STUDIES ON THE USE OF HALOPEROXIDASES IN ORGANIC SYNTHESIS

Aan iedereen van wie ik iets geleerd heb

ISN 9.96452

Promotor: Dr. H.C. van der Plas, hoogleraar in de fysisch-organische chemie
Co-promotor: Dr. C. Laane, werkgroepleider Bio-Organische Chemie,
Unilever Research, Vlaardingen

M.C.R. Franssen

STUDIES ON THE USE OF HALOPEROXIDASES IN ORGANIC SYNTHESIS

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op vrijdag 9 oktober 1987
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

ISN: 296452

VOORWOORD

Het is een goed gebruik om een proefschrift te beginnen met een woord van dank aan allen die, op wat voor wijze dan ook, een bijdrage hebben geleverd aan de totstandkoming ervan. Een aantal mensen wil ik hierbij in het bijzonder noemen.

Allereerst mijn ouders, die hun beide kinderen in staat stelden de studie van hun voorkeur te volgen.

Anne-Marie, jij was steeds een goed klankbord; jouw begrip en interesse waren een onmisbare steun voor mij.

Prof. dr. H.C. van der Plas was niet alleen initiator van het hier beschreven onderzoek maar was tijdens de hele promotieperiode een enthousiaste coach.

Colja Laane maakte mij vertrouwd met bio-elektrochemie en omgekeerde micellen. Zijn optimisme en nuttige suggesties waren zeer stimulerend.

Het onderzoek werd uitgevoerd in samenwerking met prof. dr. E.M. Meijer, dr. H.E. Schoemaker en W. Boesten (DSM) en dr. R. Wever, drs. E. de Boer en drs. B.E. Krenn (vakgroep Biochemie, Universiteit van Amsterdam). Emmo, Hans, Willy, Ron, Eize en Bea, ik dank jullie hartelijk voor de vele vruchtbare discussies die wij samen hadden. Ron en Eize ben ik zeer erkentelijk voor het ter beschikking stellen van broomperoxidase en kweekmedium van *C. fumago*. Ik dank de Naamloze Vennootschap DSM, Heerlen, voor financiële ondersteuning van het onderzoek.

Tijdens hun doktoraalstage Organische Chemie leverden een negental studenten een bijdrage aan dit proefschrift. Hans Cardinaals, Liesbeth Kilsdonk, Jan-Dirk Jansma, Jean-Paul Vincken, Joop Stoots, Henry Kamphuis, Ward Mosmuller, John Weijnen en Axel Berg, bedankt voor jullie inzet. Een groot deel van de experimenten in Hoofdstuk 3 werden uitgevoerd door Wouter Pronk en Ab Weijland tijdens hun doktoraalstage Biochemie.

In het kader van een WVM-projekt verrichtte Guus van Boven gedurende 1 jaar onderzoek aan chloorperoxidase. Een deel van zijn resultaten is verwerkt in deze dissertatie.

Mijn naaste collega's Han Naeff, Johan De Meester en Steven Angelino dank ik voor de leerzame discussies die we hadden, en voor hun vriendschap.

Ik dank Riet Hilhorst (vakgroep Biochemie, LUW) en Adriaan Fuchs (vakgroep Fytopathologie, LUW) voor het kritisch doornemen van gedeelten van het manuscript.

STELLINGEN

1 In tegenstelling tot wat vaak beweerd wordt, is het mechanisme van de halogenering door het chloorperoxidase uit *Caldariomyces fumago* nog steeds niet met zekerheid bekend.

Dit proefschrift, hoofdstuk 2 en hoofdstuk 9

2 De beschouwingen van Itoh *et al.* over de produktvorming bij de halogenering van purines en pyrimidines door chloor- en broomperoxidase zijn ernstig aan bedenkingen onderhevig.

N. Itoh, Y. Izumi en H. Yamada, *Biochemistry* 26 (1987), 282

3 Holloway *et al.* geven geen overtuigend bewijs voor de onbruikbaarheid van 4-methylmorfoline-4-oxide ten behoeve van de niet-destruktieve extraktie van arabinoxylanen uit plantecelwanden.

W.D. Holloway, J. Lelievre en E.L. Richards, *Carbohydrate Res.* 143 (1985), 271
A.G.J. Voragen, H.A. Schols, J. Marijs, F.M. Rombouts en S.A.G.F. Angelino, *J. Inst. Brew.* 93 (1987), 202

4 De veelal onjuiste interpretatie der GC/MS-gegevens van enkele aromastoffen in muskaatdruiven toont aan dat het werk van Lamikanra nog onvoldoende gerijpt is.

O. Lamikanra, *Food Chem.* 19 (1986), 299

5 Cambou en Klibanov verklaren onvoldoende waarom het lipase uit *Candida cylindracea* geen stereoselektiviteit vertoont bij de hydrolyse van de methylester van 2-chloorpropionzuur en wel bij die van de octylester.

B. Cambou en A.M. Klibanov, *Appl. Biochem. and Biotechnol.* 9 (1984), 255

6 Het is zeer merkwaardig dat Bednarski *et al.* in hun publikatie over immobilisatie van enzymen in een dialysezakje geheel voorbijgaan aan het vele werk dat aan holle vezel membraanreaktoren is verricht.

M.D. Bednarski, H.K. Chenault, E.S. Simon en G.M. Whitesides, *J. Am. Chem. Soc.* 109 (1987), 1283

7 Uit de experimentele gegevens van Matsudomi *et al.* is niet duidelijk op te maken dat er bij de deamidering van gluten met chymotrypsine bij pH 10.0 weinig splitsing van eiwitketens optreedt.

N. Matsudomi, A. Tanaka, A. Kato en K. Kobayashi, *Agric. Biol. Chem.* 50 (1986), 1989

8 Het valt te betreuren dat Schultz en Messmer bij het beantwoorden van de vraag "Are There π-Bonds in Benzene?", de in de samenvatting van hun artikel verwoorde beperking niet veel duidelijker laten doorklinken in de feitelijke tekst.

P.A. Schultz en R.P. Messmer, *Phys. Rev. Lett.* 58 (1987), 2416

9 De veronderstelling van Nishitani *et al.* dat de chinonprotonen van hun P4Q4Q'-systeem boven de porphyrinering liggen is niet gerechtvaardigd.

S. Nishitani, N. Kurata, Y. Sakata en S. Misumi, *J. Am. Chem. Soc.* 105 (1983), 7771

10 Vermoedelijk heeft de in 1976 in Midden-Limburg waargenomen *Syngrapha interrogationis* L. (Lepidoptera, Noctuidae) een andere herkomst dan de overige in Nederland gesignaleerde exemplaren van deze soort.

M. Franssen, *Ent. Ber. (Amst.)* 40 (1980), 161

11 De agrarische sektor wordt in de fiscale wet- en regelgeving op onmiskenbare wijze bevoordeeld ten opzichte van de rest van het bedrijfsleven.

12 Vooral gezien het feit dat steeds meer wetenschappelijke tijdschriften eisen dat ingediende manuscripten "camera ready" moeten zijn, is het dringend gewenst dat de voorschriften voor auteurs bij deze tijdschriften gelijkluidend worden.

M.C.R. Franssen

Wageningen, 9 oktober 1987

Studies on the use of haloperoxidases in organic synthesis.

Biotechnologische studies gaan vaak samen met lastige analyses. Daarom ben ik veel dank verschuldigd aan Pim Melger en Gerrit Lelyveld (chromatografie), Cees Landheer en Kees Teunis (massaspectrometrie), Bep van Veldhuizen en Herman Holterman (NMR) en Hugo Jongejan (element analyse). Verder stelde dr. Maarten A. Posthumus zijn kostbare GC/MS apparatuur genereus ter beschikking voor talrijke analyses.

Het strenge schoonmaakregime van Hanny Böhmer zorgde voor orde in de werkruimtes.

Almost last, but definitely not least, dank aan de mensen die direkt betrokken waren bij tekst en lay-out van het proefschift. Chris Rasmussen bracht als "linguistic adviser" de nodige correcties aan. Marieke Bosman wees mij de weg op het glibberige pad van de tekstverwerking en Fieke Wien verzorgde een deel van het typewerk en de uitdraai van het manuscript. Jurrie Menkman vervaardigde op snelle en vakkundige wijze de tekeningen.

Voorts dank ik iedereen van de vakgroepen Organische Chemie en Biochemie voor hun hulpvaardigheid, belangstelling en bijdrage aan de prettige sfeer (A! SDIMWADT, THKKKNLW).

CONTENTS

1 INTRODUCTION

1.1 ENZYMES IN ORGANIC SYNTHESIS

Enzymes are the universally occurring catalysts responsible for many chemical reactions in living systems. Their remarkable specificity has intrigued mankind for a long time: enzymes are able to react at only one site of a molecule (regiospecific) and are able to distinguish between molecules that differ in their threedimensional structure (stereospecific) giving pure products in very high yield. These properties make them unique compared to chemical catalysts, which sometimes have a high specificity but in general do not give 100% of one isomer but a mixture of wanted and unwanted products. Due to these properties there is a growing interest in the use of enzymes in organic synthesis [see refs. 1-4 for reviews]. In our laboratory this subject is under study since 1975. Free and immobilized xanthine oxidase and xanthine dehydrogenase have been used for the regiospecific hydroxylation of alkyl- and arylpteridinones [5-9] and free and immobilized aldehyde oxidase for the regiospecific hydroxylation of alkyl- and arylpyridinium halides [10,11], alkyl- and arylquinolinium chlorides [12] and N-alkylpyrimidinones [13]. Another subject in our group is the inhibition of milk xanthine oxidase by substituted hypoxanthines and pteridinones [14].

Besides the incorporation of hydroxyl groups, which is accomplished by above-mentioned oxidases, the introduction of halogen atoms into organic compounds is an interesting topic as well, because i) introduction of halogen atoms in organic compounds frequently results in enhanced physiological activity [15] and ii) halogen atoms can easily be replaced by nucleophilic substitution which makes halogenated compounds useful intermediates in organic synthesis. The subject of this thesis is the enzymatic halogenation of organic molecules; the enzymes used are called haloperoxidases.

1.2 IMMOBILIZED ENZYMES

Although enzymes can be very useful in organic synthesis, they also have some disadvantages: i) most of them are expensive, so recovery of the biocatalysts is needed for an economic process[1]; ii) soluble enzymes are rather labile, losing their activity due to autooxidation, self-digestion and

denaturation by the solvent or solutes; water-miscible organic solvents in particular are harmful to enzymes. Some of these problems can be overcome by immobilization of the enzyme. This technique involves the confinement of the enzyme to a restricted area from which it cannot leave but where it remains catalytically active [see for some recent reviews refs. 17-21].

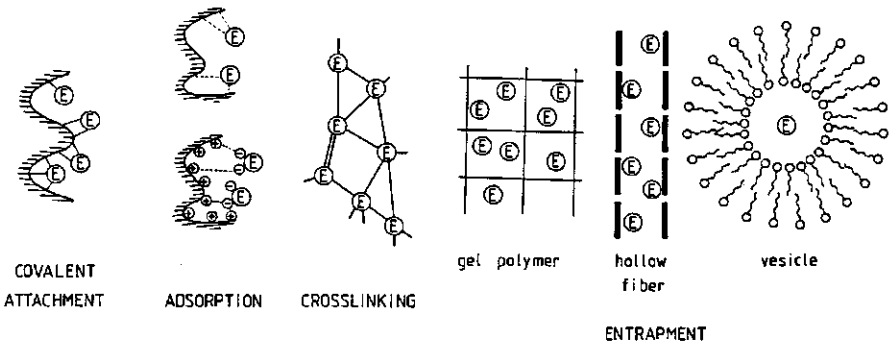


Figure 1.1 Modes of immobilization and schematic representation of immobilized enzymes [adapted from 22].

Four different immobilization techniques can be distinguished [20, modified] (see Fig. 1.1):

a) covalent attachment: an amino, thiol, hydroxyl or carboxyl group of the enzyme is covalently linked to active groups present on the surface of the solid support. This method leads to tightly bound enzymes, but some inactivation generally occurs.

b) adsorption: the enzyme is bound non-covalently to the support using Van der Waals forces, dipolar interactions, hydrophobic forces, hydrogen bonds or ionic (Coulomb) forces. In general, this is a mild procedure but the bonding is much weaker than in case a).

c) crosslinking: the enzyme molecules are crosslinked to form an insoluble polymer. The reaction is carried out under rather harsh conditions and the method is not often used.

d) entrapment (or encapsulation): the enzyme is physically or chemically locked

---

[1] However, in some cases the costs of the biocatalyst are so low that reuse is not strictly needed [16].

in a structure from which it cannot leave; interactions of the enzyme with the matrix are of minor importance. Substrates and products can diffuse in and out the matrix more or less freely, but the enzyme can not. Well-known methods are entrapment of whole cells and enzymes in a polymer (a very mild procedure) and polymerization of monomers in the presence of the enzyme. Other systems in which the enzyme is confined to a restricted area are reversed micelles (see 1.3 and fig. 1.2), vesicles and hollow fibers; incorporation of enzymes in these is called entrapment as well.

Thus, various methods are available to the organic chemist wishing to work with immobilized enzymes. However, no general guidelines can be given as to which method is best for a particular enzyme or reaction: this remains a matter of trial and error. Furthermore, the demands set by the system under study will always dictate the choice of the approach [18].

1.3 REVERSED MICELLES

It has been known for a long time that water/oil/detergent mixtures of certain compositions give transparent solutions in which the oil is the continuous phase. A model for this system was proposed for the first time in 1943 [23]. The model consists of "oleopathic hydro-micelles", nowadays called reversed micelles: tiny water droplets (their diameter is 6-40 nm [24]) embedded in an water-immiscible organic solvent (e.g. octane, toluene), stabilized by a surfactant and, when necessary, a cosurfactant (see Fig. 1.2). The water entrapped in these micelles has several chemical and physical properties that deviate from "normal" water, such as restricted molecular motion, decreased hydrogen bonding, increased viscosity and depressed freezing point [25,26]. The effect is most pronounced at low water content; on adding more water to the system the values return to normal. The transfer of material from one micelle to another occurs by means of collisions and is an extremely fast process [27].

It was found in 1974 that it is possible to entrap enzymes in the water pools of these micelles [25], and a lot of enzymes were proved to be active under these conditions [see refs. 28 and 29 for reviews]. Remarkably, enzyme activity is usually enhanced [30-32]. In some cases their specificity is changed [33,34], or their temperature stability is enhanced [35]. Most important however is the fact that in these systems compounds which are

sparingly soluble in water, such as steroids [36,37], can be converted in much higher rates than would have been possible in aqueous media. Thus, reversed micelles are a useful extension of the methodology currently used by the organic chemists and biochemists.

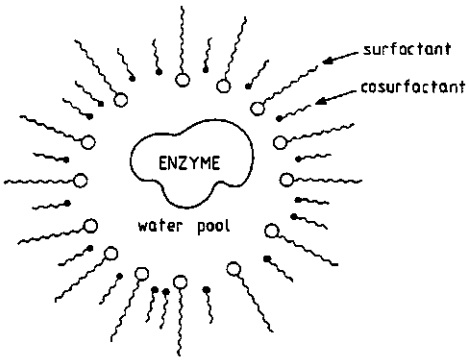


Figure 1.2 Model for an enzyme in a reversed micelle.

1.4 HALOPEROXIDASES

Haloperoxidases are enzymes which are capable of halogenating a variety of organic compounds using hydrogen peroxide and halide ions as substrates. The general overall reaction is given below:

$$AH + H_2O_2 + H^+ + X^- \longrightarrow AX + 2H_2O$$

AH = organic substrate; X = chloride, bromide or iodide ion;
AX = halogenated product.

The enzymes are called chloro-, bromo-, or iodoperoxidases, dependent on the smallest halide ion they can oxidize. For excellent reviews see Hewson and Hager [38], or, more recently, Neidleman and Geigert [15].

Some thirty years ago haloperoxidases were thought to be unique enzymes: their halogenating capacities were unusual and they were considered to be rare. Nowadays, haloperoxidases are known from almost 100 sources, including mammals,

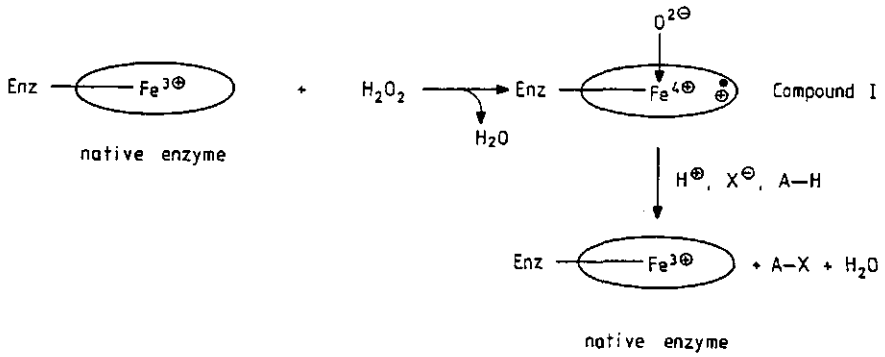
birds, plants, algae, molds and bacteria, clearly showing that these enzymes are widely occurring. The best known enzymes are mentioned in Table 1.1.

Table 1.1 List of some well-characterized haloperoxidases. See for reviews [55] *and* [15].

name	source	reference
chloroperoxidases		
chloroperoxidase	*Caldariomyces fumago* (mold)	39
myeloperoxidase	white blood cells (neutrophils)	40-42
eosinophil peroxidase	white blood cells (eosinophils)	42-44
bromoperoxidases		
lactoperoxidase	milk, saliva, tears	45
ovoperoxidase	sea urchin eggs	46
bromoperoxidase	red, green, brown algae	15,47
bromoperoxidase	bacteria: *Pseudomonas aureofaciens*	48
	Pseudomonas pyrrocinia	49
	Streptomyces phaeochromogenes	50
iodoperoxidases		
thyroid peroxidase	thyroid gland (mammals and birds)	51,52
horseradish peroxidase	horseradish root	53,54
iodoperoxidase	brown algae	48

Haloperoxidases are, however, still unique in the reaction they carry out and not much is known about the reaction mechanism. The enzymes can be divided into the heme- and non-heme haloperoxidases. The heme enzymes contain protoporphyrin IX as prosthetic group. Scheme 1.1 shows the reaction sequence in simplified form. In the first step hydrogen peroxide is bound by means of ligation to the iron ion and hydrogen bonding to certain amino acid residues of the protein backbone [56]; the iron ion and the heme group are oxidized with simultaneous cleavage of the hydrogen peroxide molecule. Then a halide ion binds with subsequent reduction of the iron ion and the heme group, after which the organic substrate is halogenated. This topic is discussed in more detail in chapter 2. Examples of non-heme haloperoxidases are a lactoperoxidase [57], an

algal bromoperoxidase [47] and a bacterial chloroperoxidase [58]; their prosthetic group is not yet known. Vanadium ion has been shown to be the prosthetic group of the bromoperoxidases from the brown alga *Ascophyllum nodosum* [59,60], and six other brown algae [61]. It was suggested that the occurrence of vanadium is a common feature of the bromoperoxidases of brown algae. Unfortunately, nothing is known about the reaction mechanism of these enzymes up to now. In the native enzyme vanadium is in the V^{5+} - state, so oxidation by hydrogen peroxide, as in the heme-enzymes is impossible. Hydrogen peroxide may be bound to the vanadium as a ligand [60,61] but the subsequent steps in catalysis remain unclear.

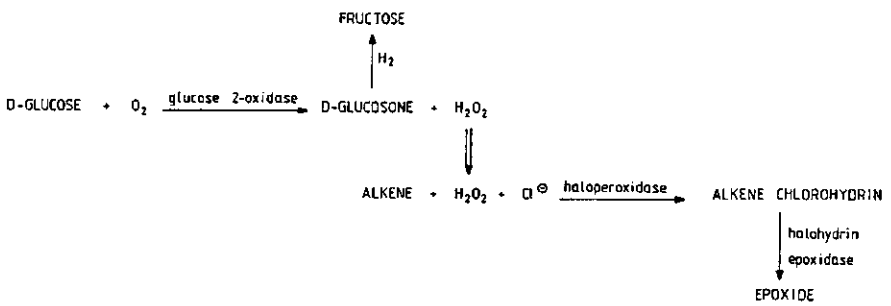


Scheme 1.1 *The reaction mechanism of heme-containing haloperoxidases. The protein part of the enzyme is represented as ENZ, the heme group is depicted as Fe^{x+} (x = 3 or 4) in the center of an ellipse, for reasons of clarity. In Compound I, the iron atom has a 4+ oxidation state and the porphyrin ring is oxidized to a radical cationic species. X = Cl, Br or I; A-H = organic substrate; A-X = halogenated product.*

The natural function of these enzymes is not yet known, but they seem to be involved in the defence mechanism of their hosts; it is known that a lot of halogen-containing compounds are physiologically more active than their non-halogenated counterparts [15]. Halometabolites have the same broad occurrence as the haloperoxidases [15,62-64] but are especially abundant in sea organisms [65,66] in which they sometimes make up 20% of the dry weight [15]. Some algae produce halometabolites which make them uneatable to predators. However, some sea hares (*Aplysia*) prefer these algae as their food and become unedible

themselves [66]. The only halometabolites that have been found in mammalian tissues are iodotyrosine and derivatives thereof which are formed by the action of thyroid peroxidase [67]. The other mammalian haloperoxidases act in a different way. It has been found that the white blood cell enzymes myeloperoxidase and eosinophil peroxidase produce HOCl and $^1O_2$ [68,69], both reactive compounds poisonous to microorganisms. Lactoperoxidase produces HOBr and oxidizes thiocyanate (SCN^-) to the antimicrobial agent hypothiocyanate ($OSCN^-$) [70]. In short, haloperoxidases play a more important role in nature than thought in the past.

It is not surprising that industrial interest in these remarkable enzymes has developed. Four patents have appeared thus far, submitted by Cetus Corporation and Standard Oil Company of California. In these patents several free and immobilized haloperoxidases were used for the production of iodine [71] or epoxides [72-74], namely chloroperoxidase (*Caldariomyces fumago*), "seaweed peroxidase" (*Corallina sp.* and *Laurencia pacifica*), bovine lactoperoxidase, thyroid peroxidase, myeloperoxidase and horseradish peroxidase. The hydrogen peroxide necessary as second substrate was produced by glucose oxidase, methanol oxidase or, preferably, glucose-2-oxidase. The process with the latter enzyme is depicted in Scheme 1.2. The haloperoxidases convert alkenes to halohydrins, which are in turn converted to epoxides by a halohydrin epoxidase from *Flavobacterium sp.* The final products are fructose and epoxides. Although it has been reported that the process was economically feasible [75] it has up to now not reached pilot plant scale which is in part due to the decreased price of fructose on the world market [76].

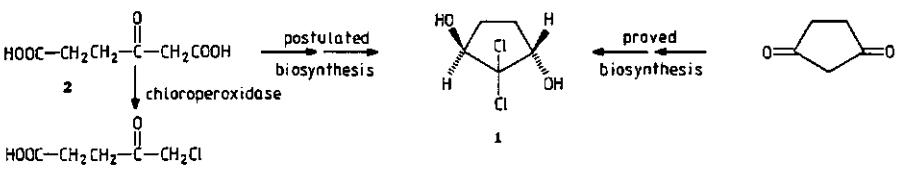


Scheme 1.2 The Cetus process in which glucose and alkenes (e.g. ethene or propene) are converted to fructose and alkene epoxides, respectively.

The haloperoxidases which are studied in this thesis will now be discussed in more detail.

1.4.1 Chloroperoxidase (Caldariomyces fumago)

In 1940 Clutterbuck and coworkers [77] studied the halogenating capacities of 139 mold species or strains. Five of these converted more than 25% of the chloride ions in the growth medium into organic chloro compounds. An organic metabolite named caldariomycin (**1**, see Scheme 1.3) was isolated and characterized from one of the molds, *Caldariomyces fumago* Woronichin. The biosynthesis of **1** was thought to occur [78] via β-keto adipic acid (**2**)[2], and the search started for the enzyme responsible for the chlorination of this compound. In 1959 an impure "β-keto adipate chlorinase" was isolated from acetone-dried mycelium [80] and in the same year it was recognized that hydrogen peroxide could replace certain cell components in the enzyme assay [81]. Thus, the enzyme is peroxidative in nature and the name chloroperoxidase was proposed [81]. The enzyme was purified [39,82,83] and finally crystallized [84]. Chloroperoxidase appears to be a complex mixture of isoenzymes due to differences in charge [85,86] and microheterogeneity in the carbohydrate part of the enzyme [87]. The enzyme is now commercially available (Sigma Chem. Co.) and techniques have been developed for large-scale production using *C. fumago* mycelium immobilized in κ-carrageenan [88]. The isolation is facilitated by the fact that the mold excretes the enzyme into the medium during its final stages of growth [84].



