometabolites. Studies on the biosynthesis of compounds like the ones depicted in Scheme 9 will reveal if stereo- and/or regioselective halogenating biocatalysts really exist in Nature.

References

Allain, E.J., L.P. Hager, L. Deng and E.N. Jacobsen, 1993. Highly enantioselective epoxidation of disubstituted alkenes with hydrogen peroxide catalyzed by chloroperoxidase. *J. Am. Chem. Soc.* **115**, 4415–4416.

Björkling, F., S.E. Godtfredsen and O. Kirk, 1990. Lipase-mediated formation of peroxycarboxylic acids used in catalytic epoxidation of alkenes. *J. Chem. Soc. Chem. Commun.*, 1301–1303.

Colonna, S., N. Gaggero, G. Carrea and P. Pasta, 1994. The microperoxidase-11 catalyzed oxidation of sulfides is enantioselective. *Tetrahedron Lett.* **35**, 9103–9104.

Colonna, S., N. Gaggero, L. Casella, G. Carrea and P. Pasta, 1992. Chloroperoxidase and hydrogen peroxide: an efficient system for enzymic enantioselective sulfoxidations. *Tetrahedron: Asymmetry* **3**, 95–106.

Cooney, C.L. and J. Hueter, 1974. Enzyme catalysis in the presence of nonaqueous solvents using chloroperoxidase. *Biotechnol. Bioeng.* **16**, 1045–1053.

Coughlin, P., S. Roberts, C. Rush and A. Willetts, 1993. Biotransformation of alkenes by haloperoxidases: regiospecific bromohydrin formation from cinnamyl substrates. *Biotechnol. Lett.* **15**, 907–912.

De Boer, E. and R. Wever, 1988. The reaction mechanism of the novel vanadium-bromoperoxidase: a steady-state kinetic analysis. *J. Biol. Chem.* **263**, 12326–12332.

De Boer, E., H. Plat, M.G.M. Tromp, R. Wever, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer and H.E. Schoemaker, 1987. Vanadium containing bromoperoxidase, an example of an oxido-reductase with high operational stability in aqueous and organic media. *Biotechnol. Bioeng.* **30**, 607–610.

De Boer, E., Y. van Kooyk, M.G.M. Tromp, H. Plat and R. Wever, 1986. Bromoperoxidase from *Ascophyllum nodosum*: a novel class of enzymes containing vanadium as a prosthetic group? *Biochim. Biophys. Acta* **869**, 48–53.

Dexter, A.F., F.J. Lakner, R.A. Campbell and L.P. Hager, 1995. Highly enantioselective epoxidation of 1,1-disubstituted alkenes catalyzed by chloroperoxidase. *J. Am. Chem. Soc.* **117**, 6412–6413.

Faber, K., 1995. Biotransformations in Organic Chemistry. 2nd Edition, Springer-Verlag, Berlin, 356 pp.

Faber, K. and M.C.R. Franssen, 1993. Prospects for the increased application of biocatalysts in organic transformations. TIBTECH **11**, 461–469.

Facey, S.J., F. Gross, L.C. Vining, K. Yang and K.-H. van Pée, 1996. Cloning, sequencing and disruption of a bromoperoxidase-catalase gene in *Streptomyces venezuelae*: evidence that it is not required for chlorination in chloramphenicol biosynthesis. *Microbiology* **142**, 657–665.

Franssen, M.C.R., 1994. Halogenation and oxidation reactions with haloperoxidases. *Biocatalysis* **10**, 87–111.

Franssen, M.C.R. and H.C. van der Plas, 1987. The chlorination of barbituric acid and some of its derivatives by chloroperoxidase. *Bioorg. Chem.* **15**, 59–70.

Franssen, M.C.R. and H.C. van der Plas, 1992. Haloperoxidases: their properties and their use in organic synthesis. *Adv. Appl. Microbiol.* **37**, 41–99.

Franssen, M.C.R., P.M.A.C. Boavida dos Santos, N.L.F.L. Camacho Mondril and Ae. de Groot, 1992. Enzymatic synthesis of a chiral building block for perhydrofuro[2,3b]furans. *Pure Appl. Chem.* **64**, 1089–1092.