*Scheme 1.3 Biosynthesis of caldariomycin (**1**) and reaction of β-keto adipic acid (**2**) with chloroperoxidase.*

[2]Proved to be incorrect, [79].

Chloroperoxidase has a molecular weight of 42,000. The amino acid composition shows relatively large amounts of serine and proline. In particular a lot of aspartic and glutamic acid residues are present [84], which is reflected by the low isoelectric point of the enzyme (± 3.7) [85]. Chloroperoxidase contains one mol of ferriprotoporphyrin IX per mol of enzyme in which the iron (III) ion is hexacoordinated. The porphyrin moiety stands for four ligands and there is growing evidence that the fifth ligand is a cysteine residue from the protein [89-96]. The sixth iron ligand is most probably a carboxyl group of the protein backbone, as was concluded from pH- [97,98] and NMR studies [99] and oxidation-reduction potential measurements [100]. The enzyme is only active when this carboxyl group is protonated, allowing the approach of hydrogen peroxide and other ligands [97,101]. Halide ions can bind to the iron instead of hydrogen peroxide, leading to reversible inactivation of the enzyme. At pH > 7.2 chloroperoxidase is irreversibly inactivated [100] because a histidine residue is deprotonated and binds to the iron ion [102,103]. Unfortunately, the three-dimensional structure of the enzyme is still not known, although some preliminary data concerning the X-ray structure have been published [104].

Chloroperoxidase is able to carry out three different reactions:

a) The chlorination, bromination or iodination of electron-rich organic compounds as mentioned earlier. The reaction occurs between pH 1.5 and 4.2, being optimal at pH 2.7. The K_ms for hydrogen peroxide, chloride and bromide ion at pH 2.7 are respectively 350 μM, 2.0 mM and 0.1 mM [105]. The enzyme does not oxidize fluoride ions because its oxidation potential is not high enough to do so; in fact, fluoride is an inhibitor of the enzyme.

b) The oxidation of electron-rich compounds according to the equation

$$BH_2 + H_2O_2 \longrightarrow B + 2\ H_2O$$

The reaction occurs between pH 4 and 7 and is optimal at pH 4.5.

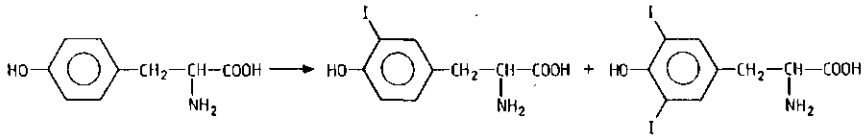
c) The decomposition of hydrogen peroxide (catalase-reaction):

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

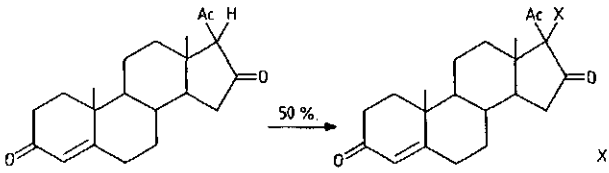
This reaction occurs in a very broad pH range.

Chloroperoxidase is an enzyme with a very broad substrate specificity; some of its reactions are depicted in Scheme 1.4. The reader is referred to Neidleman [15] for a more complete list.

REF

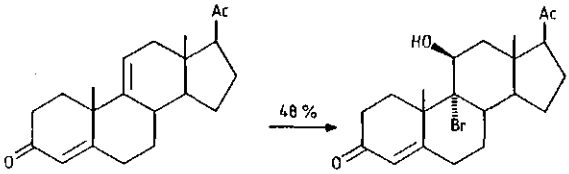


106



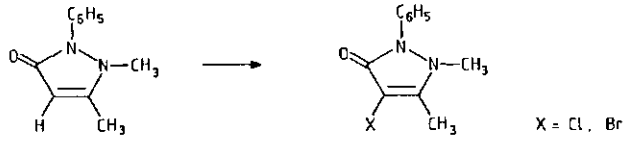
X = Cl, Br

107



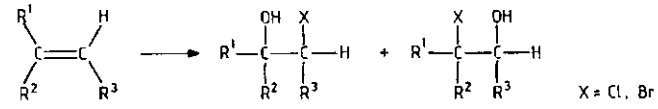
108

109



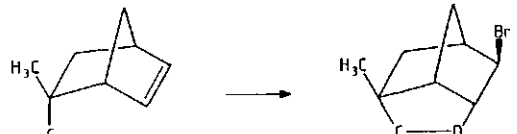
X = Cl, Br

110

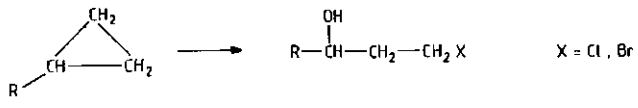


X = Cl, Br

111

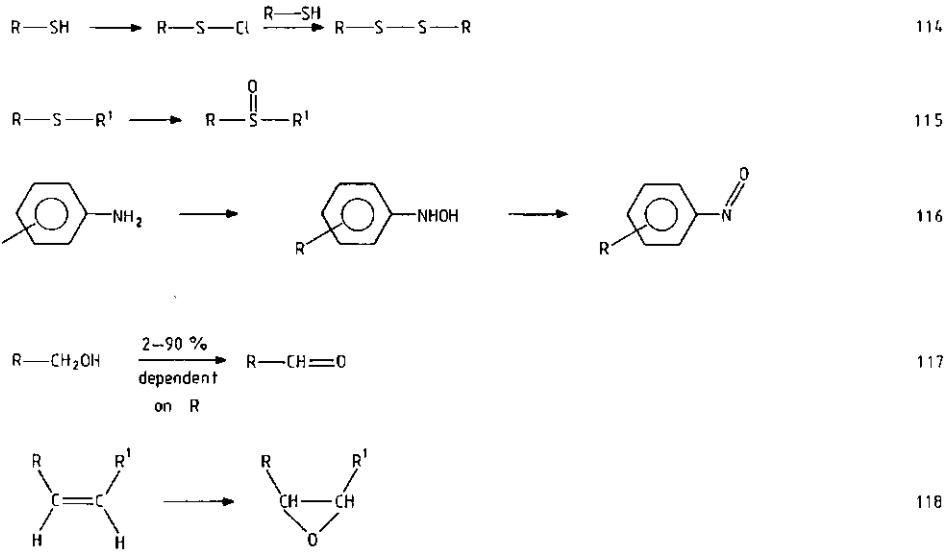


112



X = Cl, Br

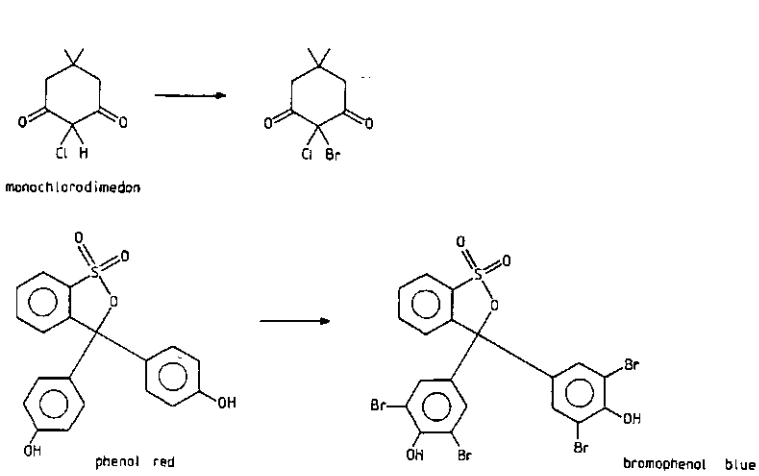
113



Scheme 1.4 *Some reactions catalyzed by the chloroperoxidase from* Caldariomyces fumago. *Reaction yields are only given when clearly stated in the reference cited.*

1.4.2 Bromoperoxidase (Ascophyllum nodosum)

The bromoperoxidase from *Ascophyllum nodosum* was detected and purified recently by a German [59] and a Dutch group [119]. This glycoenzyme has a dimeric structure consisting of two subunits with molecular weights of 45,000. Maximal brominating activity is achieved between pH 4.5 and 6.5, dependent on the concentration of hydrogen peroxide and bromide ions present [119]. The K_m for hydrogen peroxide is 0.03-3.0 mM, dependent on pH and lowest at high pH. The K_m for bromide is 12.7 mM, independent of pH [119]. This bromoperoxidase is not inhibited by hydrogen peroxide or cyanide, in contrast to chloroperoxidase. Its prosthetic group is one vanadium (V) ion per enzyme molecule [60]. A remarkable feature of the enzyme is its great stability towards elevated temperatures and organic solvents on storage [120] as well as under turnover conditions [119]. Bromoperoxidase has been used for the bromination of monochlorodimedon [119] and phenol red [120] (see Scheme 1.5).



Scheme 1.5 Reactions catalyzed by the bromoperoxidase from Ascophyllum nodosum.

1.5 OUTLINE OF THIS THESIS

The enzymatic halogenation of heterocyclic compounds has hardly been documented so far. The aim of this study is to extend the applicability of haloperoxidases in the field of synthetic heterocyclic chemistry.

Chapters 2 deals with the synthesis of chlorinated barbituric acids by soluble chloroperoxidase from *C. fumago*, the kinetics of the reactions and their implications for the reaction mechanism of the enzyme.

The electro-enzymatic synthesis of 5-chlorobarbituric acid by means of electricity and chloroperoxidase entrapped in a hollow fiber reactor is described in Chapter 3.

In Chapter 4 the reaction of chloroperoxidase with other heterocyclic compounds like pyrazoles and pyridine derivatives is discussed.

The bromination of barbituric acid and some of its derivatives by the bromoperoxidase from *Ascophyllum nodosum* is presented in Chapter 5.

A study on the activity of chloroperoxidase when confined to a restricted

area is described in Chapter 6 and 7. In Chapter 6 the enzyme is entrapped in an organic solvent by means of reversed micelles, and in Chapter 7 the enzyme is immobilized on various solid supports.

The natural function of chloroperoxidase is the subject of Chapter 8, in which some new halometabolites from *Caldariomyces fumago* are presented.

A general discussion on the contents of this thesis and some miscellaneous results are given in Chapter 9.

1.6 REFERENCES

[1] C.J. Suckling and K.E. Suckling, Chem. Soc. Rev. **3** (1974), 387-406

[2] *Biocatalysts in Organic Synthesis*, (J. Tramper, H.C. van der Plas and P. Linko, eds), Studies in Organic Synthesis **22**, Elsevier, Amsterdam, (1985), pp. 1-259

[3] J.B. Jones, Tetrahedron **42** (1986), 3351-3403

[4] S. Butt and S.M. Roberts, Nat. Prod. Rep. **3** (1986), 489-503

[5] J. Tramper, A. Nagel, H.C. van der Plas and F. Müller, Recl. Trav. Chim. Pays-Bas **98** (1979), 224-231

[6] J. Tramper, S.A.G.F. Angelino, F. Müller and H.C. van der Plas, Biotechnol. Bioeng. **21** (1979), 1767-1786

[7] J. Tramper, W.E. Hennink and H.C. van der Plas, J. Appl. Biochem. **4** (1982), 263-270

[8] H.C. van der Plas, J. Tramper, S.A.G.F. Angelino, J.W.G. De Meester, H.S.D. Naeff, F. Müller and W. Middelhoven, in *Innovations in Biotechnology* (E.H. Houwink and R.R. van der Meer, eds), Progress in Industrial Microbiology **20**, Elsevier, Amsterdam, (1984), 93-101

[9] J.W.G. De Meester, W. Kraus, W.J. Middelhoven and H.C. van der Plas, in *Bio-Organic Heterocycles: Synthesis, Mechanisms and Bioactivity* (H.C. van der Plas, M. Simonyi, F.C. Alderweireldt and J.A. Lepoivre, eds), Studies in Organic Chemistry **27**, Elsevier, Amsterdam, (1986), 243-251

[10] S.A.G.F. Angelino, D.J. Buurman, H.C. van der Plas and F. Müller, Recl. Trav. Chim. Pays-Bas **101** (1982), 342-346

[11] S.A.G.F. Angelino, D.J. Buurman, H.C. van der Plas and F. Müller, Recl. Trav. Chim. Pays-Bas **102** (1983), 331-336

[12] S.A.G.F. Angelino, B.H. van Valkengoed, D.J. Buurman, H.C. van der Plas and F. Müller, J. Heterocyclic Chem. **21** (1984), 107-112

[13] S.A.G.F. Angelino, D.J. Buurman, H.C. van der Plas and F. Müller, J. Heterocyclic Chem. **21** (1984), 749-752

[14] H.S.D. Naeff, H.C. van der Plas, J. Tramper and F. Müller, Quant. Struct.-Act. Relat. **4** (1985), 161-166

[15] S.L. Neidleman and J. Geigert, *Biohalogenation: Principles, Basic Roles and Applications*, Ellis Horwood Ltd., Chichester, (1986), pp. 1-203

[16] E.M. Meijer, W.H.J. Boesten, H.E. Schoemaker and J.A.M. van Balken, in ref. 2, pp. 135-156

[17] B.P. Sharma, L.F. Bailey and R.A. Messing, Angew. Chem. **94** (1982), 836-852

[18] J. Tramper, in *Solid Phase Biochemistry: Analytical Synthetic Aspects*, (W.H. Scouten, ed), Wiley, New York, (1983), 393-477

[19] H.O. Hultin, Food Technol. **37** (1983), 66

[20] A.M. Klibanov, Science **219** (1983), 722-727

[21] A. Rosevear, J. Chem. Technol. Biotechnol. **34** (1984), 127-150

[22] J. Tramper, PhD Thesis, Pudoc, Wageningen, The Netherlands (1979)
[23] T.P. Hoar and J.H. Schulman, Nature **152** (1943), 102-103
[24] F.J. Bonner, R. Wolf and P.L. Luisi, J. Solid Phase Biochem. **5** (1980), 255-268
[25] P.H. Poon and M.A. Wells, Biochemistry **13** (1974), 4928-4936
[26] M.A. Wells, Biochemistry **13** (1974), 4937-4942
[27] H.-F. Eicke, J.C.W. Shepherd and A. Steinemann, J. Colloid Interf. Sci. **56** (1976), 168-176
[28] K. Martinek, A.V. Levashov, N.L. Klyachko, Y.L. Khmelnitsky and I.V. Berezin, Eur. J. Biochem. **155** (1986), 453-468
[29] P.L. Luisi and C. Laane, Trends Biotechnol. **4** (1986), 153-161
[30] P. Meier and P.L. Luisi, J. Solid Phase Biochem. **5** (1980), 269-282
[31] S. Barbaric and P.L. Luisi, J. Am. Chem. Soc. **103** (1981), 4239-4244
[32] N.L. Klyachko, A.V. Levashov and K. Martinek, Mol. Biol. (Moscow), Engl. transl. **18** (1984), 830-840
[33] K. Martinek, A.N. Semenov and I.V. Berezin, Biochim. Biophys. Acta **658** (1981), 76-89
[34] K. Martinek, A.V. Levashov, Y.L. Khmelnitsky, N.L. Klyachko and I.V. Berezin, Science **218** (1982), 889-891
[35] C. Grandi, R.E. Smith and P.L. Luisi, J. Biol. Chem. **256** (1981), 837-843
[36] R. Hilhorst, R. Spruijt, C. Laane and C. Veeger, Eur. J. Biochem. **144** (1984), 459-466
[37] P.L. Luisi, P. Lüthi, I. Tomka, J. Prenosil and A. Pande, Ann. N.Y. Acad. Sci. **434** (1984), 549-557
[38] W.D. Hewson and L.P. Hager, The Porpyrins **7** (1979), 295-332
[39] P.D. Shaw and L.P. Hager, J. Biol. Chem. **236** (1961), 1626-1630
[40] A.R.J. Bakkenist, R. Wever, T. Vulsma, H. Plat and B.F. van Gelder, Biochim. Biophys. Acta **524** (1978), 45-54
[41] R.L. Olsen and C. Little, Biochem. J. **209** (1983), 781-787
[42] C.C. Winterbourn, Biochim. Biophys. Acta **840** (1985), 204-210
[43] R. Wever, H. Plat and M.N. Hamers, FEBS Lett. **123** (1981), 327-331
[44] B.G.J.M. Bolscher, H. Plat and R. Wever, Biochim. Biophys. Acta **784** (1984), 177-186
[45] K.G. Paul and P.I. Ohlsson, in *The Lactoperoxidase System, Chemistry and Biological Significance* (K.M. Pruitt and J.O. Tenovuo, eds), M. Dekker, New York, Immunol. Ser. **27** (1985), 15-29
[46] T. Deits, M. Farrance, E.S. Kay, L. Medill, E.E. Turner, P.J. Weidman and B.M. Shapiro, J. Biol. Chem. **259** (1984), 13525-13533
[47] N. Itoh, Y. Izumi and H. Yamada, J. Biol. Chem. **261** (1986), 5194-5200
[48] K.-H. van Pêe and F. Lingens, J. Bacteriol. **161** (1985), 1171-1175
[49] W. Wiesner, K.-H. van Pêe and F. Lingens, Biol. Chem. Hoppe-Seyler **366** (1985), 1085-1091
[50] K.-H. van Pêe and F. Lingens, J. Gen. Microbiol. **131** (1985), 1911-1916
[51] S. Ohtaki, H. Nakagawa, S. Nakamura, M. Nakamura and I. Yamazaki, J. Biol. Chem. **260** (1985), 441-448
[52] H. Nakagawa, T. Kotani, S. Ohtaki, M. Nakamura and I. Yamazaki, Biochem. Biophys. Res. Commun. **127** (1985), 8-14
[53] K.G. Welinder, FEBS Lett. **72** (1976), 19-23
[54] A.P. Wagner, E. Psarrou and L.P. Wagner, Anal. Biochem. **129** (1983), 326-328
[55] K.G. Paul, The Enzymes **8** (1963), 227-274
[56] D.V. Behere, E. Gonzalez-Vergara and H.M. Goff, Biochim. Biophys. Acta **832** (1985), 319-325
[57] C. Dumontet and B. Rousset, J. Biol. Chem. **258** (1983), 14166-14172
[58] W. Wiesner, K.-H. van Pêe and F. Lingens, FEBS Lett. **209** (1986), 321-324
[59] H. Vilter, Phytochemistry **23** (1984), 1387-1390

[60] E. de Boer, Y. van Kooyk, M.G.M. Tromp, H. Plat and R. Wever, Biochim. Biophys. Acta **869** (1986), 48-53
[61] E. de Boer, M.G.M. Tromp, H. Plat, G.E. Krenn and R.Wever, Biochim. Biophys. Acta **872** (1986), 104-115
[62] J.F. Siuda and J.F. DeBernardis, Lloydia **36** (1973), 107-143
[63] S.L. Neidleman, CRC Crit. Rev. Microbiol. **3** (1975), 333-358
[64] K.C. Engvild, Phytochemistry **25** (1986), 781-791
[65] W. Fenical, J. Phycol. **11** (1975), 245-259
[66] D.J. Faulkner, Nat. Prod. Rep. **1** (1984), 251-280
[67] M. Nakamura, I. Yamazaki, T. Kotani and S. Ohtaki, J. Biol. Chem. **260** (1985), 13546-13552
[68] J.E. Harrison and J. Schultz, J. Biol. Chem. **251** (1976), 1371-1374
[69] A.U. Khan, Biochem. Biophys. Res. Commun. **122** (1984), 668-675
[70] B. Reiter, Protides Biol. Fluids **32** (1984), 111-114
[71] S.L. Neidleman and J. Geigert, US 4,282,324 (1980)
[72] S.A Cerefice and D.H. Meyer, Belg. 884,004 (1980)
[73] S.L. Neidleman, W.F. Amon Jr. and J. Geigert, US 4,247,641 (1981)
[74] S.L. Neidleman, W.F. Amon Jr. and J. Geigert, US 4,284,723 (1981)
[75] G. Parkinson, Chem. Eng. (NY) **87** (1980), 37-38
[76] S.L. Neidleman, personal communication
[77] P.W. Clutterbuck Jr., S.L. Mukhopadhyay, A.E. Oxford and H. Raistrick, Biochem. J. **34** (1940), 664-677
[78] P.D. Shaw, J.R. Beckwith and L.P. Hager, J. Biol. Chem. **234** (1959), 2560-2564
[79] J.R. Beckwith, R. Clark and L.P. Hager, J. Biol. Chem. **238** (1963), 3086-3090
[80] P.D. Shaw and L.P. Hager, J. Biol. Chem. **234** (1959), 2565-2569
[81] P.D. Shaw and L.P. Hager, J. Am. Chem. Soc. **81** (1959), 6527-6528
[82] P.F. Hallenberg and L.P. Hager, Meth. Enzymol. **52** (1978), 521-529
[83] E. Gonzalez-Vergara, D.C. Ales and H.M. Goff, Prep. Biochem. **15** (1985), 335-348
[84] D.R. Morris and L.P. Hager, J. Biol. Chem. **241** (1966), 1763-1768
[85] A.S.W. Sae and B.A. Cunningham, Phytochemistry **18** (1979), 1785-1787
[86] M.A. Pickard and A. Hashimoto, Can. J. Microbiol. **28** (1982), 1382-1388
[87] A. Hashimoto and M.A. Pickard, J. Gen. Microbiol. **130** (1984), 2051-2058
[88] R.D. Carmichael, A. Jones and M.A. Pickard, Appl. Environ. Microbiol. **51** (1986), 276-280
[89] S.P. Cramer, J.H. Dawson, K.O. Hodgson and L.P. Hager, J. Am. Chem. Soc. **100** (1978), 7282-7290
[90] M. Sono, J.H. Dawson and L.P. Hager, J. Biol. Chem. **259** (1984), 13209-13216
[91] M. Sono, K.S. Eble, J.H. Dawson and L.P. Hager, J. Biol. Chem. **260** (1985), 15530-15535
[92] M. Sono, J.H. Dawson and L.P. Hager, Inorg. Chem. **24** (1985), 4339-4343
[93] D.V. Behere, E. Gonzalez-Vergara and H.M. Goff, Biochem. Biophys. Res. Commun. **131** (1985), 607-613
[94] H.M. Goff, E. Gonzalez-Vergara and M.R. Bird, Biochemistry **24** (1985), 1007-1013
[95] M. Sono, J.H. Dawson and L.P. Hager, Biochemistry **25** (1986), 347-356
[96] O. Bangcharoenpaurpong, P.M. Champion, K.S. Hall and L.P. Hager, Biochemistry **25** (1986), 2374-2378
[97] J.A. Thomas, D.R. Morris and L.P. Hager, J. Biol. Chem. **245** (1970), 3129-3134
[98] J.A. Thomas, D.R. Morris and L.P. Hager, J. Biol. Chem. **245** (1970), 3135-3142
[99] G.E. Krejcarek, R.G. Bryant, R.J. Smith and L.P. Hager, Biochemistry **15** (1976), 2508-2511

[100] R. Makino, R. Chiang and L.P. Hager, Biochemistry **15** (1976), 4748-4754
[101] A.M. Lambeir and H.B. Dunford, J. Inorg. Biochem. **19** (1983), 291-300
[102] B.N. Campbell Jr., T. Araiso, L. Reinisch, K.T. Yue and L.P. Hager, Biochemistry **21** (1981), 4343-4349
[103] A.M. Lambeir and H.B. Dunford, Arch. Biochem. Biophys. **220** (1983), 549-556
[104] B. Rubin, J. Vanmiddelsworth, K. Thomas and L.P. Hager, J. Biol. Chem. **257** (1982), 7768-7769
[105] M.C.R. Franssen, unpublished results
[106] A. Taurog and E.M. Howell, J. Biol. Chem. **241** (1966), 1329-1339
[107] S.L. Neidleman, P.A. Diassi, B. Junta, R.M. Palmere and S.C. Pan, Tetrahedron Lett. **1966**, 5337-5347
[108] S.L. Neidleman and S.D. Levine, Tetrahedron Lett. **1968**, 4057-4059
[109] S.L. Neidleman, A.I. Cohen and L. Dean, Biotechnol. Bioeng. **11** (1969), 1227-1232
[110] P.L. Ashley and B.W. Griffin, Arch. Biochem. Biophys. **210** (1981), 167-178
[111] J. Geigert, S.L. Neidleman, D.J. Dalietos and S.K. DeWitt, Appl. Environ. Microbiol. **45** (1983), 366-374
[112] K. Ramakrishnan, M.E. Oppenhuizen, S. Saunders and J. Fisher, Biochemistry **22** (1983), 3271-3277
[113] J. Geigert, S.L. Neidleman and D.J. Dalietos, J. Biol. Chem. **258** (1983), 2273-2277
[114] R.M. Silverstein and L.P. Hager, Biochemistry **13** (1974), 5069-5073
[115] D.R. Doerge, Arch. Biochem. Biophys. **244** (1986), 678-685
[116] M.D. Corbett, B.R. Chipko and D.G. Baden, Bioorg. Chem. **8** (1979), 91-95
[117] J. Geigert, D.J. Dalietos, S.L. Neidleman, T.D. Lee and J. Wadsworth, Biochem. Biophys. Res. Commun. **114** (1983), 1104-1108
[118] J. Geigert, T.D. Lee, D.J. Dalietos, D.S. Hirano and S.L. Neidleman, Biochem. Biophys. Res. Commun. **136** (1986), 778-782
[119] R. Wever, H. Plat and E. de Boer, Biochim. Biophys. Acta **830** (1985), 181-186
[120] E. de Boer, H. Plat, M.G.M. Tromp, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer, H.E. Schoemaker and R. Wever, Biotechnol. Bioeng., in press

2 THE CHLORINATION OF BARBITURIC ACID AND SOME OF ITS DERIVATIVES BY CHLOROPEROXIDASE*

2.1 INTRODUCTION

Chloroperoxidase from *Caldariomyces fumago* (EC 1.11.1.10, chloride: hydrogen-peroxide oxidoreductase) is a widely studied enzyme capable of halogenating a variety of organic compounds by means of hydrogen peroxide and chloride, bromide, or iodide ions. Examples are β-dicarbonyl species such as monochlorodimedon [1,2], alkenes [3-5] cyclopropanes [6] and the heterocycles thiazole [7], antipyrine [8] and NADH [9].

The reaction mechanism of the enzyme is still a matter of controversy. There is some evidence that a complex is formed involving an oxidized form of the enzyme, a halide ion, and the organic substrate [10,11]. Other results imply that the halogenation is performed by an enzyme-made hypohalous acid [12,13]. A third mechanism was published recently involving hypohalous acid or elemental halogens and radical intermediates [14].

Due to our continuing interest into the chemistry of heterocycles and the use of (immobilized) enzymes in organic syntheses [15] we investigated the potential application of chloroperoxidase (CPO) in heterocyclic chemistry. In this paper, we wish to present the results on the CPO-mediated halogenation of barbituric acid and some of its derivatives. Moreover, we want to report on our studies concerning the kinetics of these halogenation reactions and on their implications for the enzymatic reaction mechanism. Part of this work has already been published as a preliminary communication [16].

2.2 MATERIALS AND METHODS

General

Chloroperoxidase (the crude type) from *Caldariomyces fumago* was obtained from Sigma Chemical Company or was a gift from E. de Boer and Dr. R. Wever from the Laboratory of Biochemistry, University of Amsterdam, The Netherlands. The specific activity of the enzyme was 400-600 μmol monochlorodimedon(MCD).mg

---

* Adapted from: M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70.

protein^{-1}.min^{-1}, depending on the batch used. Protein concentrations were determined by the Lowry method [17] using bovine serum albumin as standard. Monochlorodimedon was also purchased from Sigma. 1,3-Dimethylbarbituric acid was obtained from Fluka and the other substituted barbituric acids were synthesized from the corresponding malonic ester and urea derivatives according to the procedure of Dickey and Gray [18]. 5-Chlorobarbituric acid and 5,5-dichlorobarbituric acid were obtained by means of Bock's method [19]. Sodium hypochlorite solution was purchased from Janssen Chimica and was assayed by injecting an appropriate amount in 0.1 mM MCD pH 2.7. The difference in A_{278} is proportional to the hypochlorite concentration, using $\Delta\varepsilon$=12,200 $M^{-1}.cm^{-1}$. All chemicals were of the highest commercial grade.

UV-spectra and kinetic measurements were performed on an Aminco-Chance DW-2 split-beam spectrophotometer. Mass spectra were recorded on an AEI MS 902 instrument or a VG-Micromass 7070 F apparatus (direct probe mode). Circular dichroism spectra were run on a Jobin-Yvon Auto-Dichrograph Mark V. HPLC-analysis was carried out on a Varian 5000 instrument (see below for experimental details).

HPLC-measurements

The reaction of CPO with barbituric acid and its derivatives could be monitored by HPLC using reversed phase - ion pair-chromatography. The stationary phase was a Spherisorb-S 10 ODS column. The mobile phase was a mixture of 20% methanol and 80% water containing 10 mM potassium phosphate and 5 mM nonyltrimethylammonium bromide pH 7.3 (28% methanol for **1e**). The eluent flow was 1.0 ml/min. An aliquot of the enzymatic reaction was taken and directly injected into the chromatograph. The barbituric acids were detected by means of their UV-absorption at 265 nm (245 nm for **1c**).

Kinetic measurements

Specific activities of the enzyme were determined under the standard assay conditions [2]: 100 mM H_3PO_4/KOH pH 2.7, 20 mM KCl, 0.24 mM H_2O_2, 0.1 mM MCD, 25°C. To 2.5 ml of this solution was added 100-150 ng CPO and the absorption at 278 nm was followed, using $\Delta\varepsilon$=12,200 $M^{-1}.cm^{-1}$. These conditions were also applied in all other kinetic measurements and in the experiments where there was no need for isolation of the product; for the compounds **1a-c** 0.48 mM H_2O_2 was used. All determinations were carried out in triplicate. The following molar absorption coefficients ($\Delta\varepsilon$, $M^{-1}.cm^{-1}$) were used at pH 2.7: 5-chlorobarbituric acid (13,800 at 268 nm), 5-phenylbarbituric acid (15,800 at 267 nm).

Circular dichroism (CD)

2.44 ml of a solution of 50 µM 1-methyl-5-phenylbarbituric acid in 0.1 M H_3PO_4/KOH pH 2.7 containing 20 mM KCl was mixed with 60 µl freshly prepared 10 mM H_2O_2 in doubly distilled water. To this was added 440 ng CPO and the reaction was monitored with a UV-spectrophotometer at 268 nm. When the reaction was complete, a CD-spectrum was recorded between 210 and 330 nm. A reference spectrum was made of the same solution by replacing the hydrogen peroxide solution with 60 µl 0.1 M H_3PO_4/KOH pH 2.7. This spectrum was subtracted from the first one, yielding the final CD-spectrum of the enzymatic conversion.

Isolation of enzymatic products

Since the barbituric acids are much too soluble in water, isolation of the products by direct extraction with organic solvents is not very effective; therefore the halogenation reaction was performed in the following way. The standard reaction medium was replaced by dilute HCl pH 2.7 containing 5 mM KCl and 0.24 mM H_2O_2. When needed some extra H_2O_2 was added. A suitable amount of CPO was injected into the solution, typically 0.5 µg per ml reaction medium containing 1.0 mM barbituric acid derivative. When UV-spectroscopy indicated that the reaction was complete the reaction mixture was lyophilized and the product was extracted from the solid material with distilled methanol. In this way, a more or less salt-free product was obtained, which was analyzed by mass spectroscopy. The mass spectra of all compounds were identical to those of authentic specimen.

Reactions with HOCl

To 1 mmol substrate in water/HCl pH 2.7 or buffer were added 2 mmoles (**1a–c**) or 1 mmol (**2d,e**) of HOCl in four portions with stirring. The pH of the solution was maintained at 2.7 by adding dilute HCl when needed. After 15 min incubation the reaction mixture was either lyophilized and extracted with distilled methanol or analyzed by HPLC (see above).

2.3 RESULTS

2.3.1 Reaction of CPO with barbituric acid and its derivatives

When barbituric acid (**1a**) is incubated with CPO under standard conditions, a rapid change in the UV-spectrum is visible (see Fig. 2.1). During the first

two minutes of the reaction the original absorption peak of **1a** at 258 nm shifts to a higher wavelength (268 nm) with a concomitant increase of the absorption intensity. After this period the peak at 268 nm slowly disappears, indicating the consecutive formation of a second product (peak at 210 nm, not shown in the Figure). The same observation is made when monitoring the reaction by reversed-phase ion-pair HPLC (see Fig. 2.2). At t=0 min, only a peak of **1a** appears (t_r=4.3 min). After 0.5 min reaction time, the peak of the first product becomes visible (t_r=5.2 min) and at t=2 min, the second product starts to form (t_r=13.6 min). After 8 min reaction time no further changes in the chromatogram are observed, and only a large peak of the second product together with a small one of the primary compound are visible [20].

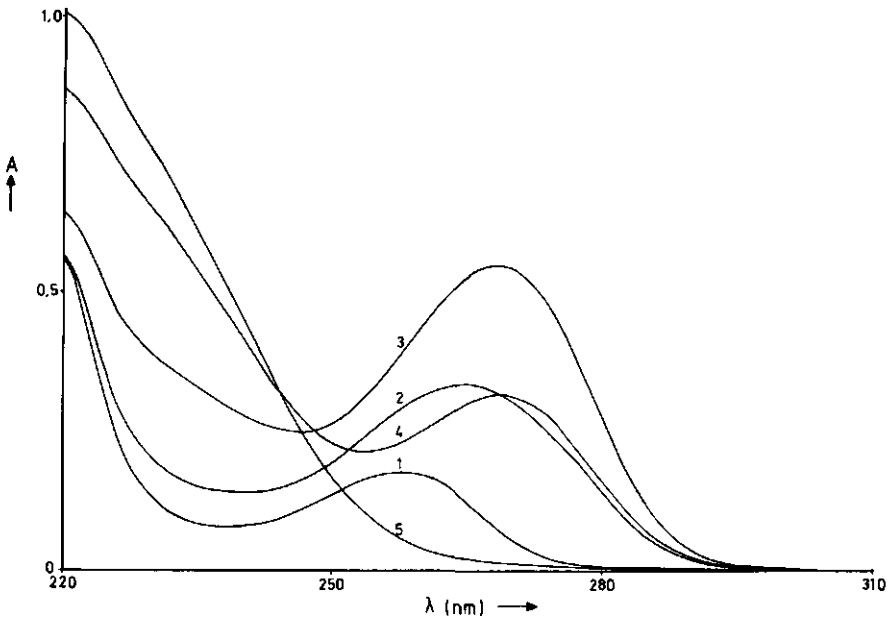


*Figure 2.1 UV-absorption spectra, as recorded during the reaction of barbituric acid (**1a**) with chloroperoxidase. The quartz cuvette was filled with 2.5 ml of a solution containing 0.1 mM **1a**, 0.48 mM H_2O_2, 20 mM KCl and 0.1 M H_3PO_4/KOH pH 2.7. To this, 114 ng of CPO was added and UV-spectra were recorded with appropriate time intervals. Trace 1: t=0 min (pure **1a**); trace 2: t=0.5 min; trace 3: t=2.0 min; trace 4: t=5.0 min; trace 5: t=8.0 min (reaction is complete).*

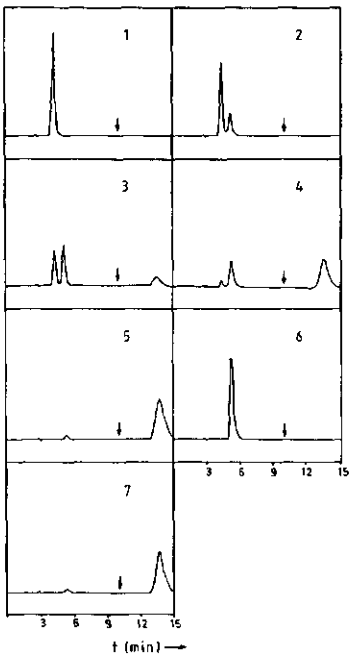
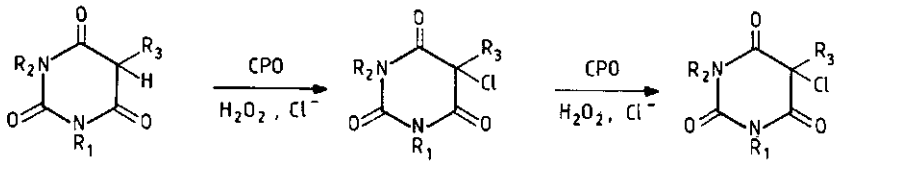


Figure 2.2 *HPLC-chromatograms, as recorded during the reaction of* ***1a*** *with CPO. See Figure 2.1 for experimental details. 10 µl of the enzymatic reaction medium was injected directly into the chromatograph (see Materials and Methods for chromatographic conditions). Eight minutes after each injection, the absorption scale was changed from 0.055 to 0.005 AUFS (indicated by arrow). Explanation of the chromatograms: 1: t=0 min (pure* ***1a****); 2: t=0.5 min; 3: t=2.0 min; 4: t=5.0 min; 5: t=8.0 min (reaction is complete); 6: authentic* ***2a****; 7: authentic* ***3a****.*

The UV-spectra and the retention times of the products coincide perfectly with authentic 5-chlorobarbituric acid (**2a**) and 5,5-dichlorobarbituric acid (**3a**), indicating that **1a** undergoes chlorination at C-5. That these compounds are indeed formed is confirmed by the mass spectra of **2a** and **3a**, isolated from the enzyme-mediated chlorination of **1a**, which were fully identical to those obtained from authentic samples. The reaction sequence is depicted in Scheme 2.1. Additional evidence for the suggested reaction path is given by the fact

that authentic **2a** is converted by the enzyme into **3a** at a high rate, showing that the first chlorination takes place at C-5 and a subsequent chlorination at the same position. The yield of **3a** is 99%, based on the HPLC-measurements. No N-halogenated product is detectable; this is supported by our observation that cyanuric acid (**4**) shows no reactivity towards the enzyme.



1

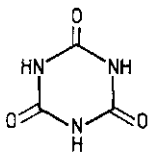
a. R_1, R_2, R_3= H
b. R_1 = CH_3, R_2, R_3 = H
c. R_1, R_2 = CH_3, R_3 = H
d. R_1, R_2 = H, R_3 = C_6H_5
e. R_1 = CH_3, R_2 = H, R_3 = C_6H_5

2

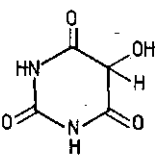
a. R_1, R_2, R_3 = H
b. R_1 = CH_3, R_2, R_3 = H
c. R_1, R_2 = CH_3, R_3 = H
d. R_1, R_2 = H, R_3 = C_6H_5
e. R_1 = CH_3, R_2 = H, R_3 = C_6H_5

3

a. R_1, R_2 = H, R_3 = Cl
b. R_1 = CH_3, R_2 = H, R_3 = Cl
c. R_1, R_2 = CH_3, R_3 = Cl



4



5

*Scheme 2.1 Reaction pattern of the halogenation of barbituric acid (**1a**) and some of its derivatives by CPO/H_2O_2/Cl^-. Cyanuric acid (**4**) and 5-hydroxybarbituric acid (**5**) were not chlorinated by the enzyme.*

Comparison of the enzyme-mediated chlorination with the standard chemical procedures for chlorination of **1a** [19,21,22] show that the yield is higher in the enzymatic reaction [23]. However, pure 5-monochlorobarbituric acids like **2a-c** cannot be prepared directly by the enzymatic procedure: if the reaction is stopped halfway or if only one equivalent of H_2O_2 is used a mixture of starting material, monochloro and dichloro product is obtained. It has been reported that by combining the enzymatic conversion with an electrochemical reduction, the initially produced **3a** can be reduced quantitatively into **2a** in one step,

allowing the isolation of pure **2a** [24,25;Chapter 3 of this thesis].

The enzymatic chlorination reaction was also investigated with substituted barbituric acids as substrates. 1-Methylbarbituric acid (**1b**) and 1,3-dimethylbarbituric acid (**1c**) are converted via their 5-chloro derivatives **2b** and **2c** into the 5,5-dichloro compounds **3b** and **3c** (see Scheme 2.1) in yields comparable to those obtained with **1a** (see Table 2.1). The 5-phenylbarbituric acids **1d** and **1e** are also found to be very good substrates for the enzyme, giving the corresponding 5-chloro derivatives **2d** and **2e** respectively, in very good yields. Remarkably, 5-hydroxybarbituric acid (**5**) seems to resist the enzymatic chlorination reaction, since the UV-spectrum of **5** did not show any change.

*Table 2.1 Yields of the enzymatic and chemical chlorination of **1a** and some of its derivatives.*[a]

Starting material	Product	Yield (%) CPO/H_2O_2/Cl^-	Yield (%) HOCl
1a	**3a**	99	90
1b	**3b**	98	85
1c	**3c**	94	67
1d	**2d**	98	50[b]
1e	**2e**	98	94

[a] The yields are determined by HPLC.

[b] Could be increased to 72% by adding extra 0.4 equiv. HOCl.

2.3.2 *Kinetics of the CPO-mediated chlorination of barbituric acids*

To gain further insight into the mechanism of the enzyme-mediated reaction, we studied the kinetics of the chlorination of barbituric acid (**1a**) and some of its derivatives (**1b-e**).

Comparison of the enzymatic chlorination rate of MCD with those determined with **1d** and **2a** shows that the velocities of the three reactions differ only to a very small extent (see Table 2.2). The presence of methyl groups on the barbituric acid nitrogen atoms also has no effect on the rate of chlorination (Table 2.3). Since we deal with two competing reactions (**1** → **2** and **2** → **3**) we determined the total time in which the reaction **1** → **3** is completed. The results thus obtained indicate that the barbituric acids show considerable flexibility

in their structure regarding their reactivity towards CPO. It appears that they do not influence the reaction rate, suggesting that the active site of the enzyme can accommodate compounds with rather different electronic and steric influences. This result is further supported by the fact that the concentration of neither MCD, nor of **1d** and **2a** in the range of 2 to 100 μM has any effect on the enzymatic reaction rate, so no K_m value could be obtained for these substrates.

Table 2.2 Relative reaction rates of the enzymatic chlorination of various substrates.[a]

Compound	rel. reaction rate[b]
MCD	100±3[c]
1d	98±6
2a	110±5

[a] Determined under standard assay conditions (see Materials and Methods)

[b] Arbitrary units; the value for MCD was set at 100; n=2; each value is the mean of three determinations.

[c] Corresponds to 400 μmol MCD.mg protein^{-1}.min^{-1}.

*Table 2.3 Time needed for complete conversion (**1** → **3**) of some barbituric acids.*[a]

compound	conversion time[b]
1a	100±3[c]
1b	101±4
1c	111±4

[a] Determined under standard assay conditions by monitoring the UV-absorption at 268 nm, except that $[H_2O_2]$=0.48 mM.

[b] Arbitrary units; the value for **1a** was set at 100; n=2; each value is the mean of three determinations.

[c] Corresponds to 150 sec.

Additional information is obtained from inhibition experiments. As mentioned previously, cyanuric acid (**4**) and 5-hydroxybarbituric acid (**5**) are not reactive towards the enzyme. Since both compounds contain the β-dicarbonyl function common to all good substrates for CPO, there is the possibility of a non-productive binding. However, we find that **4** and **5** in concentrations up to 200 μM are not able to inhibit the enzymatic chlorination of MCD.

2.3.3 Stereochemistry of the chlorination reaction

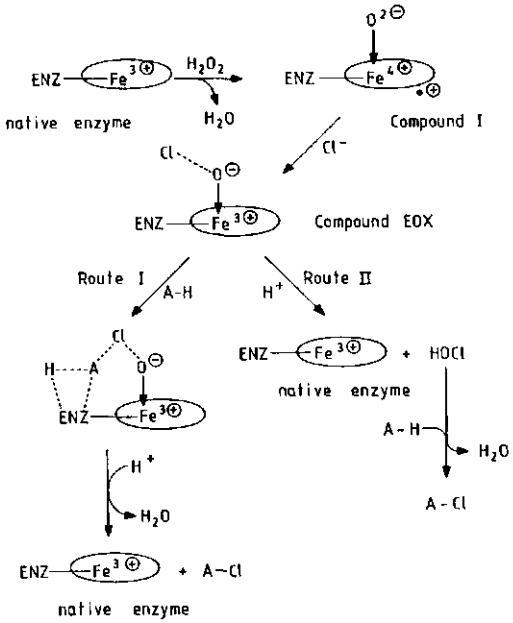
To determine whether the enzyme-mediated halogenation shows some stereoselectivity, we investigated the halogenation of 1-methyl-5-phenylbarbituric acid (**1e**). Compound **1e** has a chiral C5-atom. Resolution of the two enantiomers in **1e** is not possible, however, because of the rapid enolization at C5 in protic solvents. When the hydrogen at C5 in **1e** is replaced by a halogen atom, the configuration is fixed and possibly one isomer is formed preferentially in a stereoselective reaction. To examine whether CPO is capable of stereoselective halogenation, the enzymatic chlorination of **1e** is monitored by means of circular dichroism. However, the CD spectrum shows no absorption, clearly indicating that a racemic mixture is obtained and thus no stereoselective reaction has occurred. This result is in agreement with investigations performed by other authors [3,4,26].

2.3.4 Reactions with HOCl

When the barbituric acids are incubated with HOCl, we find that the products are the same as in the enzymatical reactions, but yield and purity are generally much lower: **1a-c** give mixtures of **2a-c** and **3a-c**. The results are summarized in Table 2.1. The yields of the 5,5-dichlorobarbituric acids are not improved by adding more than 2 equivalents HOCl, because this results in partial degradation of the heterocyclic compounds.

2.4 DISCUSSION

The chloroperoxidase from *C. fumago* is, as shown here, capable of smoothly converting barbituric acid and some of its derivatives into the corresponding 5-chloro or 5,5-dichloro compounds. The very high yields in which the products are obtained make the enzymatic reaction competitive with chemical syntheses.



Scheme 2.2 *The reaction mechanism of* Caldariomyces fumago *chloroperoxidase. The protein part of the enzyme is represented by ENZ, the heme group is depicted as* Fe^{x+} *(x=3 or 4) in the centre of an ellipse, for reasons of clarity. In Compound I, the iron atom has the 4+ oxidation state and the porphyrin ring is oxidized to a radical cationic species. Covalent bonds which are generally accepted in literature are indicated by solid lines between the atoms; if there is any doubt dashed lines are used. A-H = organic substrate, A-Cl = chlorinated product.*

The reaction mechanism of the enzyme is depicted in Scheme 2.2. Native CPO is transformed by H_2O_2 to an enzyme form named Compound I. This highly oxidized species contains an iron (IV) ion and a radical cation located in the porphyrin ring of the heme group [27]. Compound I is reduced by Cl^- to a species called Compound EOX, containing an oxygen atom which is attached to the iron and a chlorine atom which is located in the proximity of the heme, but is not an iron ligand [28]. Two alternative pathways are advanced concerning the following

steps: route I, suggesting that the organic substrate (AH) binds to the enzyme to form a complex which splits into the native enzyme, the organic product and a molecule of water, and/or route II, in which Compound I decomposes to native CPO and a molecule of hypochlorous acid, being the active halogenating species in this reaction.

Evidence for the occurrence of route I was based on kinetic measurements: i) the ratio of the reaction velocities of thiourea and MCD was different for $CPO/H_2O_2/Cl^-$ and HOCl [11]; ii) the reaction constant for the enzymatic conversion of MCD was 200 times higher than the reaction constant for MCD and HOCl [10]; iii) a K_m value was found for MCD and 2-methyl-4-propylcyclopentanedione [4], indicating some kind of interaction between the organic substrate and the enzyme active site [29].

Support for Route II comes from the fact that the products obtained from both enzymatic and HOCl reactions [2,5,6] as well as their ratios in case of mixture [13], are identical. No stereoselectivity was found with the enzymatic reactions [3,4,26]. Without addition of an organic substrate, the enzyme produces HOCl [11] and studies on the catalase reaction of the enzyme also pointed to HOCl-involvement [12]. Recently, a combination of free radicals and molecular halogens was suggested to play a role in the halogenation reaction [14].

The results, presented in this publication, show that CPO is an enzyme with a broad substrate specifity. Barbituric acid (**1a**) and a number of its derivatives are chlorinated by the enzyme in very good yields. HOCl has the same regioselectivity as CPO, since with HOCl the same chloro compounds are obtained, although both yield and purity of the products are usually far lower than in the enzymatic reaction. In contrast to what is reported in the literature [4] the concentration of MCD (or any barbituric acid derivative, not shown) has no influence on the enzymatic reaction rate in the range 2-100 μM. Moreover, even the nature of the organic substrate is not important to the enzyme, as can be concluded from the results mentioned in Tables 2.2 and 2.3 [30]. Potential inhibitors such as **4** and 5 have no effect, and the enzyme-mediated chlorination of **1e** is not stereoselective.

Although it is tempting to conclude from our work that CPO from *Caldariomyces fumago* reacts via route II, generating free HOCl, caution should be exercised. The fact that both CPO and HOCl show the same regioselectivity and complete lack of stereoselectivity cannot be considered as a definite proof for enzymatically generated free HOCl, since it remains possible that the enzyme has a HOCl-like reactivity, for instance via heme-bound hypochlorite. In addition, most studies mentioned in the literature deal with substrates which are

far less reactive than MCD and which use much more enzyme and longer reaction times than is necessary for standard assay conditions. It is possible that under reaction conditions in which the enzyme cannot find a suitable substrate, HOCl is produced, as if there were no substrate at all.