Franssen, M.C.R., J.D. Jansma, H.C. van der Plas, E. de Boer and R. Wever, 1988. Enzymatic bromination of barbituric acid and some of its derivatives. *Bioorg. Chem.* **16**, 352–363.

Franssen, M.C.R., H. Jongejan, H. Kooijman, A.L. Spek, N.L.F.L. Camacho Mondril, P.M.A.C. Boavida dos Santos and Ae. de Groot, 1996. Resolution of a tetrahydrofuran ester by *Candida rugosa* lipase (CRL) and an examination of CRL's stereochemical preference in organic media. *Tetrahedron: Asymmetry* **7**, 497–510.

Franssen, M.C.R., H.G. van Boven and H.C. van der Plas, 1987. Enzymatic halogenation of pyrazoles and pyridine derivatives. *J. Heterocyclic Chem.* **24**, 1313–1316.

Fu, H., H. Kondo, Y. Ichikawa, G.C. Look and C.-H. Wong, 1992. Chloroperoxidase-catalyzed asymmetric synthesis: enantioselective reactions of chiral hydroperoxides with sulfides and bromohydration of glycals. *J. Org. Chem.* **57**, 7265–7270.

Hecht, H.J., H. Sobek, T. Haag, O. Pfeifer and K.-H. van Pée, 1994. The metal-ion-free oxidoreductase from *Streptomyces aureofaciens* has an α/β hydrolase fold. *Nat. Struct. Biol.* **1**, 532–537.

Hoeft, E., H.-J. Hamann, A. Kunath, W. Adam, U. Hoch, C.R. Saha-Moeller and P. Schreier, 1995. Enzyme-catalyzed kinetic resolution of racemic secondary hydroperoxides. *Tetrahedron: Asymmetry* **6**, 603–608.

Ishihara, J., N. Kanoh and A. Murai, 1995. Enzymatic reaction of (3E, 6S, 7S)-laurediol and the molecular modeling studies on the cyclization of laurediols. *Tetrahedron Lett.* **36**, 737–740.

Itoh, N., Y. Izumi and H. Yamada, 1987. Haloperoxidase-catalyzed halogenation of nitrogen-containing aromatic heterocycles represented by nucleic bases. *Biochemistry* **26**, 282–289.

Kollonitsch, J., S. Marburg and L.M. Perkins, 1970. Enzymatic formation of chiral structures in racemic form. *J. Am. Chem. Soc.* **92**, 4489–4490.

Lakner, F.J. and L.P. Hager, 1996. Chloroperoxidase as enantioselective epoxidation catalyst: an efficient synthesis of (R)-(-)-mevalonolactone. *J. Org. Chem.* **61**, 3923–3925.

Libby, R.D., N.S. Rotberg, J.T. Emerson, T.C. White, G.M. Yen, S.H. Friedman, N.S. Sun and R. Goldowski, 1989. The chloride-activated peroxidation of catechol as a mechanistic probe of chloroperoxidase reactions: competitive activation as evidence for a catalytic chloride binding site on Compound I. *J. Biol. Chem.* **264**, 15284–15292.

Libby, R.D., A.L. Shedd, A.K. Phipps, T.M. Beachy and S.M. Gerstberger, 1992. Defining the involvement of hypochlorous acid or chlorine as enzyme-generated intermediates in chloroperoxidase-catalyzed reactions. *J. Biol. Chem.* **267**, 1769–1775.

Liu, T.E., T. M'Timkulu, J. Geigert, B. Wolf, S.L. Neidleman, D. Silva and J.C. Hunter-Cevera, 1987. Isolation and characterization of a novel nonheme chloroperoxidase. *Biochem. Biophys. Res. Commun.* **142**, 329–333.

Messerschmidt, A. and R. Wever, 1996. X-ray structure of a vanadium-containing enzyme: chloroperoxidase from the fungus *Curvularia inaequalis. Proc. Natl. Acad. Sci. USA* **93**, 392–396.

Neidleman, S.L. and J. Geigert, 1986. Biohalogenation: principles, basic roles and applications. Ellis Horwood Ltd., Chichester, 203 pp.