The fact that **1e** is not chlorinated stereoselectively cannot be considered as evidence for route I, because the difference between the enantiotopic faces of the substrate is possibly too small to exert influence. Moreover, it is questionable whether stereoselectivity is an intrinsic property of the enzyme, as shown in the conversion of cyclopentanedione (its natural substrate [31]) to caldariomycin, in which there is no need for stereoselective control.

The kinetic measurements, presented in this study, do not allow a decision whether route I or II is most favoured either. The fact that the concentration of the organic substrate has no influence on the rate-determining step does not exclude route II. The reaction between MCD or barbiturates and HOCl is possibly so fast that the first two steps in the enzymatic reaction sequence, which are independent of the organic substrate concentration, determine the overall reaction velocity. The same reasoning is valid for route I: if step 1 or 2 is much slower than the subsequent steps, the concentration of the organic substrate will not affect the overall reaction velocity. The fact that the rate constant for the enzymatic chlorination step could only be determined under non-standard assay conditions with stopped-flow techniques is evidence for this view [10]. A K_m value for MCD is reported [4], but our results indicate that it cannot have been determined at pH 2.7 using 20 mM KCl and 0.24 mM H_2O_2.

We conclude that most of the literature data concerning the reaction mechanism of CPO, including our own, do not permit a choice between route I or route II. However, even if route II is proven to be correct, the steady-state concentration of HOCl in the reaction medium is so low that CPO is still a smooth halogenating agent allowing the synthesis of various chlorinated barbituric acids in very high yields. Therefore the use of chloroperoxidase as a synthetic tool in organic chemistry will remain a subject of current investigation.

2.5 REFERENCES AND NOTES

[1] L.P. Hager, D.R. Morris, F.S. Brown and H. Eberwein, J. Biol. Chem. **241** (1986), 1769-1777

[2] P.F. Hollenberg and L.P. Hager, Meth. Enzymol. **52** (1978), 521-529

[3] J. Geigert, S.L. Neidleman, D.J. Dalietos and S.K. DeWitt, Appl. Environ. Microbiol. **45** (1983), 366-374
[4] K. Ramakrishnan, M.E. Oppenhuizen, S. Saunders and J. Fisher, Biochemistry **22** (1983), 3271-3277
[5] H. Yamada, N. Itoh and Y. Izumi, J. Biol. Chem. **260** (1985), 11962-11969
[6] J. Geigert, S.L. Neidleman and D.J. Dalietos, J. Biol. Chem. **258** (1983), 2273-2277
[7] S.L. Neidleman, A.I. Cohen and L. Dean, Biotechnol. Bioeng. **11** (1969), 1227-1232
[8] P.L. Ashley and B.W. Griffin, Arch. Biochem. Biophys. **210** (1981), 167-178
[9] B.W. Griffin and R. Haddox, Arch. Biochem. Biophys. **239** (1985), 305-309
[10] A.M. Lambeir and H.B. Dunford, J.Biol.Chem. **258** (1983), 13558-13563
[11] R.D. Libby, J.A. Thomas, L.W. Kaiser and L.P. Hager, J. Biol. Chem. **257** (1982), 5030-5037
[12] B.W. Griffin, Biochem. Biophys. Res. Commun. **116** (1983), 873-879
[13] T.D. Lee, J. Geigert, D.J. Dalietos and D.S. Hirano, Biochem. Biophys. Res. Commun. **110** (1983), 880-883
[14] B.W. Griffin and P.L. Ashley, Arch. Biochem. Biophys. **233** (1984), 188-196
[15] *Viz.* our work on aldehyde oxidase: S.A.G.F. Angelino and H.C. van der Plas, *"Bio-Organic Heterocycles"*, pp. 285-317 (H.C. van der Plas, L. Ötvös, and M. Simonyi, Eds.), Akadémiai Kiadó, Budapest, 1984, and our work on xanthine oxidase: H.S.D. Naeff, H.C. van der Plas, J. Tramper and F. Müller, Quant. Struct.-Act. Relat. **4** (1985), 161-166, and references cited therein.
[16] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas, **103** (1984), 99-100
[17] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J.Biol.Chem. **193** (1951), 265-275
[18] J.B. Dickey and A.R. Gray, Org. Syn. **18** (1938), 8-9
[19] W. Bock, Ber. Dtsch. Chem. Ges. **56** (1923), 1222-1227
[20] It should be noted that the described chromatographic system separates various barbituric acid derivatives excellently, whereas other stationary phases (silica, silica modified with propionitril or octadecyl groups, or ion exchange columns) either give no separation or unacceptably long retention times.
[21] A.K. Macbeth, T.H. Numan and D. Traill, J. Chem. Soc. (1926), 1248-1253
[22] E. Ziegler, R. Salvador and Th. Kappe, Monatsh. Chem. **93** (1962), 1376-1382
[23] E.g., **3a** was synthesized by treating **1a** with chlorine in water [19,21]. No yield was given, but in our hands it was 56%.
[24] C. Laane, W. Pronk, M.C.R. Franssen and C. Veeger, Enzyme Microb. Technol. **6** (1984), 165-168
[25] C. Laane, A. Weyland and M.C.R. Franssen, Enzyme Microb. Technol. **8** (1986), 345-348
[26] J. Kollonitsch, S. Marburg and L.M. Perkins, J. Am. Chem. Soc. **92** (1970), 4489-4490
[27] R. Rutter, L.P. Hager, H. Dhonan, M. Hendrich, M. Valentine and P. Debrunner, Biochemistry **23** (1984), 6809-6816
[28] G.E. Krejcarek, R.G. Bryant, R.J. Smith and L.P. Hager, Biochemistry **15** (1976), 2508-2511
[29] These data are most probably incorrect, *vide infra*.
[30] The fact that **4** and **5** are not converted by the enzyme seems contradictory to this conclusion; however, neither are these compounds halogenated by HOCl, so these results do not support any mechanism.
[31] J.R. Beckwith and L.P. Hager, J. Biol. Chem. **238** (1963), 3091-3094

3 BIOELECTROSYNTHESIS OF 5-CHLOROBARBITURIC ACID USING CHLOROPEROXIDASE*

3.1 INTRODUCTION

To date, the main objective of bioelectrochemical devices is either the generation of electricity from relatively cheap organic compounds [1], the development of sensitive detectors for (bio)chemicals [2] or the synthesis of (bio)chemicals. With respect to the last application two types of bioelectrochemical systems can be distinguished: the bioelectrochemical fuel cell and the bioelectrolytical cell (Fig. 3.1). In both cell systems an enzymatic reaction in one of the half cells is coupled to redox reactions in the other half cell via an electron mediator.

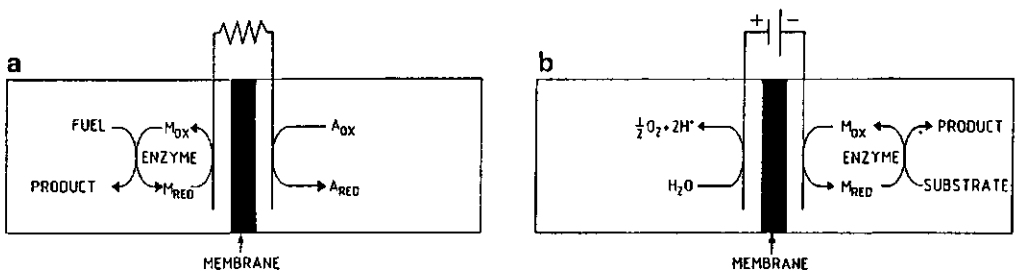


Fig. 3.1 *Types of bioelectrochemical cells. a). Bioelectrochemical fuel cell; b) Bioelectrolytical cell. M, electron mediator; A, electron acceptor; ox, oxidized; red, reduced.*

Basically, a fuel cell consists of two compartments, each with an electrode and separated by an ion-conducting medium. In an electrochemical fuel cell compounds such as H_2, CH_3OH, NH_2-NH_2, NH_3 or CO are oxidized at the anode; the electrons thus generated are used for the reduction of O_2 or H_2O_2 at the cathode [1,3]. These cells have mostly been used for the generation of elec-

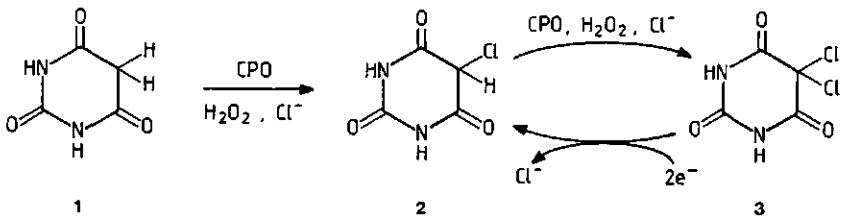
---

* Adapted from:
C. Laane, W. Pronk, M.C.R. Franssen and C. Veeger, Enzyme Microb. Technol. **6** (1984), 165 - 168, and
C. Laane, A. Weijland and M.C.R. Franssen, Enzyme Microb. Technol. **8** (1986), 345 - 348

tricity [1,4]. In a bioelectrochemical fuel cell biochemicals such as glucose are oxidized by means of free or immobilized biocatalysts, yielding not only electricity but also useful chemical products such as gluconic acid.

The main difference between a bioelectrochemical fuel cell and a bioelectrolytical cell is that in the former the electric energy is generated *in situ*, whereas in the latter electricity is supplied externally by a potentiostat, which makes the system more versatile.

In this study we demonstrate the electro-enzymatic conversion of barbituric acid (**1**, see Scheme 3.1) to 5-chlorobarbituric acid (**2**) in a bioelectrochemical fuel cell and a bioelectrolytical cell using the chloroperoxidase from the mold *Caldariomyces fumago*.



Scheme 3.1 *Barbituric acid (**1**) is chlorinated by chloroperoxidase to 5-chlorobarbituric acid (**2**) which is subsequently converted by the same enzyme into 5,5-dichlorobarbituric acid (**3**). The enzyme needs hydrogen peroxide and chloride ions as cosubstrates. The final product is **2** because **3** is reduced at the electrode. CPO, chloroperoxidase.*

3.2 MATERIALS AND METHODS

Materials.

Aspergillus niger D-glucose oxidase (α-D glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) and all enzymes required for analytical purposes were purchased from Boehringer. *Caldariomyces fumago* chloroperoxidase (chloride:hydrogen peroxide oxidoreductase, EC 1.11.1.10) was obtained from Sigma. 5-chloro- and 5,5-dichlorobarbituric acid were synthesized according to Bock [5]. All other (bio)chemicals were analytical grade and obtained from commercial sources. The gold electrode (99.99%, 50x30x2 mm) and the platinum-gauze electrode (50 mesh,

50x15 mm) were supplied by Drijfhout & Zoon's, Amsterdam, The Netherlands, and graphite felt (RVG 2000) was from Le Carbone-Lorraine. The Nafion perfluorinated membrane and the hollow fibre unit (TAF 100 from Terumo, number 82310A) were generous gifts of Dupont and dr. P. Kerkhof of the Department of Process Engineering, respectively. The membrane surface of the hollow fibre was 0.8 m^2, the total volume ± 80 ml.

Bioelectrochemical fuel cell.

The cell consisted of two 7 ml gastight compartments made of glass and interconnected by a 3 cm long salt bridge (3 M KCl in 1.5% agar-agar). The electrodes were rolled into cylinders and thoroughly cleaned with acetone prior to use. The anaerobic anode compartment contained a solution (4 ml) of 0.1 M potassium phosphate, 0.1 mM dichlorophenol indophenol (DCIP) and 0.4 mg D-glucose oxidase, final pH 8.0. The aerobic cathode compartment contained 0.1 M potassium phosphate and 20 mM KCl, final pH 2.7. Various amounts of barbituric acid and 1 µg chloroperoxidase were added when indicated. Both compartments were stirred rapidly with a magnetic stirrer and kept at 25°C. Reactions were started by adding the appropriate substrate to the anode compartment.

Bioelectrolytical cell.

The electrolytic cell consisted of two 22x1.5x1.5 cm perspex, flow-through compartments filled with graphite felt and separated by a Nafion cation-permeable membrane. To prevent leakage, rubber seals were installed between the compartments and the membrane. The graphite electrodes were soaked for 1 week in a solution of EDTA in doubly distilled water prior to use to remove traces of metal ions. The electrodes were rinsed thoroughly with doubly distilled water afterwards and further cleaned by switching the potential difference ± 40 times from -2.0 V to +2.0 V ending with -2.0 V. The anodic compartment contained 20 mM H_3PO_4/KOH pH 2.75 and the cathodic compartment a solution of barbituric acid which was brought to pH 2.75 by HCl. The cathodic potential was set at -0.5 V unless stated otherwise with a Bank MP 81 potentiostat versus a standard saturated calomel electrode connected to the cathodic compartment via a salt bridge (3 M KCl in 1.5% agar-agar). Under these circumstances the potential difference between the cathode and the anode was -1.65 V.

Analytical methods.

Hydrogen peroxide measurements were performed at pH 5.0 by recording the oxygen concentrations with a Clark oxygen electrode or in a Rank Brothers

Oxygraph, before and after the addition of catalytic amounts of catalase. The concentration of glucose and gluconic acid were determined enzymatically as described earlier [6,7]. The activity of chloroperoxidase was measured using monochlorodimedon as a substrate [8] and the concentrations of barbituric acid and its chloro derivatives were determined spectroscopically or by HPLC [9,10]. The current produced by the fuel cell was measured as described in [2]. The current through the electrolytic cell was measured by a recorder attached to the current output of the potentiostat.

3.3 RESULTS AND DISCUSSION

3.3.1 Bioelectrochemical fuel cell

Our enzyme-containing fuel cell was constructed as follows. In the anode compartment D-glucose oxidase oxidizes D-glucose to D-glucono-1,5-lactone which is non-enzymatically hydrolyzed to gluconic acid, a valuable industrial chemical currently produced by fermentation [11]. The reduced enzyme in turn donates its reducing equivalents to the electron carrier DCIP, which is oxidized at the anode. The electrons thus generated flow through the external circuit to the cathode, where oxygen is reduced to water. Other electron mediators like methylene blue [12] and phenazine ethosulphate [2] can be used instead of DCIP.

Fig. 3.2a shows the time course for current output of the cell under operational conditions. Routinely, currents of ± 350 μA were drawn from the cell but under optimum conditions currents greater than 1 mA can be reached. Factors that influence the current flow are the ohmic resistance of the cell, the electrode surface, the mediator concentration and the stirring rate. The open circuit potential of the cell is 0.6 V. Fig. 3.2b shows the time course for D-glucose conversion. These results demonstrate that D-glucose is converted exclusively and completely into gluconic acid and that the total amount of current passing through the circuit is directly proportional to the amount of D-glucose added.

When we used a gold instead of a platinum electrode, hydrogen peroxide was generated at the cathode instead of water at a rate of 0.1 μmol H_2O_2 $h^{-1}.ml^{-1}$. This gave the opportunity to add another enzymatic reaction to the system because hydrogen peroxide is a substrate for some oxidoreductases. We chose the enzyme chloroperoxidase and barbituric acid (**1**, see Scheme 3.1) was taken as a

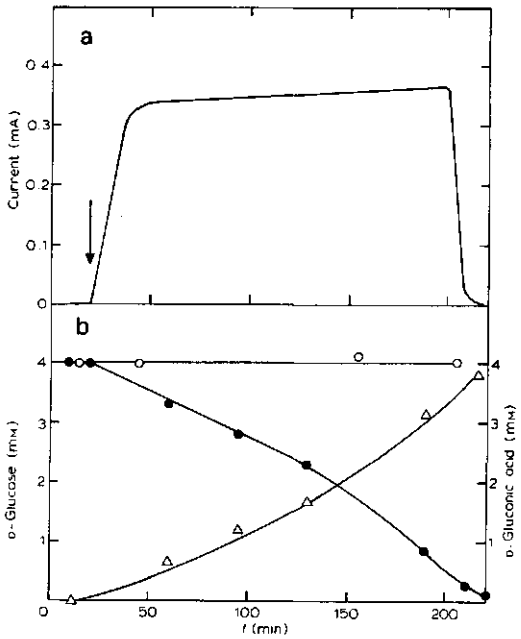


Fig. 3.2 *Performance of a D-glucose oxidase containing bioelectrochemical cell. Experiments were performed as described in the Materials and Methods section. D-glucose was added to the anode compartment at the time indicated by the arrow. a). Time course of current output. b) Time course of: ●, D-glucose consumption; △, D-gluconic acid formation; o, control, D-glucose concentration in the absence of DCIP.*

model substrate for our purpose, because it is known that chloroperoxidase chlorinates this compound and some of its derivatives very rapidly and efficiently [9,10]. Fig. 3.3 shows the reactions taking place in such a combined enzymatic fuel cell. With D-glucose as fuel, three products are formed: gluconic acid, 5-chlorobarbituric acid (**2**) and electricity. Other (bio)chemicals can also be prepared simply by using a different oxidase and peroxidase, together with their respective substrates. Other, cheaper, fuels like sucrose and cellulose can also be used when additional enzymes are added to the anodic compartment, e.g. invertase resp. cellulase [13]. The fact that the chemicals are produced in different compartments facilitates their

isolation although separation of products and enzymes remains necessary. Summarizing, in a bioelectrochemical cell optimum use of a fuel can be made due to a combination of chemical, biochemical and electrochemical reactions.

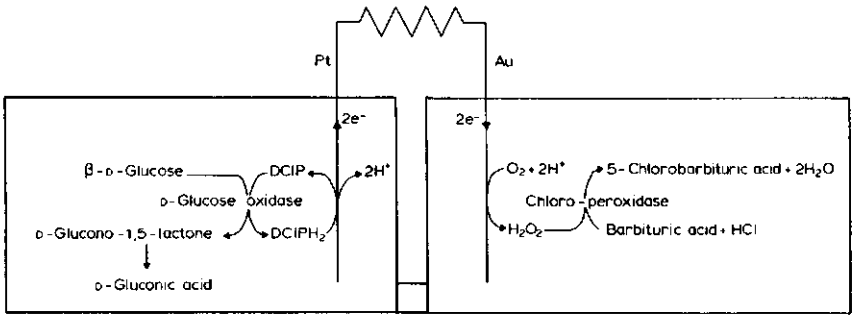


Fig. 3.3 Reactions taking place in a bioelectrochemical cell containing D-glucose oxidase in the anode compartment and chloroperoxidase in the cathode compartment. For explanation see text.

Normally the product of chloroperoxidase action is dichlorobarbituric acid (**3**)[9,10]. Surprisingly, in our cell only the monohalogenated compound could be detected by various techniques such as UV-spectroscopy, TLC and HPLC (Fig. 3.4). A reasonable explanation for this phenomenon is that the enzyme does produce 5,5-dichlorobarbituric acid (**3**) which undergoes a reductive electrochemical dehalogenation. This hypothesis is substantiated by the following observations: i) 3 is rapidly converted in the cathode compartment to **2** in the absence of enzyme; ii) when all 1 has been converted the current is maintained and no hydrogen peroxide is accumulated in the cathodic compartment.

After three days of reaction our small model system had produced 10 mg of gluconic acid and 8 mg of **2**. During this period the current of 60 μA slowly decreased to ± 50% of the original level. Determination of turnover numbers (moles of product formed per mole of enzyme) after three days reaction time gave values of 1.8×10^4 and 1×10^7 for D-glucose oxidase and chloroperoxidase, respectively. These results demonstrate that the enzymes in the redox cycle in Fig. 3.3 operate catalytically.

The great operational stability of our bioelectrochemical cell is in part due to the fact that in the anode compartment no oxygen is present and an artificial electron acceptor is used. The natural acceptor for oxidases is namely oxygen, which is reduced to hydrogen peroxide. Glucose oxidase is

inactivated by the hydrogen peroxide [14] and by a reactive species not yet identified [15]. In our system hydrogen peroxide is formed in the cathode compartment resulting in an increased operational lifetime of the glucose oxidase. The same observations were made by others with glucose oxidase [16] and glucose dehydrogenase [17] using benzoquinone resp. Meldola Blue as artificial electron acceptor.

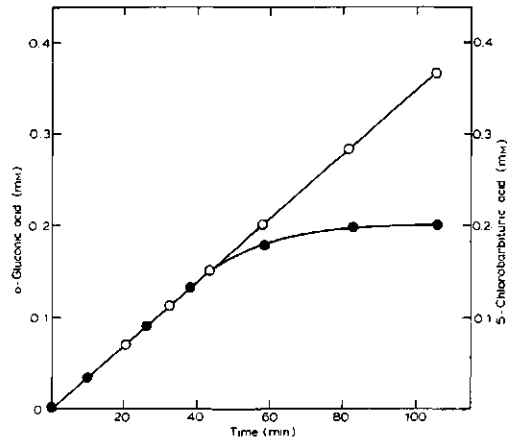


Fig. 3.4 *Performance of a bioelectrochemical cell containing D-glucose oxidase and chloroperoxidase. Experiments were performed as described in the Materials and Methods section. o, Time course of D-gluconic acid production; •, time course of* **2** *formation.*

In 1980 the Cetus Corporation together with the Standard Oil Company described a multi-enzyme process that produces propylene oxide and D-fructose or furfural as coproducts [18,19] (see Ch. 1 Scheme 1.2). D-glucose is used to generate hydrogen peroxide, which in turn is consumed by haloperoxidase for the production of propene resp. ethene halohydrin. In a second step D-glucosone and the halohydrins are converted to D-fructose and propene oxide or ethene oxide, respectively. Although details of this process are not available, some potential disadvantages are: i) limited stability of the oxidase due to the formation of hydrogen peroxide in the presence of the enzyme; ii) a difficult isolation procedure in cases where neither product is gaseous; iii) enzyme mixtures will not operate with maximum activity due to differences in pH optima. These problems could be overcome in our bioelectrochemical cell since the enzymatic reactions occur in different compartments and hydrogen peroxide

is not produced in the vicinity of the hydrogen peroxide-sensitive oxidases.

3.3.2 The bioelectrolytical system

A disadvantage of the bioelectrochemical fuel cell is the low current density that can be obtained, leading to relatively slow conversion rates. A second disadvantage, as already noted above, is the need for separation of the chlorinating enzyme and the formed product in the final reaction mixture. As organic solvents are involved, this process will certainly lead to inactivation of the enzyme. Therefore, another approach was studied, in which the current was applied externally by means of a potentiostat and the enzyme and the electrode processes are separated. The complete, so-called bioelectrolytical system is depicted in Fig. 3.5. The system consists of three interconnected units: a) an electrolytical cell that oxidizes water to oxygen at the anode and reduces oxygen to hydrogen peroxide and **3** to **2** at the cathode using electricity as energy source; b) a hollow-fibre membrane reactor in which the electrolytically produced hydrogen peroxide is used by chloroperoxidase for the chlorination of **1** giving a mixture of **2** and **3**; and c) an anion exchanger that scavenges the **2** produced in the membrane reactor and the anode compartment.

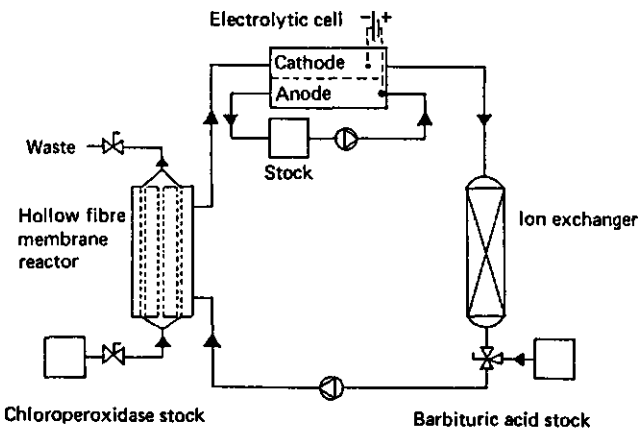


Fig. 3.5 Schematic representation of the complete chloroperoxidase containing bioelectrolytical system producing **2**.

The rate of hydrogen peroxide production by the electrolytical cell was optimized by varying the cathode potential and the flow rate of the medium

through the cell (Fig. 3.6). It was found that the amount of hydrogen peroxide produced per mA is optimal at a cathode potential of -0.5 V and increases with increasing flow rate up to ± 6-8 ml. min^{-1}. Under these circumstances the current efficiency of the cell is between 90 and 95% and the concentration of hydrogen peroxide in the medium is ± 0.07 mM.

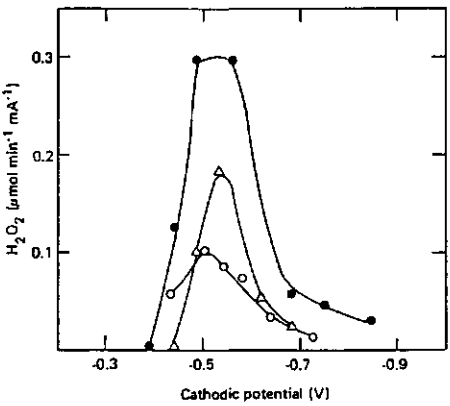


Fig. 3.6
Dependence of the rate of hydrogen peroxide production in the electrolytical cell on the cathodic potential and the flow rate through the cell. Flow rate: o, 1.5 ml.min^{-1}; Δ, 4.3 ml. min^{-1}; •, 5.6 ml.min^{-1}.

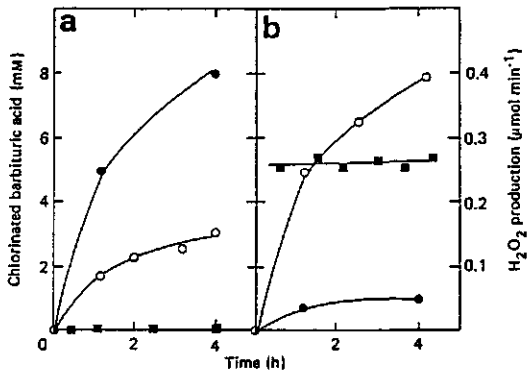


Fig. 3.7
*Time course of the formation of **2**, **3** and hydrogen peroxide. a) After the hollow fibre reactor; b) After passage through the electrolytical cell. The complete system was used without an anion exchanger. o, **2**; •, **3**; ▪, hydrogen peroxide.*

The enzyme-containing hollow fibre membrane reactor was then connected to the electrolytical cell. Within the fibres chloroperoxidase first converts **1** into **2** and subsequently into **3** (Fig. 3.7a). The ratio of **3** to **2** appeared to depend strongly on the concentration of hydrogen peroxide in the system as well as on the residence time of the medium in the reactor and increased with rising hydrogen peroxide concentration and residence time. In practice a residence time of 25 min was taken which corresponds exactly to the time needed to consume all hydrogen peroxide entering the hollow fibre unit. Under these circumstances the concentration of hydrogen peroxide in the system was relatively low (± 0.07 mM) and the flux of reactants through the system was relatively high

(4 ml.min^{-1}). Hence chloroperoxidase catalysis was least hindered by hydrogen peroxide inactivation [20].

Fig. 3.7b shows the time course of the formation of **2**, **3** and hydrogen peroxide after passage of the solution leaving the reactor through the electrolytic cell. As expected the rate of hydrogen peroxide production returned to the original level of 0.27 μmol.min^{-1}. After one passage through the cell the concentration of **3** had dropped 10-fold whereas the concentration of **2** had increased by the same magnitude. Thus, it appeared that the electrochemical reduction of **3** to **2**, which was previously observed when using a gold electrode, also occurred at the surface of a carbon felt electrode. All reactions occurring in the system are summarized in Scheme 3.2. The net result is that one mol of **1** is converted to one mol of **2** using half a mol of oxygen and one mol of hydrogen chloride; besides **2** one mol of water is produced.

CATHODIC REACTIONS

$4e^- + 4H^+ + 2O_2 \longrightarrow 2H_2O_2$

$1 + H_2O_2 + H^+ + Cl^- \xrightarrow{CPO} 2 + 2H_2O$

$2 + H_2O_2 + H^+ + Cl^- \xrightarrow{CPO} 3 + 2H_2O$

$3 + 2e^- + H^+ \longrightarrow 2 + Cl^-$

overall

$1 + 2O_2 + 7H^+ + Cl^- + 6e^- \longrightarrow 2 + 4H_2O$

ANODIC REACTIONS

$3H_2O \longrightarrow 1\frac{1}{2}O_2 + 6H^+ + 6e^-$

OVERALL

$1 + \frac{1}{2}O_2 + H^+ + Cl^- \xrightarrow{CPO} 2 + H_2O$

Scheme 3.2 Reactions taking place in the complete bioelectrolytical system. CPO, chloroperoxidase.

In the complete system an anion exchanger was introduced to scavenge the produced 2, thereby facilitating the isolation procedure and preventing the product from entering in the wasteful halogenation-dehalogenation cycle. Specific binding of **2** was achieved at the operational pH of 2.75, since its pK_a value is 0.00 [21], while the pK_a values of **1** and 3 are 4.05 [21] and 5.55 [22], respectively. This enables us to synthesize pure **2** in high yield using chloroperoxidase. Since running the reaction of the enzyme with **1** in water only gives **3** without the ability to stop the reaction at the intermediate stage, we consider the electrochemical procedure to be a useful addition to the methodology of enzymatic organic synthesis.

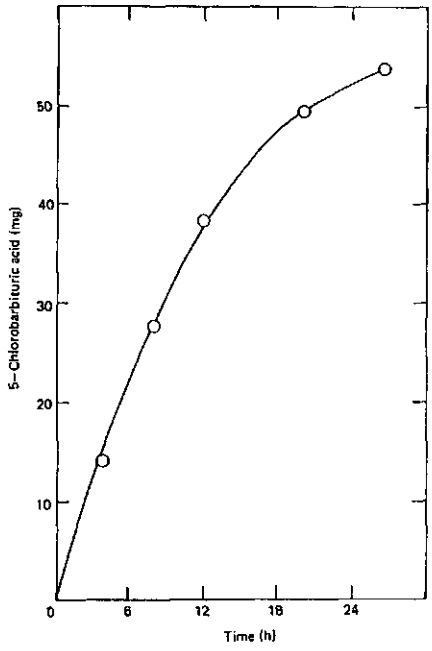


Fig. 3.8 *Time course of **2** production in the complete bioelectrolytical system. The enzyme load was 40 U (1 U represents the amount of enzyme that chlorinates 1 μmol of monochlorodimedon in 1 min at 25°C.).*

Fig. 3.8 shows the time course of the production of **2** in the complete system. The conversion was completed after 24 h and more than 96% of the product could be recovered from the column by applying a salt gradient. During these 24 h the medium circulated through the system 19 times. Based on the

amount of product per mol enzyme, the turnover of chloroperoxidase was about $5x10^5$ times. However, the actual turnover of the enzyme must have been higher, when the turnover of the halogenation-dehalogenation cycle is taken into account. It was estimated from the amount of current passing through the system that approximately half of the electrical energy was spent on the halogenation-dehalogenation process. Hence the actual turnover number of this relatively labile enzyme is $\pm 10^6$ times.

3.3.3. Conclusion

The results obtained with the biochemical fuel cell and the bioelectrolytical system, although preliminary, are encouraging and indicate that combinations of enzymatic and electrochemical reactions can be a useful extension of organic synthetic methodology. Future work will show the scope of the system presented here.

3.4 REFERENCES

[1] J. O'm. Bockris, B.E. Conway, E. Yeager, and R.E. White, in *Comprehensive Treatise of Electrochemistry*, Plenum Press, New York, 1981, vol.3

[2] E.V. Plotkin, I.J. Higgins, and H.A.O. Hill, Biotechnol. Lett. **3** (1981), 187-192

[3] L.B. Wingard Jr., C.H. Shaw, and J.F. Castner, Enzyme Microb. Technol. **4** (1982), 137-142

[4] K. Lewis, Bacteriol. Rev. **30** (1966), 101-112

[5] W. Bock, Ber. Dtsch. Chem. Ges. **56** (1923), 1222-1227

[6] H.U. Bergmeyer, E. Bernt, F. Schmidt, and H. Stork, in *Methods in Enzymatic Analysis* (H.U. Bergmeyer, ed.), Verlag Chemie, Weinheim, and Academic Press, New York and London, 1974, pp. 1196

[7] H. Möllering, and H.U. Bergmeyer, in *Methods in Enzymatic Analysis* (H.U. Bergmeyer, ed.), Verlag Chemie, Weinheim, and Academic Press, New York and London, 1974, vol. 3, pp. 1243-1292

[8] P.F. Hollenberg, and L.P. Hager, Meth. Enzymol. **52** (1978), 521-529

[9] M.C.R. Franssen, and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100

[10] M.C.R. Franssen, and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70

[11] J. Kominek, in *Biotechnology: A Comprehensive Treatise* (H.J. Rehm, and G. Reed, eds.), Verlag Chemie, Weinheim, 1983, pp. 355-465

[12] J.B. Davis, and H.F. Yarbrough, Science **137** (1962), 615-616

[13] C. Laane, K. Dekker, and C. Veeger, Proc. Third Eur. Congr. on Biotechnol., vol. 1, 1984, Verlag Chemie, Weinheim, 395-399

[14] K. Kleppe, Biochemistry **5** (1966), 139-143

[15] C. Bourdillon, V. Thomas and D. Thomas, Enzyme Microb. Technol. **4** (1982), 175-180

[16] C. Bourdillon, C. Hervagault, and D. Thomas, Biotechnol. Bioeng. **27** (1985), 1619-1622

[17] B. Persson, L. Gorton, G. Johansson, and A. Torstensson, Enzyme Microb. Technol. **7** (1985), 549-552
[18] G. Parkinson, Chem. Eng. (NY) **87** (1980), 37-38
[19] S.L. Neidleman, W.F. Amon Jr., and J. Geigert, US 4,247,641 (1981)
[20] P.D. Shaw, and L.P. Hager, J. Biol. Chem. **236** (1961), 1626-1630
[21] V.I. Slesarev, and B.A. Ivin, J. Org. Chem. USSR **10** (1974), 113-117
[22] R.H. McKeown, J. Chem. Soc. Perkin Trans. II **1980**, 504-514

4 ENZYMATIC HALOGENATION OF PYRAZOLES AND PYRIDINE DERIVATIVES*

4.1 INTRODUCTION

Chloroperoxidase from *Caldariomyces fumago* (CPO; EC 1.11.1.10; chloride: hydrogen peroxide oxidoreductase) is a well-known enzyme, capable of halogenating a great variety of organic compounds by means of hydrogen peroxide and chloride, bromide or iodide ions [1]. However, until recently only a few heterocyclic substrates have been studied: thiazole [2], antipyrine [3], NADH [4] and barbituric acid and some of its derivatives [5]. Since we have an ongoing interest in the use of enzymes in heterocyclic organic synthesis [5,6] we studied the enzymatic chlorination of pyrazole (**1a**), 1-methylpyrazole (**1b**), 3-methylpyrazole (**1c**) and 2-amino-pyridine (**4**) and the bromination of 8-hydroxyquinoline (**6**) [7]. The publication of our results was induced by the appearance of a study on the CPO-mediated halogenation of nucleic bases, in which also pyrazole was mentioned as one of the substrates used [8].

4.2 MATERIALS AND METHODS

General

Chloroperoxidase from *Caldariomyces fumago* (the crude type) was obtained from Sigma Chemical Company or from the Laboratory of Biochemistry, University of Amsterdam, the Netherlands (Dr. Wever). Monochlorodimedon was also purchased from Sigma. 1-Methylpyrazole and the chlorinated pyrazoles were synthesized according to Hüttel [9,10]. Sodium hypochlorite was purchased from Janssen Chimica and was assayed by injecting an appropriate amount of the solution in 0.1 mM monochlorodimedon pH 2.7. The difference in A_{278} is proportional to the hypochlorite concentration, using $\Delta\varepsilon$=12,200 $M^{-1}.cm^{-1}$. All chemicals were of the highest commercial grade, but 2-aminopyridine and 8-hydroxyquinoline were purified, by sublimation and recrystallization from ethanol/water, respectively.

UV-spectra and kinetic measurements were performed on an Aminco-Chance DW-2 split-beam spectrophotometer. Mass spectra were recorded on an AEI MS 902 instrument or a VG-Micromass 7070 F apparatus (direct probe mode). GC/MS

---

* Adapted from: M.C.R. Franssen, H.G. van Boven and H.C. van der Plas, J. Heterocyclic Chem., in press.

spectra were obtained using the VG-Micromass 7070 F gaschromatograph-mass spectrometer. The reaction of pyrazole with CPO was monitored with a Bruker CXP-300 ^{1}H-NMR spectrometer operating at 300.066 MHz, with suppression of the HOD signal. Gaschromatography was performed on a Varian 3000 apparatus, equipped with a fused silica capillary column (L = 30 m); the stationary phase was DB17, film thickness 0.25 μm.

Enzymatic halogenation

For UV-monitoring of the enzymatic halogenation the reaction was performed as follows. 2.44 ml of a solution containing 0.1 mM (0.05 mM for **6**) of organic substrate and 20 mM of potassium halide in 0.1 M H_3PO_4/KOH pH 2.7 was placed in a 3.0 ml quartz cuvette. 60 μl 10 mM H_2O_2 in doubly distilled water (final concentration 0.24 mM) and 280 ng CPO [11] were added and the UV-spectra were recorded at 25°C. It was necessary in the reaction of **1a** to add extra 840 ng of CPO after 2.5 h.

For GLC-analysis the procedure was as follows: 2.5 ml of a solution containing 0.5 mM of organic substrate and 20 mM of potassium halide in 0.1 M H_3PO_4/KOH pH 2.7 was placed in a 10 ml test tube. 300 μl 10 mM H_2O_2 in doubly distilled water (final concentration 1.07 mM) and 1.5 μg of CPO were added. After 20 min incubation at 25°C a 0.5 μl sample was taken and directly injected into the gaschromatograph. It was necessary in the reaction of **1a** to add extra 1.5 μg of CPO after 60 min and again after 120 min; after 135 min a sample was taken for analysis. The GLC oven temperature was 120°C for **1a-c** and 160°C for **4**. The adjusted retention times were: **1a**: 1.0 min; **1b**: 0.6 min; **1c**: 2.6 min; **2a**: 4.4 min; **2b**: 2.1 min; **2c**: 6.5 min; **4**: 1.2 min; **5**: 1.9 min. Analysis of **7** with GLC or HPLC was not possible due to its strong interactions with all tested column materials.

Isolation of enzymatic products

The reaction with the relatively apolar compounds like the pyrazoles and 8-hydroxyquinoline were carried out as follows. To a solution of the substrate (0.5 mM) in 0.1 M potassium phosphate buffer (pH 2.7) which contained 20 mM of potassium halide and 1.07 mM of hydrogen peroxide was added 0.6 μg CPO per ml reaction medium. The conversion of the substrate was monitored by means of UV-spectroscopy or GLC. In the case of **1a-c** the reaction mixture was worked up by adjusting the pH of the solution to 6.0 by adding a dilute solution of potassium hydroxide. The reaction medium was extracted continuously overnight with distilled diethyl ether and after drying and evaporation of the ether, the pure

product was isolated and characterized. In the case of **6** the product separates as a greyish precipitate during the reaction which can be easily filtered off, dried and analyzed.

Since **5** is too water-soluble for direct extraction with organic solvents at low pH and unstable at neutral pH the halogenation reaction was performed in the following way. The standard reaction medium described above was replaced by dilute HCl (pH 2.7) containing 5 mM of potassium chloride and 1.07 mM of hydrogen peroxide. 0,6 µg CPO per ml reaction medium was injected into the solution. During the reaction some extra hydrogen peroxide and CPO were added. After 3 hours of incubation the reaction mixture was lyophilized and the product was extracted from the solid material with distilled methanol. A more or less salt-free product was obtained which could be analyzed.

Analysis data of **5**: MS m/e 128-130 (100%), 101-103 (27), 93 (43), 66 (19). ^{1}H-NMR (in $CDCl_3$): δ 4.8 (br s, NH_2), δ 6.62 (dd, H_5, J_{45} = 8.1 Hz, J_{56} = 5.2 Hz), δ 7.48 (dd, H_4, J_{46} = 1.5 Hz), δ 7.97 (dd, H_6).

GC/MS study on the enzymatic chlorination of pyrazole

The enzymatic reaction was performed as described in the previous section. The ethereal solution of the extracted product was injected into the gaschromatograph. Column dimensions: 2 mm ID x 1.5 m, filling 3% OV-17 on Chromosorb WHP 100/120. The initial temperature (100°C) was maintained for 1 min, and then raised to 250° with a rate of 8°C/min. The adjusted retention times were: pyrazole: 1.5 min, 4-chloropyrazole: 2.1 min, 4-bromopyrazole: 4.0 min.

^{1}H-NMR study on the enzymatic chlorination of pyrazole

To 5 ml of D_2O was added 5 µl 1 M H_2SO_4 (in D_2O), 100 µl 10 mM pyrazole (in D_2O) and 7,4 mg of potassium chloride (Solution A). The apparent pH of this solution was 2.53, corresponding to a pD of 2.93 [12], well within the optimal activity region of the enzyme [1]. 30% of hydrogen peroxide was diluted with D_2O to a final concentration of 10 mM (Solution B).

2.44 ml of Solution A was mixed with 60 µl of Solution B and 330 ng of CPO was added. Part of this mixture was placed in an NMR-tube and spectra were recorded using 30 min.-pulse periods. When the reaction was complete the apparent pH of the solution was checked and proved to be 2.67.

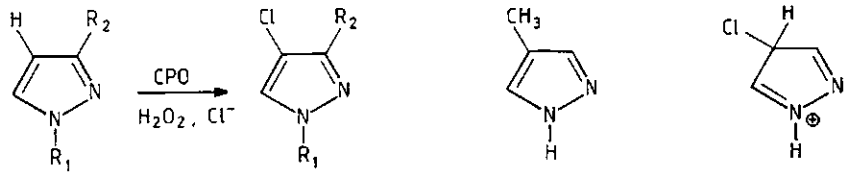
Reactions with hypochlorous acid

To 2.5 ml of a 0.5 mM solution of substrate in buffer pH 2.7 was added 1 equivalent of hypochlorous acid in 4 portions with stirring. After 15 min.

incubation the reaction mixture was analyzed as described above.

4.3 RESULTS AND DISCUSSION

When pyrazole (**1a**, see Scheme 4.1) was incubated with CPO under conditions which are optimal for the enzyme (0.1 M H_3PO_4/KOH pH 2.7, 20 mM KCl, 0.24 mM H_2O_2) and the reaction was monitored by UV-spectroscopy we observed that the maximum in the UV-spectrum shifted rapidly from 214 to 218 nm (see Fig. 4.1).



1 2 1d 3

a. $R_1 = R_2 = H$
b. $R_1 = CH_3$, $R_2 = H$
c. $R_1 = H$, $R_2 = CH_3$

*Scheme 4.1 Conversion of pyrazole (**1a**) and two derivatives thereof by CPO/H_2O_2/Cl^-. 4-Methylpyrazole (**1d**) is not a substrate for the enzyme. The 4H-pyrazole **3** is postulated as an intermediate in the reaction (see text).*

This UV-absorption slowly decreased but readdition of fresh enzyme caused a rapid shift of the λ_{max} to 224 nm; this peak did not change on further addition of enzyme. After isolation the product appeared to be 4-chloropyrazole (**2a**). The structure of the intermediate with λ_{max} = 218 nm is possibly the positively charged 4-chloro-4*H*-pyrazole **3**, since 4*H*-pyrazolium salts are known to have maximal UV-absorption around 220 nm [13]. 4*H*-pyrazoles have been observed as intermediates during reactions of pyrazoles with chlorine or *t*-butylhypochlorite, but isomerize to 3*H*-pyrazoles or form trimers when they contain hydrogen atoms attached to the ring carbon atoms [14]. Attempts to detect **3** by GC/MS inspection of the enzymatic reaction mixture failed; only **2a** was found [15]. Monitoring the enzymatic reaction by ^{1}H-NMR spectroscopy in D_2O was also not successful, since only signals of starting material **1a** and product **2a** were

observed. Probably the concentration of intermediate **3** is too low for detection; the fact that **1a** and **2a** only became visible after a 30 min. pulse period indicates that this method is unfavourable for this particular study. Although it is reasonable to assume that 4*H*-pyrazoles are short living intermediates in halogenation reactions of 1*H*-pyrazoles, more concrete evidence is needed.

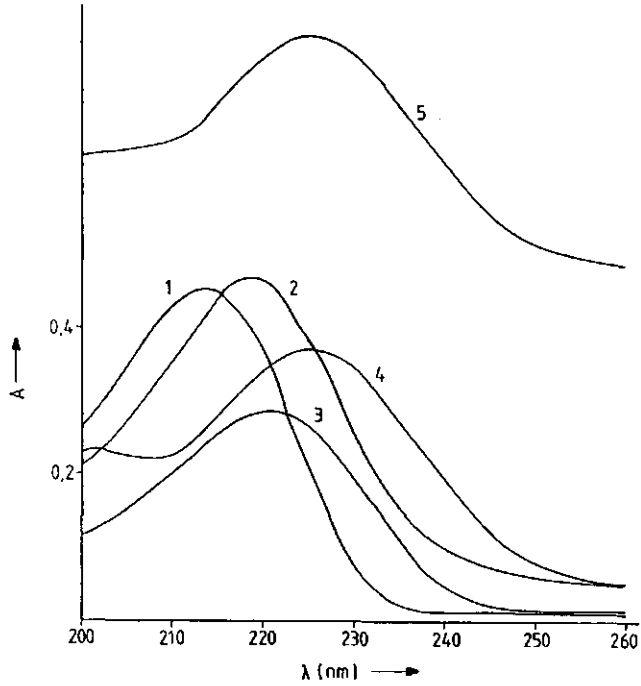


Fig. 4.1 *UV-absorption spectra, as recorded during the reaction of pyrazole (**1a**) with CPO. The quartz cuvette was filled with 2.5 ml of a solution containing 0.1 mM **1a**, 0.24 mM H_2O_2, 20 mM KCl and 0.1 M H_3PO_4/KOH pH 2.7. 280 ng of CPO was added to this and UV-spectra were recorded with appropriate time intervals. Trace 1: t=0 (pure **1a**); trace 2: t=0.5 min; trace 3: t=2.5 h. 840 ng additional CPO was subsequently added. Trace 4: t=3.0 h (reaction is complete); Trace 5: authentic **2a** (offset + 0,4 Abs).*

CPO reacts with the substituted pyrazoles 1-methylpyrazole (**1b**) and 3-methylpyrazole (**1c**) in a comparable manner as with **1a**, giving the 4-chloro-

derivatives **2b** resp. **2c** in very good yields (see Table 4.1). It is interesting that the regiochemistry of this reaction is the same as in the CPO-mediated halogenation of 1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazol-3-one (antipyrine) [3]. As expected, 4-methylpyrazole (**1d**) is not a suitable substrate for chloroperoxidase, because position 4 is blocked.

Table 4.1 Yields of the CPO-mediated chlorination of several heterocyclic substrates, compared to those obtained with HOCl.[a]

Compound	Yield (%)	
	$CPO/H_2O_2/X^-$	HOCl
4-chloropyrazole (**2a**)	68	20
4-chloro-1-methylpyrazole (**2b**)	83	68
4-chloro-3-metnylpyrazole (**2c**)	91	67
2-amino-3-chloropyridine (**5**)	18	11
5,7-dibromo-8-hydroxyquinoline (**7**)	79[b]	-

[a] See Materials and Methods for details; yields were determined by GLC unless indicated otherwise.

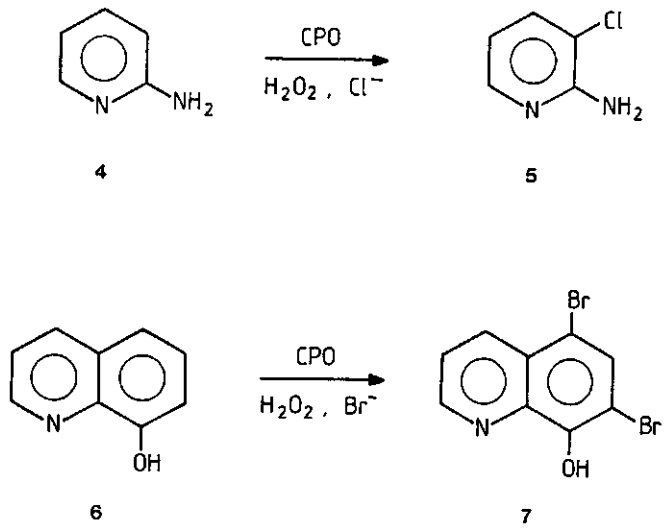
[b] Yield of isolated product.

Because there is some evidence that CPO generates free hypochlorous acid, which could act as the active chlorinating species in solution [16], we also studied the chlorination of pyrazoles with this reagent. We found that hypochlorous acid, when slowly added to solutions of **1** at pH 2.7, indeed reacts with **1a-c** giving **2a-c**, but only in moderate yields (see Table 4.1).