Newmyer, S.L. and P.R. Ortiz de Montellano, 1995. Horseradish peroxidase his-42 → ala, his-42 → val and phe-41 → ala mutants. *J. Biol. Chem.* **270**, 19430–19438.

Ozaki, S.-I. and P.R. Ortiz de Montellano, 1994. Molecular engineering of horseradish peroxidase. Highly enantioselective sulfoxidation of aryl alkyl sulfides by the phe-41 → leu mutant. *J. Am. Chem. Soc.* **116**, 4487–4488.

Ozaki, S.-I. and P.R. Ortiz de Montellano, 1995. Molecular engineering of horseradish peroxidase: thioether sulfoxidation and styrene epoxidation by phe-41 leucine and threonine mutants. *J. Am. Chem. Soc.* **117**, 7056–7064.

Pelletier, I., J. Altenbuchner and R. Mattes, 1995. A catalytic triad is required by the non-heme haloperoxidase to perform halogenation. *Biochim. Biophys. Acta* **1250**, 149–157.

Pickard, M.A., T.A. Kadima and R.D. Carmichael, 1991. Chloroperoxidase, a peroxidase with potential. *J. Ind. Microbiol.* **7**, 235–241.

Poppe, L. and L. Novak, 1992. Selective Biotransformations. VCH Verlag Chemie, Weinheim, 319 pp.

Ramakrishnan, K., M.E. Oppenhuizen, S. Saunders and J. Fisher, 1983. Stereoselectivity of chloroperoxidase-dependent halogenation. *Biochemistry* **22**, 3271–3277.

Rush, C., A. Willetts, G. Davies, Z. Dauter, H. Watson and J. Littlechild, 1995. Purification, crystallization and preliminary X-ray analysis of the vanadium-dependent haloperoxidase from *Corallina officinalis.* FEBS *Lett.* **359**, 244–246.

Sheffield, D.J., T. Harry, A.J. Smith and L. Rogers, 1992. Purification and characterization of the bromoperoxidase from the macroalga *Corallina officinalis. Phytochemistry* **32**, 21–26.

Sundaramoorthy, M., J. Terner and T.L. Poulos, 1995. The crystal structure of chloroperoxidase: a heme peroxidase-cytochrome P450 functional hybrid. *Structure* **3**, 1367–1377.

Tschirret-Guth, R.A. and A. Butler, 1994. Evidence for organic substrate binding to vanadium bromoperoxidase. *J. Am. Chem. Soc.* **116**, 411–412.
Van Deurzen, M.P.J., B.W. Groen, F. van Rantwijk and R.A. Sheldon, 1994. A simple purification method for chloroperoxidase and its use in organic media. *Biocatalysis* **10**, 247–255.
Van Pée, K.-H., 1990. Bacterial haloperoxidases and their role in secondary metabolism. *Biotech. Adv.* **8**, 1–9.
Van Schijndel, J.W.P.M., P. Barnett, J. Roelse, E.G.M. Vollenbroek and R. Wever, 1994. The stability and steady-state kinetics of vanadium chloroperoxidase from the fungus *Curvularia inaequalis*. *Eur. J. Biochem.* **225**, 151–157.
Wever, R., 1988. Ozone destruction by algae in the arctic atmosphere. *Nature* **335**, 501.
Wiesner, W., K.-H. van Pée and F. Lingens, 1986. Detection of a new chloroperoxidase in *Pseudomonas pyrrocinia*. FEBS *Lett.* **209**, 321–324.
Wuosmaa, A.M. and L.P. Hager, 1990. Methyl chloride transferase: a carbocation route for biosynthesis of halometabolites. *Science* **249**, 160–162.
Zaks, A. and D.R. Dodds, 1995. Chloroperoxidase-catalyzed asymmetric oxidations: substrate specificity and mechanistic studies. *J. Am. Chem. Soc.* **117**, 10419–10424.
Zong, Q., P.A. Osmulski and L.P. Hager, 1995. High-pressure assisted reconstitution of recombinant chloroperoxidase. *Biochemistry* **34**, 12420–12425.

Wageningen Agricultural University, Department of Organic Chemistry, Dreijenplein 8, 6703 HB Wageningen, the Netherlands.