The non-enzymatic chlorination of pyrazoles as published in the literature usually takes place by bubbling an appropriate amount of dry chlorine gas into a solution of the compound in tetrachloromethane or by treatment with sulfurylchloride [10]. In the first case **2a** was synthesized with a yield of 55%. The yield of **2c** was not given and **2b** was prepared by a different route. Itoh *et al.* [8] converted **1a** by means of CPO giving **2a** and its bromo analogue in yields of resp. 7 and 14%. Their low yields are most probably due to their unconventional workup procedure and the high concentration of hydrogen peroxide they use, which is known to inactivate CPO [17]. This is further illustrated by the

turnover number of the reaction, which is 32,000 in our case while a figure of about 7,700 can be calculated from the studies cited above.

We believe that our enzymatic procedure is competitive with the non-enzymatical synthesis, since the yields are higher and the products obtained are more pure.



4 5 6 7

*Scheme 4.2 Reactions of CPO/H_2O_2/X^- (X = Cl or Br) with two pyridine derivatives, e.g. 2-aminopyridine (**4**) and 8-hydroxyquinoline (**6**).*

2-Aminopyridine (**4**, see Scheme 4.2) was converted by CPO into 2-amino-3-chloropyridine (**5**). No 5-chloro- or 3,5-dichloro-2-aminopyridine could be detected in the reaction mixture. This regiospecific chlorination was slow and inactivation of the enzyme occurred during incubation. It was not possible to obtain complete conversion by repeatedly adding fresh enzyme to the reaction mixture. This is in part due to the very low stability of **5** under the conditions used; the reaction mixture immediately turns brown upon neutralization. The structure of the product was proved by mass spectroscopy and ^{1}H-NMR. When the hypochlorous acid method was applied **5** was obtained in very low yield together with starting material (see Table 4.1). **5** Is described in the literature [18] and was synthesized by aminolysis of 2,3-dichloropyridine or from 2-amino-4-bromo-3-chloropyridine by reduction with hydrogen; yields are

not given. The regiospecific enzymatic chlorination at C-3 described here contrasts interestingly with the different regiospecificity observed in the chlorination of **4** in 20% sulfuric acid using chlorine, 2-amino-5-chloropyridine being obtained in 54% yield [19]. The same product was also obtained when using hydrogen peroxide or potassium chlorate in concentrated hydrochloric acid [20].

8-Hydroxyquinoline (**6**, see Scheme 4.2) was found to be a more suitable substrate for the enzyme than **4**. When reacting with CPO, hydrogen peroxide and potassium bromide, 5,7-dibromo-8-hydroxyquinoline (**7**) was formed in a yield of 79%. **7** was already synthesized previously in high yields by reacting **6** with bromine [21]. However, our enzymatic synthesis can be performed much more smoothly, without the need of molecular halogens and/or hypohalous acid. For this reason, the scope of enzymatic halogenations will be intensively studied at our laboratory.

4.4 REFERENCES AND NOTES

[1] L.P. Hager, D.R. Morris, F.S. Brown and H. Eberwein, J. Biol. Chem. **241** (1966), 1769-1777

[2] S.L. Neidleman, A.I. Cohen and L. Dean, Biotechnol. Bioeng. **11** (1969), 1227-1232

[3] P.L. Ashley and B.W. Griffin, Arch. Biochem. Biophys. **210** (1981), 167-178

[4] B.W. Griffin and R. Haddox, Arch. Biochem. Biophys. **239** (1985), 305-309

[5] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100; M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70

[6] See, for example: S.A.G.F. Angelino and H.C. van der Plas, in *Bio-Organic Heterocycles*, Proceedings of the third FECHEM Conference in Bio-Organic Chemistry, H.C. van der Plas, L. Ötvös and M. Simonyi, Eds, Akadémiai Kiadó, Budapest, Hungary, 1984, p. 285 and references cited therein.

[7] We also studied the enzymatic chlorination of pyrrole, but this resulted in a complex mixture of poorly separable compounds.

[8] N. Itoh, Y. Izumi and H. Yamada, Biochemistry **26** (1987), 282-289

[9] R. Hüttel and M.E. Schön, Liebigs Ann. Chem. **625** (1959), 55-65

[10] R. Hüttel, O. Schäfer and G. Welzel, Liebigs Ann. Chem. **598** (1956), 186-197

[11] 100 ng CPO corresponds to ± 0,04 U; 1 U catalyzes the chlorination of 1 µmol of monochlorodimedon in 1 minute.

[12] P.K. Glasoe and F.A. Long, J. Phys. Chem. **64** (1960), 188-190

[13] J. Elguero, R. Jacquier and D. Tizané, Bull. Soc. Chim. Fr. (1968), 3866-3868; W.J. Leigh and D.R. Arnold, Can. J. Chem. **57** (1979), 1186-1200

[14] M.P. Sammes and A.R. Katritzky, Adv. Heterocycl. Chem. **34** (1983), 53-78

[15] Traces of starting material and 4-bromopyrazole were also detectable. The brominated pyrazole is probably formed from small amounts of bromide in the (*pro analyse*!) potassium chloride.

[16] T.D. Lee, J. Geigert, D.J. Dalietos and D.S. Hirano, Biochem. Biophys. Res. Commun. **10** (1983), 880-883

[17] P.D. Shaw and L.P. Hager, J. Biol. Chem. **236** (1961), 1626-1630

5 ENZYMATIC BROMINATION OF BARBITURIC ACID AND SOME OF ITS DERIVATIVES*

5.1 INTRODUCTION

Haloperoxidases are enzymes capable of halogenating several organic substrates using hydrogen peroxide and halide anions [1]. Three different types of haloperoxidases are recognized, depending on the halide ion they are able to oxidize: chloroperoxidases, bromoperoxidases and iodoperoxidases. Thus, a chloroperoxidase oxidizes Cl^-, Br^- and I^--ions, a bromoperoxidase oxidizes bromide and iodide ions and an iodoperoxidase can only act on the iodide ion. The chloroperoxidase from *Caldariomyces fumago* is one of the best studied enzymes in this field; its biochemistry is well explored [1,2] as well as its application to synthetic organic chemistry [1,3-5]. Several bromoperoxidases have been isolated: e.g., from the eggs of the sea-urchin (ovoperoxidase; [6]), from green algae [7-9], red algae [9-12] and bacteria [13-16] and, thus, are much more common than thought in the past. Most of these enzymes feature a heme-protein structure. Recently a novel non-heme-containing bromoperoxidase was detected in the brown alga *Ascophyllum nodosum* [17,18]. It was found that the isolated enzyme contained vanadium as a prosthetic group and exhibited an unusual stability for an oxido-reductase [19,20]. The enzyme was found to brominate monochlorodimedon (MCD) and the dye phenolred. We wish to report in this paper the results of our study on the bromination of barbituric acid (**1a**) and some of its derivatives (**1b-f**) by this enzyme and on the kinetics of the enzymatic conversions.

5.2 MATERIALS AND METHODS

General

Bromoperoxidase (BPO) from *Ascophyllum nodosum* was isolated as described previously [18,19] and had a specific activity of 8-10 μmol MCD.mg protein^{-1}.min^{-1} depending on the batch used. Protein concentrations were determined by the Lowry method [21] using bovine serum albumin as standard. Monochlorodimedon was purchased from Sigma. 1,3-Dimethylbarbituric acid (**1c**)

---

* Adapted from: M.C.R. Franssen, J.D. Jansma, H.C. van der Plas, E. de Boer and R. Wever, Bioorg. Chem., submitted

was obtained from Fluka and the other alkyl- and arylbarbituric acids (**1b,1d-e**) were synthesized from the corresponding malonic ester and urea derivatives according to the procedure of Dickey [22]. **1f** was prepared by chlorination of barbituric acid [23]. An authentic sample of 5,5-dibromobarbituric acid (**3a**) was obtained using Bock's method [24]. All chemicals were of the highest commercial grade.

UV spectra and kinetic measurements were performed on an Aminco-Chance DW-2 split-beam spectrophotometer or on a Varian DMS-100 spectrophotometer equipped with a DS-15 data station, using the Varian programs Kinetics Storage DMS-100/DS-15 (85-100541-00) and Enhanced Kinetics Calculations (85-100542-00). Initial velocities were determined by the Direct Linear Plot Method, and apparent affinity values by means of Hanes-Woolf plots [25]. Mass spectra were recorded on an AEI MS-902 instrument or a VG-Micromass 7070 F apparatus (direct probe mode). Circular dichroism spectra were run on a Jobin-Yvon Auto-Dichrograph Mark V. HPLC analysis was carried out on a Varian 5000 instrument (see below for experimental details).

HPLC-measurements

The reactions of BPO with barbituric acid and its derivatives were monitored by HPLC as described earlier for chloroperoxidase [4]. However, the eluent was slightly modified for the bromobarbituric acids: 15% methanol and 85% water containing 10 mM potassium phosphate and 5 mM nonyltrimethylammonium bromide pH 7.3. The eluent flow was 1.5 ml/min. The compounds were detected by means of their UV absorption at 254 nm.

Synthesis of 5-bromobarbituric acid (**2a**)

This reference compound was prepared from the phenyl iodonium betaine of barbituric acid [26], in a modified procedure. 0.8 g of this betaine was dissolved in a mixture of 5 ml of ethanol, 1 ml of concentrated HBr and 6 ml of hexane. This two-phase system was stirred vigorously for 30 min at 50°C. The mixture was cooled and stored overnight at 5°C. The white precipitate formed was dried in vacuo over phosphorous pentoxide. The yield of **2a** was 0.43 g (2.07 mmol, 86%), m.p. 210-215°C (dec.), litt. 212-215°C [24], 205-207 [26]. Elemental analysis: calcd. for $C_4H_3N_2O_3Br$: C 23.21%, H 1.46%;found: C 22.94%, H 1.60%.

Kinetic measurements

Specific activities of the enzyme were determined under the standard assay conditions [18]: 100 mM KH_2PO_4/K_2HPO_4 pH 6.5, 100 mM KBr, 2.0 mM H_2O_2, 50 µM

MCD, 25°C. The reaction was started by the addition of 8.4 nM of BPO and the absorption at 293 nm was followed, using $\Delta\varepsilon = 21{,}150\ M^{-1}.cm^{-1}$. These conditions were also used in all other kinetic measurements and in the experiments, where isolation of the product was not required. All determinations were carried out in triplicate. The following molar extinction coefficients ($\Delta\varepsilon$, $M^{-1}.cm^{-1}$) were used at pH 6.5: 5-phenylbarbituric acid (**1d**; 15,300 at 268 nm), 1-methyl-5-phenylbarbituric acid (**1e**; 15,500 at 268 nm), and 5-chlorobarbituric acid (**1f**; 11,600 at 269 nm).

Circular dichroism (CD)

The possible stereochemical conversion of 1-methyl-5-phenylbarbituric acid by BPO was studied in the following way. To 100 μM 1-methyl-5-phenylbarbituric acid (**1e**) in 100 mM KH_2PO_4/K_2HPO_4 pH 6.5 containing 100 mM KBr was added 2 mM H_2O_2. The reaction was monitored with a UV-spectrophotometer at 268 nm after addition of 68 nM BPO to this solution. When the reaction was complete, a CD-spectrum was recorded between 230 and 350 nm. A reference spectrum was made of the same solution to which buffer was added instead of H_2O_2. This spectrum was subtracted from the first one, yielding the final CD-spectrum of the enzymatically synthesized product.

Isolation of enzymatic products

To prove the structure of the enzymatic products they were isolated and analysed by mass spectroscopy. Since 5-bromobarbituric acids are too much water-soluble, their isolation by direct extraction with organic solvents is not very effective; therefore the bromination reaction was performed in the following way. The standard reaction medium was replaced by 0.1 M acetate buffer pH 4.5 containing 1 mM **1**, 20 mM KBr and 2.4 mM H_2O_2 and a suitable amount of BPO (0.17 μM) was added to the solution. Upon reaching maximal UV-absorption at 268 nm, the reaction mixture was quickly frozen in liquid nitrogen and lyophilized. The 5-monobromobarbituric acids were extracted from the solid material with distilled methanol.

5,5-Dibromobarbituric acids could be easily isolated from the standard enzymatic medium by extraction with distilled ethyl acetate.

The mass spectra of all compounds were identical to those of authentic specimen. 5-Bromo-5-chlorobarbituric acid (**2f**) has not been synthesized before; its mass spectrum shows peaks at m/e 244 (3%), 242 (12), 240 (8), 201 (8; -HNCO), 199 (30), 197 (23), 158 (30; -HNCO), 156 (100), 154 (74), 130 (2; -CO), 128 (8), 126 (6). Calcd. for $C_4H_2N_2O_3{}^{79}Br^{35}Cl$: 239.8938, found 239.8938.

5.3 RESULTS

5.3.1 *Reaction of* Ascophyllum nodosum *bromoperoxidase (BPO) with barbituric acid and its derivatives*

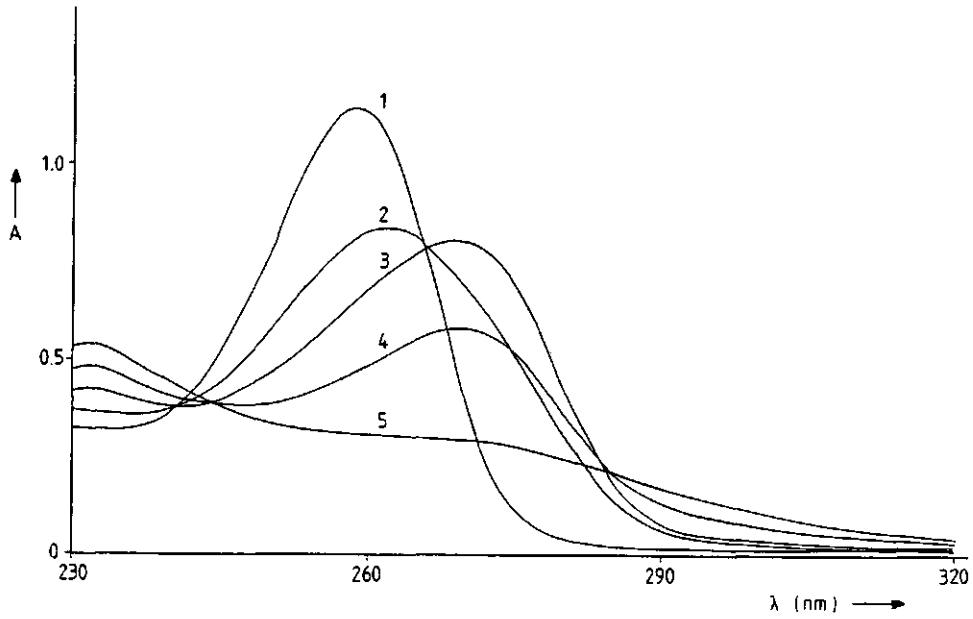
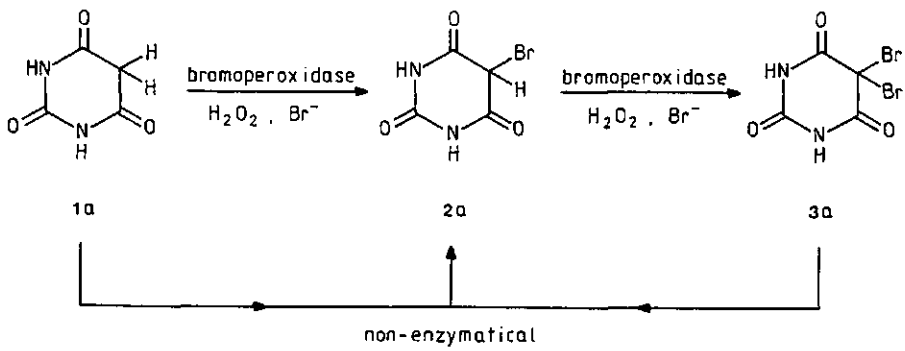


*Figure 5.1 UV-absorption spectra, as recorded during the reaction of barbituric acid (**1a**) with the bromoperoxidase from* Ascophyllum nodosum. *The quartz cuvette was filled with 50 µM **1a**, 2 mM H_2O_2, 100 mM KBr and 100 mM KH_2PO_4/K_2HPO_4 pH 6.5. To this, 2.8 nM of bromoperoxidase was added and UV-spectra were recorded at appropriate time intervals. Trace 1: t=0 min (pure **1a**); trace 2: t=1.0 min; trace 3: t=2.6 min; trace 4: t=4.2 min; trace 5: t=6.6 min (reaction is complete)*

When barbituric acid (**1a**) was incubated with BPO under standard conditions, a rapid change in the UV spectrum occurred (see Figure 5.1). During the first 2½ minutes the original absorption peak of **1a** at 258 nm shifted to a higher wavelength (268 nm) with an isosbestic point at 266 nm. After this period the peak at 268 nm disappeared indicating the consecutive formation of a second product (peak at 232 nm, isosbestic point at 285 nm). The UV spectra of

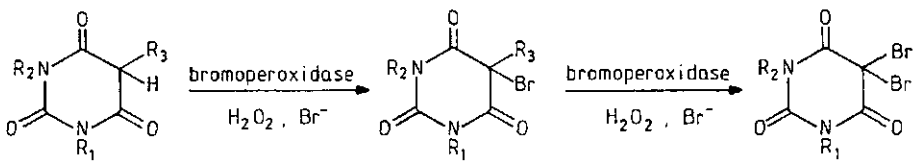
the products perfectly match those from authentic samples of 5-bromobarbituric acid (**2a**) and 5,5-dibromobarbituric acid (**3a**), indicating that **1a** underwent bromination at C5. That these compounds are indeed formed was confirmed by the mass spectra of **2a** and **3a**, isolated from the enzyme-mediated bromination of **1a**, which were fully identical to those obtained from authentic samples. When monitoring the reaction with reversed phase- ion pair HPLC it became clear that these reactions were consecutive: the peak of **3a** only appeared when **1a** has quantitatively been converted into **2a**. A possible explanation for the accumulation of **2a**, namely that the rate of the reaction **1a** → **2a** is much higher than that of **2a** → **3a** is not very likely since the rate of bromination of **2a** is about equal to that of MCD. It was published recently that **3a** is a brominating agent [27] and this must be taken into account when studying the kinetics of this reaction. When equal amounts of **1a** and **3a** were mixed only **2a** was found on analysis of the reaction mixture by means of UV-spectroscopy and HPLC. So **2a** is formed enzymatically as well as non-enzymatically; the conversion **2a** → **3a** is exclusively enzyme-mediated. This was proved by the fact that authentic **2a** was converted by the enzyme into **3a** under standard conditions. The reaction sequence is depicted in Scheme 5.1.



Scheme 5.1 *Reaction pattern of the bromination of barbituric acid (**1a**) by the bromoperoxidase from* Ascophyllum nodosum, H_2O_2 *and bromide ions. The reaction between **1a** and **3a** proceeds non-enzymatically.*

The enzymatic bromination reaction has also been investigated with substituted barbituric acids as substrates. 1-Methylbarbituric acid (**1b**) and 1,3-dimethylbarbituric acid (**1c**) were converted via their 5-bromo derivatives

2b and **2c** into the 5,5-dibromo compounds **3b** and **3c** (see Scheme 5.2). The 5-phenylbarbituric acids **1d** and **1e** were also very good substrates for the enzyme and gave the corresponding 5-bromo derivatives **2d** and **2e** respectively. Interestingly, 5-chlorobarbituric acid (**1f**) was converted to 5-bromo-5-chlorobarbituric acid (**2f**), showing the potentiality of this procedure to synthesize compounds with two different halogen atoms attached to the same carbon atom. As can be concluded from UV spectroscopy the yields of all the brominated compounds are high.



1 2 3

a. $R_1, R_2, R_3 = H$
b. $R_1 = CH_3$, $R_2, R_3 = H$
c. $R_1, R_2 = CH_3$, $R_3 = H$
d. $R_1, R_2 = H$, $R_3 = C_6H_5$
e. $R_1 = CH_3$, $R_2 = H$, $R_3 = C_6H_5$
f. $R_1, R_2 = H$, $R_3 = Cl$

a. R_1 , $R_2 = H$
b. $R_1 = CH_3$, $R_2 = H$
c. R_1 , $R_2 = CH_3$

*Scheme 5.2 General reaction scheme for the bromination of barbituric acid (**1a**) and some of its derivatives by bromoperoxidase, H_2O_2 and bromide ions.*

5.3.2 Kinetics of the BPO-mediated bromination of barbituric acids and monochlorodimedon

In contrast to the chloroperoxidase from *Caldariomyces fumago*, the mechanism of the reaction between appropriate substrates and the *Ascophyllum nodosum* bromoperoxidase has hardly been studied. As a first step in the elucidation of this mechanism we present some kinetic data on the bromination of MCD, barbituric acid (**1a**) and its derivatives **1b-1f**.

Table 5.1 Relative reaction rates of the enzymatic bromination of various substrates[a]

Compound	Relative reaction rate[b] pH 4.5	pH 6.5
MCD	100[c]	100[d]
1d	102	107
1f	103	102
2a	99	N.D.

[a] The following conditions were used: 100 mM KH_2PO_4 buffer pH 4.5 containing 20 mM KBr, 2.4 mM H_2O_2 and 50 μM organic substrate; or, 100 mM KH_2PO_4/K_2HPO_4 buffer pH 6.5 containing 100 mM KBr, 2 mM H_2O_2 and 50 μM organic substrate. See Materials and Methods for further details. n = 3.

[b] Arbitrary units, the value for MCD was set at 100. N.D. = not determined.

[c] Corresponds to 8.0 μmoles MCD.mg protein^{-1}.min^{-1}.

[d] Corresponds to 8.2 μmoles MCD.mg protein^{-1}.min^{-1}.

Comparison of the enzymatic bromination rate of MCD with those determined with **1d**, 1f and **2a** at pH 4.5 or 6.5 show that the rates of the four reactions only differ to a very small extent (see Table 5.1). Also the presence of methyl groups on the nitrogen atoms of barbituric acid has no effect on the rate of bromination (see Table 5.2) as determined by the total time in which the reaction **1** → **3** is completed. However, the differences in reaction rates may in this case be masked by the non-enzymatic side-reaction (*vide supra*).

These results are in full agreement with those found for chloroperoxidase: both enzymes seem to have little specificity regarding the structure of their organic substrates. Contrastingly, however, the concentration of the organic substrate has a marked influence on the bromination rate with BPO. In the range 2-50 μM the reaction rate increases in a hyperbolic fashion with increasing concentration of the organic substrate, as is shown in Figure 5.2a for 5-phenylbarbituric acid (**1d**). The obtained data show linear relationship when put in a Hanes-Woolf plot (see Figure 5.2b). In this way, apparent affinity constants and maximal rates were obtained for MCD and **1d-f** (Table 5.3). The maximal rates of bromination of the various substrates at high substrate concentration are practically identical. However, the apparent affinity

constants varied somewhat, although they are quite independent of the structure of the rest of the molecule being carbocyclic or heterocyclic. Also the nature of the substituent between the two carbonyl groups is of minor importance, although the presence of a 5-phenyl substituent seems to lower the interaction somewhat. However, the magnitude of the apparent affinity constants increased when more H_2O_2 was present (see Table 5.3).

*Table 5.2 Time needed for complete conversion (**1** → **3**) of some barbituric acids*[a]

Compound	Conversion time[b]
1a	100[c]
1b	95
1c	98

[a] Determined under standard assay conditions by monitoring the UV-absorption at 270 nm. n = 3.

[b] Arbitrary units; the value for **1a** was set at 100.

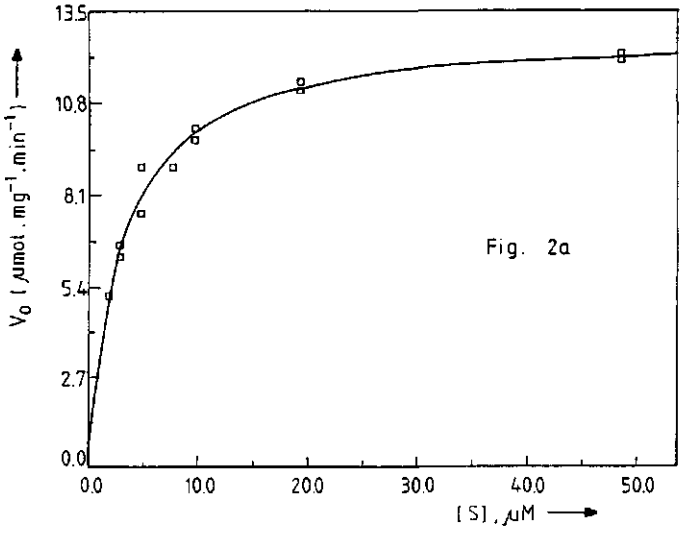
[c] Corresponds to 145 sec.

5.3.3 Stereochemistry of the bromination reaction

To determine whether the enzyme-mediated bromination reaction shows any stereoselectivity, we investigated the bromination of 1-methyl-5-phenylbarbituric acid (**1e**). Monitoring the reaction by circular dichroism clearly showed that a racemic mixture was obtained.

5.4 DISCUSSION

The non-enzymatic synthesis of the compounds **2a**, **3a** and **3c** have been reported. **2a** was prepared by the reaction of bromine with **1a** in water [24] or in dimethylformamide [28]. The latter authors reported a yield of 78%, but in our hands only undefined products were obtained. The method described by Neiland [26] involving the reaction of the phenyliodonium betaine of barbituric acid with hydrobromic acid gave good results in preparing **2a**, although we found that the addition of some hexane to the reaction medium in this conversion is



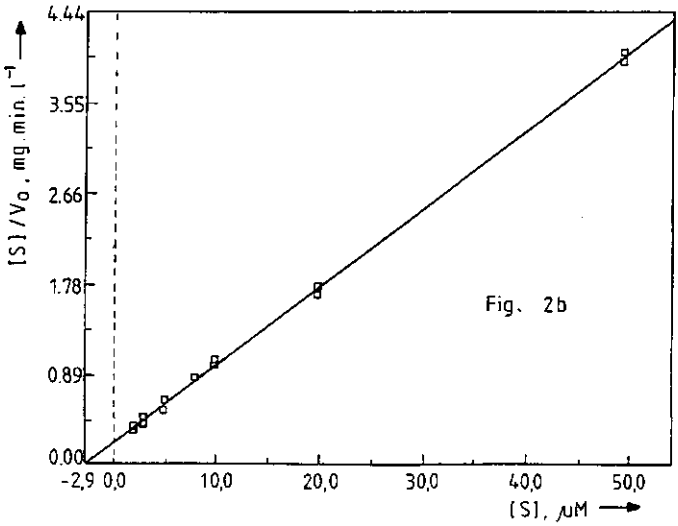


Figure 5.2 *a) The effect of the concentration of* **1d** *on the reaction rate of bromoperoxidase. Each dot represents the mean of average value obtained from three experiments.*
b) The same data used in Figure 5.2a, depicted in a Hanes-Woolf plot. The intercept on the [S]-axis gives the apparent affinity constant, the slope equals the reciproke of the maximal bromination rate.

Table 5.3 Apparent affinity constants and maximal bromination rates for some bromoperoxidase substrates at three different H_2O_2 concentrations[a]

3a Apparent affinity constants

Compound	$[H_2O_2]$, mM		
	0.8	2.0	3.0
MCD	1.8	4.0	4.7
1d	1.9	1.9	2.9
1e	0.6	1.6	2.3
1f	1.6	2.0	3.5

3b Maximal bromination rates

Compound	$[H_2O_2]$, mM		
	0.8	2.0	3.0
MCD	10.9	12.7	11.5
1d	13.7	13.1	12.9
1e	12.6	12.7	12.4
1f	12.1	11.6	12.4

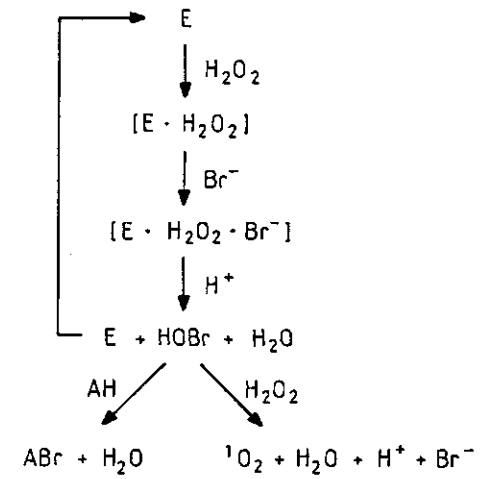
[a] See text for experimental details. Apparent affinity constants are expressed in μM and maximal bromination rates in μmoles.mg protein^{-1}.min^{-1}.

essential. The reason for this is not clear; perhaps the reaction is facilitated by the fact that iodobenzene, one of the products, is removed from the reaction equilibrium by the organic solvent. Another possibility is that the presence of hexane increases the solubility of one of the starting materials. **3a** and **3c** were synthesized from the corresponding violuric acids and molecular bromine in yields of about 85% [29]. When these compounds were treated with ammonia, the 5-monobromo compounds were obtained in yields of 50%. Later syntheses of **3a** all used molecular bromine as reagent ([24], yield not given; [27], yield 92%).

The bromoperoxidase from the brown alga *Ascophyllum nodosum* converts barbituric acid and some of its derivatives smoothly into their 5-bromo- or 5,5-dibromo compounds. The yields and the reaction rates were high, so we consider our enzymatic procedure to be competitive with the chemical syntheses described above. The occurrence of a non-enzymatic side-reaction poses no problem in this respect and affords the isolation of the intermediate products **2a–c** when the reaction is stopped halfway. **3a** is a smooth brominating agent for saturated and α,β-unsaturated carbonyl compounds [27], which further supports the reaction pattern as shown in Scheme 5.1.

There is a lot of literature on the reaction mechanism of haloperoxidases, but as yet not everything is fully understood. There is agreement in the literature about lactoperoxidase and the chloride-metabolizing enzyme myeloperoxidase: both enzymes most probably form hypohalous acids (HOCl, HOBr), which are the active halogenating species in their reactions [1]. The data concerning the chloroperoxidase from *Caldariomyces fumago* are still confusing (see [4] for a discussion on this topic), and virtually nothing is known about the other haloperoxidases. The *Ascophyllum nodosum* bromoperoxidase is unique in the sense that it is not a heme-protein, but contains a vanadium (V) ion in its active centre. In addition, unlike heme-containing peroxidases, this enzyme does not oxidize electron-rich organic compounds in the presence of hydrogen peroxide [30]. Thus, the reaction mechanism of this enzyme must be different from that of the other haloperoxidases. In the work presented here it is shown that the enzyme closely resembles chloroperoxidase with respect to substrate specificity and stereochemical behaviour [4]: i) in case of cyclic β-dicarbonyl compounds the enzymatic activity is not affected by the structure of the ring being carbocyclic or heterocyclic; ii) the enzymatic reaction rate is not affected by the presence of methyl or phenyl substituents attached to the substrate barbituric acid; iii) 1-methyl-5-phenylbarbituric acid (**1e**) is not halogenated in a stereospecifical manner. However, for chloroperoxidase no hyperbolic relation between the rate of chlorination and the concentration of the organic substrate could be found, whereas for the bromoperoxidase studied here the concentration of the organic substrate has a marked influence on the bromination rate, yielding apparent affinity constants in the range of 0.6-5 μM (see Table 5.3). That the nature of the various organic substrates does not largely affect the rate of bromination suggests that under our experimental conditions, formation of a rate-limiting ternary complex between a halogenating enzyme intermediate and an organic substrate does not occur. However, it is noteworthy that at low substrate concentrations the bromination rate is a

function of the substrate concentration. Although the latter may indicate the existence of such a ternary complex, it is more likely that we deal with a reaction in which the enzyme produces free HOBr. The hypobromous acid formed may then react with the organic substrate or in a competitive reaction with H_2O_2 to give singlet oxygen [31]. Thus, at increased concentration of H_2O_2 there is less HOBr available for the organic substrate. This suggestion explains why at higher H_2O_2 concentrations the apparent affinity constants increase (Table 5.3) and why, at higher concentrations of the organic substrates, the maximal bromination rate is not affected by H_2O_2. This phenomenon is not observed in chloroperoxidase-catalyzed reactions, probably because of differences in experimental conditions. The experiments reported here are done at pH 6.5 whereas the studies on chloroperoxidase were performed at pH 2.7, the pH optimum of this enzyme. As shown by Kanofski [32] the rate of the reaction between HOBr and H_2O_2 at neutral pH is very high whereas it is low at acid pH [33]. This is substantiated by our observation that at pH 4.5 the apparent affinity constants for the BPO-mediated bromination of MCD decreased markedly.



Scheme 3. Tentative reaction mechanism of the bromoperoxidase-induced bromination of organic compounds. E=bromoperoxidase from Ascophyllum nodosum; *AH=organic substrate; ABr=organic product.*

We have to conclude therefore that at pH 6.5 the rate of the reaction of HOBr with H_2O_2 is of the same magnitude as that between our organic substrates

and HOBr. The small differences in the apparent affinity constants of the various substrates may reflect differences in rate constants of the reaction between HOBr and the various organic substrates. Thus our experimental data favour a mechanism (see Scheme 5.3) in which bromoperoxidase generates free hypobromous acid, although we cannot rule out a short-lived enzyme-bound halogenating intermediate.

That **1e** is in fact not brominated stereoselectively is due to the brominating properties of compounds like **3** and **2d,e** themselves which in fact non-stereoselectively introduce bromo atoms into the organic substrates in solution, giving a racemic mixture.

Itoh et al. [34] very recently reported on the bromination of nucleobases by a non-heme bromoperoxidase from *Corallina pilulifera*. Some of the heterocyclics were brominated by the enzyme but not by molecular bromine. The authors therefore concluded that these substrates were brominated at the active site of the enzyme, which indicates that the reaction mechanism of the *Corallina* bromoperoxidase may differ from that of our enzyme.

The bromoperoxidase from *Ascophyllum nodosum* is an interesting enzyme, because of its unique structure, its selectivity for bromide and its remarkable stability towards organic solvents [18,20]. In this study we showed that the enzyme is a smooth brominating agent because it produces HOBr in a very low concentration. Therefore it will be studied more extensively in the near future.

5.5 REFERENCES

[1] S.L. Neidleman and J. Geigert, *Biohalogenation: principles, basic roles and applications*, Ellis Horwood Ltd., Chichester (1986), 1-203
[2] L.P. Hager, D.R. Morris, F.S. Brown and H. Eberwein, J. Biol. Chem. **241** (1966), 1769-1777
[3] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100
[4] M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70
[5] M.C.R. Franssen, H.G. van Boven and H.C. van der Plas, J. Heterocyclic Chem., in press
[6] T. Deits, M. Farrance, E.S. Kay, L. Medill, E.E. Turner, P.J. Weidman, and B.M. Shapiro, J. Biol. Chem. **259** (1984), 13525-13533
[7] D.G. Baden and M.D. Corbett, Biochem. J. **187** (1980), 205-211
[8] J.A. Manthey and L.P. Hager, J. Biol. Chem. **256** (1981), 11232-11238
[9] H. Yamada, N. Itoh, S. Murakami and Y. Izumi, Agric. Biol. Chem. **49** (1985), 2961-2967
[10] T.J. Ahern, G.G. Allan and D.G. Medcalf, Biochim. Biophys. Acta **616** (1980), 329-339
[11] N. Itoh, Y. Izumi and H. Yamada, J. Biol. Chem. **261** (1986), 5194-5200

[12] B.E. Krenn, H. Plat and R. Wever, Biochim. Biophys. Acta **912** (1987), 287-291
[13] K.-H. van Pée and F. Lingens, FEBS Lett. **173** (1984), 5-8
[14] W. Wiesner, K.-H. van Pée and F. Lingens, Biol. Chem. Hoppe-Seyler **366** (1985), 1085-1091
[15] K.-H. van Pée and F. Lingens, J. Bacteriol. **161** (1985), 1171-1175
[16] K.-H. van Pée and F. Lingens, J. Gen. Microbiol. **131** (1985), 1911-1916
[17] H. Vilter, Phytochemistry **23** (1984), 1387-1390
[18] R. Wever, H. Plat and E. de Boer, Biochim. Biophys. Acta **830** (1985), 181-186
[19] E. de Boer, Y. van Kooyk, M.G.M. Tromp, H. Plat and R. Wever, Biochem. Biophys. Acta **869** (1986), 48-53
[20] E. de Boer, H. Plat, M.G.M. Tromp, R. Wever, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer and H.E. Schoemaker, Biotechnol. Bioeng., in press
[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem. **193** (1951), 265-275
[22] J.B. Dickey and A.R. Gray, Org. Syn. **18** (1938), 8-9
[23] W. Bock, Ber. Dtsch. Chem. Ges. **56** (1923), 1222-1227
[24] W. Bock, Ber. Dtsch. Chem. Ges. **55** (1922), 3400-3405. The reported yield was 70%, but this figure could not be reproduced by us.
[25] I.H. Segel, *Enzyme kinetics. Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, J. Wiley & Sons, New York (1975), 1-957
[26] O. Ya. Neiland and D.É Neiman, J. Org. Chem. USSR **6** (1970), 2522-2525
[27] G. Grundke, W. Keese and M. Rimpler, Chem. Ber. **118** (1985), 4288-4291
[28] R. Granados, F. Marquez and M. Melgarejo, Anales Real Soc. Espan. Fis. Quim. Ser. B **58** (1962), 479-482; Chem. Abstr. **59** (1963), 16316
[29] H. Biltz and T. Hamburger, Ber. Dtsch. Chem. Ges. **49** (1916), 635-655
[30] E. de Boer, H. Plat and R. Wever, in *Biocatalysis in Organic Media* (C. Laane, J. Tramper and M.D. Lilly, eds), Elsevier, Amsterdam, The Netherlands, Stud. Org. Syn. **29** (1987), 317-322
[31] J.R. Kanofski, J. Biol. Chem. **259** (1984), 5596-5600
[32] J.R. Kanofski, Biochem. Biophys. Res. Commun. **134** (1986), 777-782
[33] R.D. Libby, J.A. Thomas, L.W. Kaiser and L.P. Hager, J. Biol. Chem. **257** (1982), 5030-5037
[34] N. Itoh, Y. Izumi and H. Yamada, Biochemistry **26** (1987), 282-289

6 CHLOROPEROXIDASE-CATALYSED HALOGENATION OF APOLAR COMPOUNDS USING REVERSED MICELLES*

6.1 INTRODUCTION

Haloperoxidases are enzymes which are capable of halogenating a large variety of organic compounds using hydrogen peroxide and either chloride, bromide or iodide ions, depending on the enzyme under study [1]. We have reported the use of the chloroperoxidase from the mold *Caldariomyces fumago* for the halogenation of barbituric acid [2,3] and some pyridine and pyrazole derivatives [4]. These compounds are rather soluble in water and good conversion rates were obtained. However, apolar, water-insoluble compounds are difficult to halogenate in this way; increasing the solubility by the addition of a water-miscible organic solvent leads to inactivation of chloroperoxidase [5].

Enzymes can be very active in some organic solvents, at least when these solvents are water-immiscible, for instance in the case of reversed micelles. These transparent systems consist of tiny water droplets embedded in water-immiscible organic solvents and stabilized by surfactants (see [6,7] for reviews). Enzymes have been entrapped into reversed micelles for the synthesis of apolar compounds. Examples are the hydroperoxidation of linoleic acid by lipoxygenase [8], the synthesis of Δ^4-cholestenone by cholesterol oxidase [9], and the reduction of ketosteroids by 20β-hydroxysteroid dehydrogenase [10]. In the latter case the micellar system was composed of cetyltrimethylammonium bromide (CTAB), hexanol and octane. The hexanol was added as cosurfactant to stabilize the micelle and to regulate the polarity of the micellar interphase; it was recognized that enzyme activity is maximal when the solubility of the substrate in the interphase is maximal [11,12].

The only haloperoxidase which has been studied in reversed micelles (with AOT as surfactant) so far is horseradish peroxidase [13]. The reactions studied were the tetramerisation of pyrogallol to purpurogallin [13,14] and the dimerisation of o-phenylene diamine giving 2,2'-diaminoazobenzene [15]. The enzymatic activity in reversed micelles increased by a hundredfold compared to aqueous media [14]; one of the reasons is the absence of substrate inhibition in reversed micelles [13]. However, halogenation reactions, which are the most

---

* Adapted from: M.C.R. Franssen, J.G.J. Weijnen, J.P. Vincken, C. Laane and H.C. van der Plas, Biocatalysis, submitted

interesting feature of haloperoxidases, have not been studied up to now.

Studying halogenation reactions in reversed micelles as a model system is interesting, because i) the substrates required, halide and hydrogen peroxide, are available in large quantities in the proximity of the enzyme; ii) the enzymatic activity can be regulated by adjusting the polarity of the micellar interphase. We here present the halogenation of two relatively apolar compounds i.e. monochlorodimedon (**1**) and 1,3-dihydroxybenzene (**3**) in reversed micelles using the chloroperoxidase from *Caldariomyces fumago*. The system is optimised with respect to enzyme activity. The micellar medium consists of phosphate buffer, cetyltrimethylammonium bromide or chloride (CTAB resp. CTAC), pentanol and octane. A novel aspect of this system is that the surfactant serves a dual function: i) it stabilizes the reversed micelle, and ii) the counter ion (chloride, bromide) is used as a substrate by the enzyme.

6.2 MATERIALS AND METHODS

Theory[11]

log P is the logarithm of the partition coefficient between water and octanol for a certain compound. It was assumed that log P values remain unchanged when using 0.1 M potassium phosphate buffer pH 3.0 instead of water.

$$w_0 = \frac{[\text{water}]}{[\text{surfactant}]} \quad (1) \qquad a_0 = \frac{[\text{cosurfactant}]_{\text{interphase}}}{[\text{surfactant}]_{\text{interphase}}} \quad (2)$$

$$\log P_{\text{interphase}} = \frac{a_0}{a_0 + 1} \times \log P_{\text{cosurfactant}} + \frac{1}{a_0 + 1} \times \log P_{\text{surfactant}} \quad (3)$$

a_0 can only be measured at the phase boundary, that means at those compositions of the reversed micellar system that just give transparent solutions. The reason is that only there the composition of the interphase is known.

General

Chloroperoxidase (the crude type) was obtained from Sigma or was a gift from Mr. E. de Boer and Dr. R. Wever (Lab. of Biochemistry, Univ. of Amsterdam, The Netherlands). Cetyltrimethylammonium bromide (CTAB) was obtained from Merck, cetyltrimethylammonium chloride (CTAC) was from Kodak, 1-pentanol and n-octane were from BDH, monochlorodimedon (MCD) was from Sigma and 1,3-dihydroxybenzene (DHB) was from Baker. DHB was recrystallized before use. The UV-absorbance was measured with a Varian DMS-100 spectrophotometer equipped with a DS-15 data station using the Varian Kinetics Storage DMS-100/DS-15 program.

Preparation of the reversed micelles

To a 0.2 M suspension of CTAX (X=Cl or Br) in octane containing 0.1 mM of the organic substrate were successively added suitable amounts of pentanol, 0.1 M potassium phosphate buffer pH 3.0 and H_2O_2 dissolved in the same buffer. The suspension was mixed on a Vortex; only compositions which gave clear solutions within 5 sec. were used. A suitable amount of enzyme was then injected into the solution and the UV-absorbance was monitored to follow the enzymatic conversion, at 279 nm ($\Delta\varepsilon$=10,100 $1.mol^{-1}.cm^{-1}$) for MCD and at 292 nm ($\Delta\varepsilon$=2680 $1.mol^{-1}.cm^{-1}$) for DHB. For a system with w_0=25 and 11% pentanol (w/w) the medium was composed as follows: 2064 µl 0.1 mM organic substrate/0.2 M CTAX/octane; 250 µl pentanol; 123 µl buffer; 60 µl 10 mM H_2O_2 in buffer and 3 µl (150 ng) chloroperoxidase. The specific activity of the enzyme was 400-450 µmol MCD.mg $protein^{-1}.min^{-1}$. It should be noted that the concentration of the substrate used was only 0.1 mM to facilitate the monitoring of the reaction. The maximal solubility of MCD in the reversed micellar system is more than 30 mM.

Determination of log P-values

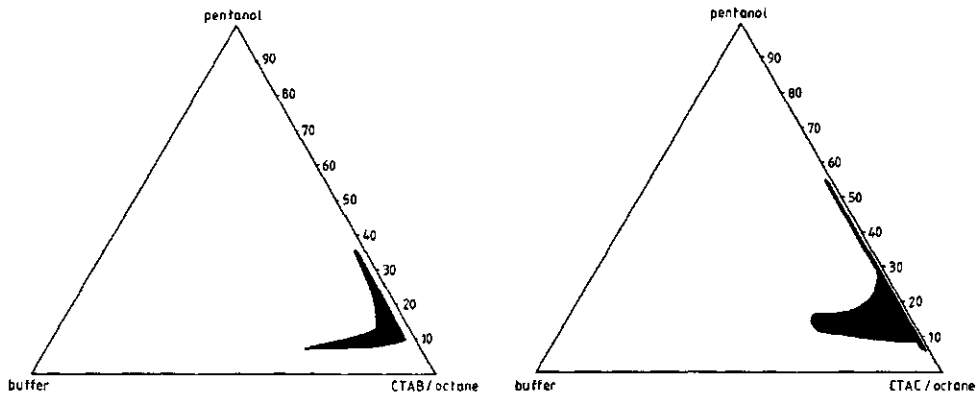
The log P-values of octane, pentanol and DHB were calculated using hydrophobic fragmentation constants [16] and for CTAB the log P value given by Hilhorst [11] was used. log P (CTAC) was assumed to be equal to that of its bromo analogue (-0.66) and log P (MCD) was determined as follows. 3 ml 0.1 M H_3PO_4/KOH pH 3.0 containing 20 mM KBr was shaken with 3 ml of n-octanol containing 5, 25 or 100 µM MCD. After shaking for 16 hours the concentration of MCD in the aqueous layer was determined by means of HPLC (Varian 5000 Liquid Chromatograph equipped with a 25 cm x 4.6 mm Chrompack Standard Column with a Nucleosil 10 CN-filling). MCD was detected with a Kratos Spectroflow 773 Absorbance Detector operating at 278 nm. A log P value of 1.1 was obtained.

Isolation of enzymatic products

The enzymatic reaction was run in 100 ml reversed micellar medium. After termination of the reaction the organic solvent was removed by means of a rotary evaporator and the thick remaining cake of CTAX was extracted with three 50 ml portions of distilled ether. The surfactant is completely insoluble in ether in contrast to the reaction products. The CTAX is filtered off and the ether is evaporated giving the pure product. This procedure is an improvement on the one described earlier for the isolation of steroids [10].

6.3 RESULTS AND DISCUSSION

6.3.1 Bromination reactions



a *b*

Fig. 6.1 *Pseudo-ternary phase diagrams for the system CTAX/octane/pentanol/ 0.1 M H_3PO_4 pH 2.7. The black area reflects those compositions which gave clear solutions within 5 sec mixing time.*
a: X=Br; b: X=Cl

When buffer, cetyltrimethylammonium bromide (CTAB), pentanol and octane are mixed in suitable concentrations a transparent solution is obtained. This solution can be regarded as a dispersion of tiny water droplets in a water-immiscible organic liquid, stabilized by surfactants, in short reversed micelles. This reversed micellar system is only obtained within a small area of the pseudo-ternary phase diagram that can be constructed by varying the concentration of buffer and pentanol with respect to a fixed ratio of octane/CTAB [11] (see Fig. 6.1a). When the chloroperoxidase from *Caldariomyces fumago* was added to the micellar system supplied with hydrogen peroxide and monochlorodimedon (MCD), the organic substrate was completely converted into its brominated analogon (see Scheme 6.1). It should be noted that it is not necessary to add bromide ions to the aqueous phase; the enzyme uses the bromide counter ion of the surfactant as a substrate. Thus the surfactant serves a dual function: i) it stabilizes the reversed micelles, and ii) the bromide counter ion is used for the enzymatic reaction.

6.3.2 Optimisation of the brominating system

Some parameters were varied and the effect on the enzyme activity recorded to determine the optimal conditions for catalysis. With 11% pentanol and 0.2 M CTAB in octane the w_0 can be varied between 5 and 25. Outside this region phase separation occurs. The enzyme activity increases with increasing water content, as shown in Figure 6.2a (trace 1). Typically for apolar substrates enzyme activity is maximal at low w_0 when the concentration of these substrates is rate-determining. For instance, horseradish peroxidase is most active at w_0=10-13 [14,15]. The unusual behaviour of chloroperoxidase might be explained by considering the fact that bromide ions are inhibitory to the enzyme at low pH-values [17] and the bromide concentration in the water pool decreases with increasing water content of the micelles. This phenomenon was not found earlier with horseradish peroxidase because the authors did not study halogenation reactions.

It was recognized that optimal enzyme activity in reversed micelles can be achieved by adjusting the polarity of the micellar interphase and the continuous phase with respect to the polarity of the substrate [11,18]. This was formulated in the following equations: $|\log P_{interphase} - \log P_{substrate}|$ should be minimal and $|\log P_{continuous\ phase} - \log P_{substrate}|$ should be maximal for optimal enzyme activity. The polarity of the interphase can be calculated using equation (3) and the log P values can be calculated by Rekker's method [16].

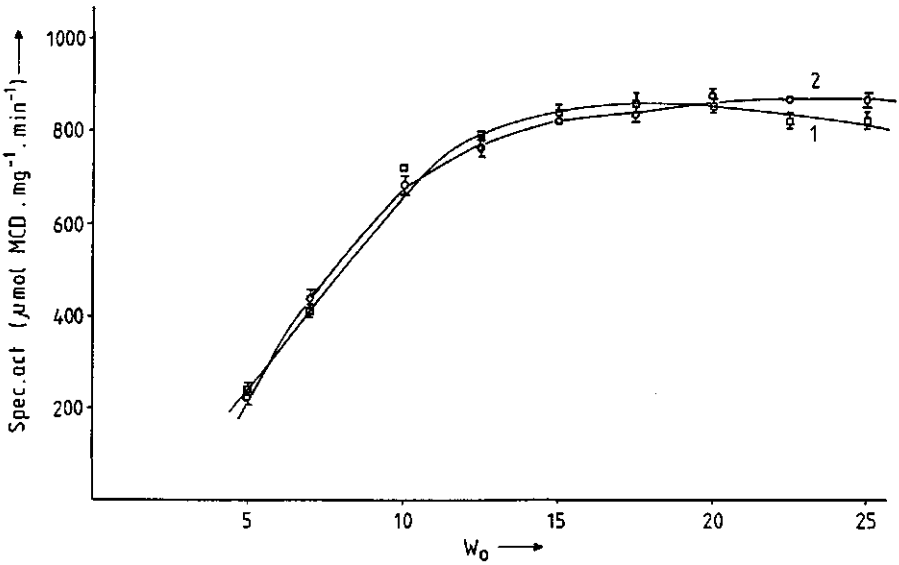
However, one of the parameters in eq. (3) is a_0 (eq. (2)) and the concentration of cosurfactant in the interphase can only be determined at the boundary of the area in the phase diagram where stable reversed micelles are formed. Thus, only empirical considerations are allowed in our case. When we varied the concentration of pentanol in our system we found that the enzymatic bromination rate decreased strongly upon addition of more than 10% pentanol (see Fig. 6.2b, trace 1). Unfortunately it was not possible to study systems with less than 10% pentanol because no stable reversed micellar solutions were obtained. The results obtained are in accordance with those obtained for 20β-hydroxysteroid dehydrogenase [11]. Increasing the pentanol content of the medium makes the interphase more apolar relative to the continuous phase, causing an extraction of the substrate into the bulk continuous phase. This makes the substrate less accessible to the enzyme and decreased activity is observed.

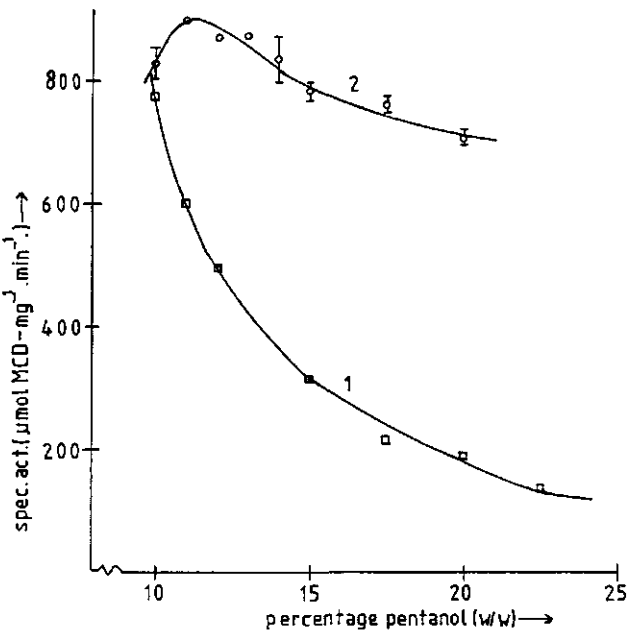
The enzymatic activity in reversed micelles as a function of the pH of the buffer used is plotted in Fig. 6.2c. The shape of the curve is steeper, more bell-shaped than in water, although the position of the optimum is about the same. It is not uncommon that the pH-dependence of enzymes is altered upon entrapment in reversed micelles: for α-chymotrypsin and lysozyme the optimum is shifted 1.5-2.0 pH-units towards basic media, caused by a change in the pK_a of the active site due to the charge of an anionic surfactant [19,20]. However, caution should be exercised in interpreting these results because it is very difficult to measure the pH inside the micelle exactly [21].

The initial rate of the bromination of **1** by CPO in reversed micelles under optimised conditions (11% pentanol, w_0=25, pH=3.0) is about 80% higher than in water (see Table 6.1), clearly showing the advantages of this system.

Fig. 6.2a *(p. 73, upper diagram)*
The effect of w_0 on the enzyme activity. The reversed micelles contain 0.2 M CTAX and 11% (w/w) pentanol. Trace 1: X=Br; Trace 2: X=Cl

Fig. 6.2b *(p. 73, lower diagram)*
The effect of the pentanol concentration on the enzyme activity. The reversed micelles contain 0.2 M CTAX. Trace 1: X=Br, w_0=10; Trace 2: X=Cl, w_0=17.5


Spec.act (µmol MCD . mg⁻¹ . min⁻¹) ⟶
1000
800
600
400
200
5
10
15
20
25
W₀ ⟶
2
1


spec.act.(µmol MCD-mg⁻¹.min⁻¹.)⟶
800
600
400
200
10
15
20
25
percentage pentanol (w/w)⟶
2
1

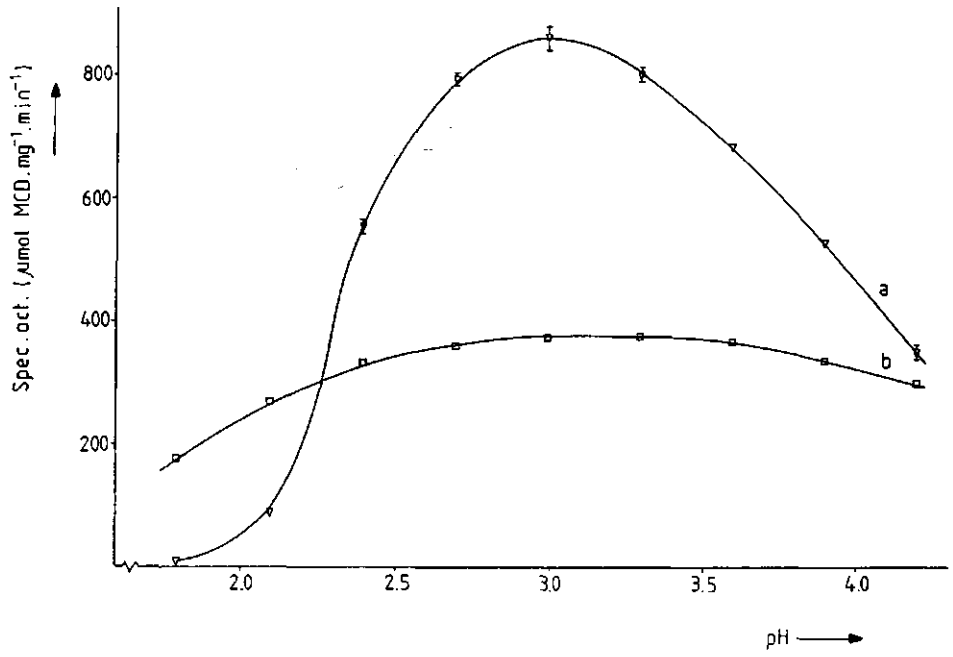
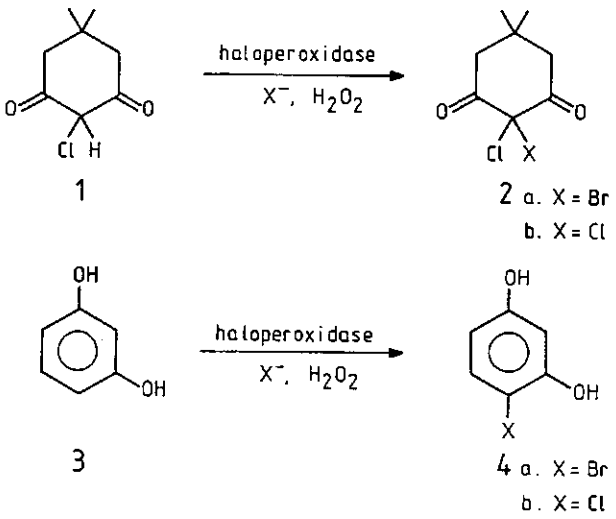


Fig. 6.2c *Specific activity of chloroperoxidase in reversed micelles (a) and aqueous medium (b) as a function of the original pH of the buffer. The reversed micelles were composed of 0.2 M CTAB and 11% pentanol; w_0=25.*

6.3.3 Other substrates

We were also interested in the conversion of other apolar substrates by chloroperoxidase in reversed micelles. It appeared that 1,3-dihydroxybenzene (resorcinol, **3**, see Scheme 6.1) was smoothly converted into its 4-bromo derivative **4a**. The reaction rate in reversed micelles is approximately 2.5 times higher than in aqueous buffer (see Table 6.1). Methoxybenzene, 4-methoxyphenol and 1,4-dimethoxybenzene were not brominated in micellar media although they are (slowly) converted in water. Adjustment of the micellar interphase by using various amounts of n-heptanol or n-octanol had no effect on the results. The reason for this resistance towards reaction in reversed micelles is unknown.



1

2 a. X = Br
b. X = Cl

3

4 a. X = Br
b. X = Cl

Scheme 6.1 Reactions catalyzed by chloroperoxidase in reversed micelles.

*Table 6.1 Rates for the chloroperoxidase-mediated conversion of monochlorodimedon (**1**) and resorcinol (**3**) in aqueous and micellar media.*

	buffer[a]	reversed micelles
chlorination of **1**	408±6	885±6[b]
bromination of **1**	489±4	872±9[c]
chlorination of **3**	196±3	478±18[b]
bromination of **3**	199±10	508±5[c]

[a] Reaction conditions: 0.1 M potassium phosphate buffer pH 3.0, 20 mM KX (X=Br or Cl).

[b] Reaction conditions: 11% pentanol, w_0=17.5.

[c] Reaction conditions: 11% pentanol, w_0=25.

6.3.4 *Chlorination reactions*

The natural function of the chloroperoxidase of *Caldariomyces fumago* is the chlorination of organic substrates [22]. Hence, we were interested in chlorination reactions using chloroperoxidase in reversed micelles composed of buffer, cetyltrimethylammonium chloride (CTAC), pentanol and octane. The new pseudo-ternary phase diagram is depicted in Fig. 6.1b. The shape of the area where stable reversed micelles are obtained deviates from that obtained with CTAB (Fig. 6.1a), indicating that the physical properties of the surfactants become different upon changing solely the counter ion. The degree of hydration of the counter ion has a strong influence on the shape and the size of the reversed micelle [23]. As chloride ions are more hydrated than bromide ions, this fact could explain the differences between Fig. 6.1a and 6.1b. MCD (**1**) and resorcinol (**3**) were smoothly chlorinated with this system giving **2b** and **4b** respectively (see Scheme 6.1). Methoxybenzene, p-methoxyphenol and p-dimethoxybenzene were not converted by this system, as was found earlier for CTAB.

6.3.5 *Optimisation of the chlorinating system*

Fig. 6.2a (trace 2) shows that the enzymatic activity increases with increasing w_0. However, in contrast to the case of CTAB there seems to be an optimum in the curve. This can be explained by considering the fact that chloride ions are inhibitors of the enzyme, just like bromide ions. Their concentration decreases at increasing w_0 causing enhanced enzymatic activity. However, the concentration of hydrogen peroxide inside the reversed micelles decreases too, so these effects will compensate each other at a certain w_0. For chloride this is around w_0=19 and for bromide it is apparently above w_0=25 because bromide is a stronger inhibitor for chloroperoxidase than chloride.

The effect of the concentration of pentanol on the enzymatic activity is shown in Fig. 6.2b (trace 2). Remarkably, an optimum was observed, at 11% pentanol, whereas the optimal concentration of pentanol in the case of CTAB was below 10%. According to the theory of Hilhorst and Laane [11,18] this means that in the case of CTAC there is more pentanol needed to make log $P_{interphase}$ equal to log P_{MCD} then when CTAB is the surfactant. In other words, log P_{CTAC} is more negative than log P_{CTAC}, probably because the chloride ion is more hydrated.

The rates of chlorination of **1** and **3** in reversed micelles at optimised

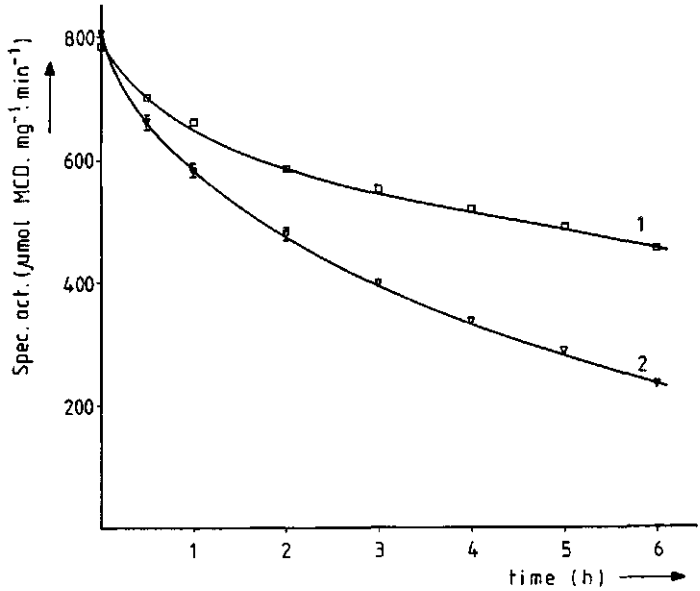
conditions (11% pentanol, w_0=17.5) were approximately 2.2 times higher than in aqueous buffer (see Table 6.1).

6.3.6 Stability of chloroperoxidase in reversed micelles

Chloroperoxidase is known to be a labile enzyme, losing its activity rather rapidly during catalysis [5]. When the enzyme was entrapped into reversed micelles activity was lost at a comparative rate when the surfactant was CTAC (see Figure 6.3a) but about two times faster than in water when CTAB was used. This observation is hard to explain; perhaps some bromide ions are oxidized by oxygen dissolved in the octane giving bromine, which could inactivate the enzyme.

Chloroperoxidase is inactivated irreversibly by large amounts of hydrogen peroxide [24]. This effect is even more pronounced in the micellar system because hydrogen peroxide is almost completely confined to the water pool, in the immediate vicinity of the enzyme. The initial enzymatic activity increases with a higher hydrogen peroxide concentration (not shown), but the activity decreases more rapidly on standing. This is shown in Fig. 6.3b where the percentage of the initial enzyme activity is plotted against time for different concentrations of hydrogen peroxide. The same phenomenon was observed under turnover conditions: the turnover number of the enzyme is about 90,000 in reversed micelles (0.5 mM **1**, 0.2 M CTAC, 11% pentanol, w_0=17.5) and about 550,000 in buffered water (2.0 mM **1**, 0.1 M phosphate buffer pH 2.7). This is not caused by a shortage of bromide ions, since their concentration is 0.2 M, compared to maximally 2 mM of substrate.

Summarizing, the enzyme chloroperoxidase from the mold *Caldariomyces fumago* is able to work efficiently in reversed micelles composed of aqueous buffer, CTAX (X=Cl or Br), pentanol and octane. The initial enzymatic activity is about twice as high as in water, improving the enzymatic conversion of apolar compounds like **1** and 3. However, enzyme stability is not enhanced by entrapment, especially when hydrogen peroxide is present. It should be noted that recently a bromoperoxidase from a brown alga (*Ascophyllum nodosum*) was isolated which is very stable in mixtures of water and water-miscible organic solvents and which is not inactivated by high concentrations of hydrogen peroxide [25,26].


Spec. act. (µmol MCD. mg⁻¹. min⁻¹)
800
600
400
200
1
2
3
4
5
6
time (h)
1
2

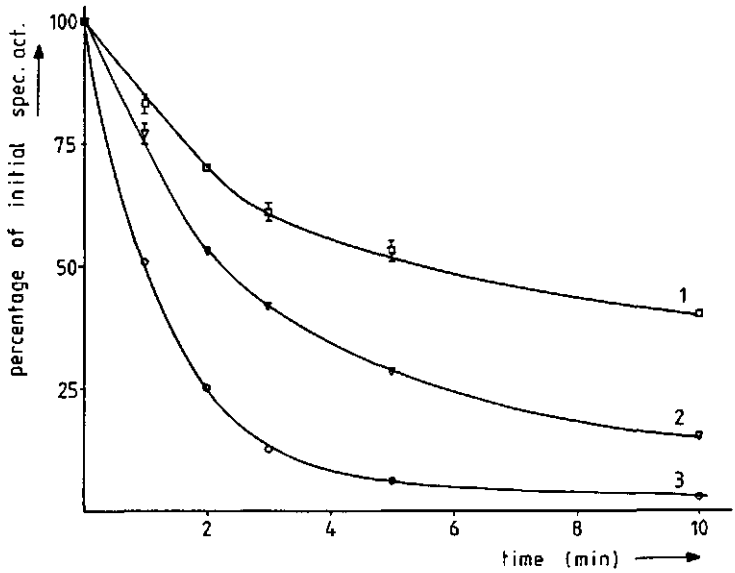

percentage of initial spec. act.
100
75
50
25
2
4
6
8
10
time (min)
1
2
3

Fig. 6.3a *(p. 78, upper diagram)*
Stability of chloroperoxidase in reversed micelles, without hydrogen peroxide. The systems were composed as follows: trace 1: 0.2 M CTAC, 11% pentanol, w_0=17.5; trace 2: 0.2 M CTAB, 11% pentanol, w_0=25.

Fig. 6.3b *(p.78, lower diagram)*
Stability of chloroperoxidase in reversed micelles with added hydrogen peroxide. The overall concentration of hydrogen peroxide is: trace 1: 0.04 mM; trace 2: 0.20 mM; trace 3: 0.40 mM. The vertical coordinate reflects the activity at t=t compared to t=0. The medium is composed of 0.2 M CTAB and 11% pentanol; w_0=25.

6.4 REFERENCES

[1] S.L. Neidleman and J. Geigert: *Biohalogenation: principles, basic roles and applications*, Ellis Horwood Ltd., Chichester (1986), 1-203
[2] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100
[3] M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70
[4] M.C.R. Franssen, H.G. van Boven and H.C. van der Plas, J. Heterocyclic Chem., in press
[5] C.L. Cooney and J. Hueter, Biotechnol. Bioeng. **16** (1974), 1045-1053
[6] K. Martinek, A.V. Levashov, N. Klyachko, Y.L. Khmelnitski and I.V. Berezin, Eur. J. Biochem. **155** (1986), 453-468
[7] P.L. Luisi and C. Laane, Trends Biotechnol. **4** (1986), 153-161
[8] P.L. Luisi, P. Lüthi, I. Tomka, J. Prenosil and A. Pande, Ann. N.Y. Acad. Sci. **434** (1984), 549-557
[9] K.M. Lee and J.-F. Biellmann, Bioorg. Chem. **14** (1986), 262-273
[10] R. Hilhorst, C. Laane and C. Veeger, FEBS Lett. **159** (1983), 225-228
[11] R. Hilhorst, R. Spruijt, C. Laane and C. Veeger, Eur. J. Biochem. **144**, (1984), 459-466
[12] C. Laane, S. Boeren and K. Vos, Trends Biotechnol. **3** (1985), 251-252
[13] K. Martinek, A.V. Levashov, N.L. Klyachko, V.I. Pantin and I.V. Berezin, Biochim. Biophys. Acta **657** (1981), 277-294
[14] K. Martinek, N.L. Klyachko, A.V. Levashov and I.V. Berezin, Dokl. Akad. Nauk SSSR (Engl. Transl.) **269** (1983), 69-71
[15] A.N. Eremin and D.I. Metelitsa, Biokhimiya (Engl. Transl.) **50** (1985), 91-97
[16] R.F. Rekker, Eur. J. Med. Chem. **14** (1979), 479-488
[17] J.A. Thomas, D.R. Morris and L.P. Hager, J. Biol. Chem. **245** (1970), 3135-3142
[18] C. Laane, S. Boeren, K. Vos and C. Veeger, Biotechnol. Bioeng., in press
[19] F.M. Menger and K. Yamada, J. Am. Chem. Soc. **101** (1979), 6731-6734
[20] C. Grandi, R.E. Smith and P.L. Luisi, J. Biol. Chem. **256** (1981), 837-843
[21] R.E. Smith and P.L. Luisi, Helv. Chim. Acta **63** (1980), 2302-2311
[22] J.R. Beckwith and L.P. Hager, J. Biol. Chem. **238** (1963), 3091-3094

[23] H.-F. Eicke and P. Kvita, in *Reverse Micelles: Biological and Technological Relevance of Amphiphilic Structures in Apolar Media* (P.L. Luisi and B.E. Straub, eds), Plenum Press, New York (1984), 21-35
[24] P.D. Shaw and L.P. Hager, J. Biol. Chem. **236** (1961), 1626-1630
[25] R. Wever, H. Plat and E. de Boer, Biochim. Biophys. Acta **830** (1985), 181-186
[26] E. de Boer, H. Plat, M.G.M. Tromp, R. Wever, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer and H.E. Schoemaker, Biotechnol. Bioeng., in press

7 THE IMMOBILIZATION OF CHLOROPEROXIDASE

7.1 INTRODUCTION

The use of enzymes as catalysts in organic synthesis is a growing field of interest because of the unique properties that enzymes possess: they are specific, react fast and under mild conditions. Drawbacks are however their cost which makes it necessary to re-isolate them from the reaction mixture, and their limited stability. A way to overcome these problems is immobilization: the confinement of the enzyme to a restricted area (see for recent reviews [1-5]).

We are interested in the use of haloperoxidases in organic synthesis. Haloperoxidases are enzymes that are capable of halogenating a large variety of electron-rich organic compounds, for example barbituric acid [6,7] and pyrazole derivatives [8]. Large scale conversions of these compounds necessitate immobilization of the enzyme. In this study we present our results on the immobilization of the chloroperoxidase (CPO) from the mold *Caldariomyces fumago*. This is an extracellular, polar glycoprotein, with an isoelectric point of about 3.7 [9] and an optimal pH of 2.7. Taking these facts into account possible immobilization methods are adsorption to hydrophilic neutral or charged supports. The latter may be positively or negatively charged; at pH 2.7 the net charge of the enzyme will be positive but the charge may be unequally distributed over the surface of the enzyme. Another possible immobilization method is covalent binding via amide linkages because CPO has a large amount of acidic residues in its protein backbone [10]. An alternative is the binding via Schiff bases using the sugar part of the enzyme [11]. Since entrapment is also known as a mild method resulting in high yields [12], the following five methods were investigated with CPO:

1) adsorption to a polar neutral support
2) ionic binding to charged supports
3) covalent binding to supports, containing an alkylamino group
4) entrapment in calcium alginate
5) entrapment/covalent binding in an acrylic polymer.

7.2 MATERIALS AND METHODS

General

Crude grade *Caldariomyces fumago* chloroperoxidase with a specific activity of 400-500 μmol of monochlorodimedon (MCD).mg^{-1}.min^{-1} was obtained from Sigma, as was MCD. The coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), azobisisobutyronitril (AIBN), 3-aminopropyltriethoxysilane and triethylamine were all purchased from Merck; *N*-hydroxysuccinimide, acryloylchloride and triethylenetetramine (TET) were from Janssen Chimica; acrylamide and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) were from BDH. *N*-acryloxysuccinimide was synthetized according to Pollak [13]. Hydroxyapatite (HAP) was obtained from Bio-Rad, DE-32 DEAE-Cellulose from Whatman, Controlled-Pore Glass (CPG) from Corning Glassworks and sodium alginate from Fluka. Silicaspheres S 970h 1.6 and S 970b 1.5 were generous gifts from Shell, UK. Bio-Rad Standard Econo-Columns, dimensions 1.0 cm ID x 10 cm and the Pharmacia peristaltic pump P-1 were used unless indicated otherwise. The dialysis tube was from Spectrapor, type 3787D12, 6 mm ID; it was boiled in 0.01 M EDTA before use. UV-spectroscopy was performed on an Aminco-Chance DW-2 split-beam spectrophotometer, a Beckman DU-7, a Varian DMS-90 or on a Varian DMS-100 apparatus equipped with a DS-15 data station. Fast Protein Liquid Chromatography (FPLC) was conducted with a Pharmacia apparatus consisting of two P-500 pumps and a GP-250 Gradient Programmer.

Aminopropylation of CPG and Silicaspheres

CPG was cleaned by refluxing with concentrated nitric acid during 8 h at 140°C. After thorough washing with doubly distilled water the support was treated with 3-aminopropyltriethoxysilane in water according to Weetall [14].

Two methods were used for the aminopropylation of the Shell Silicaspheres:

a) [15]; 2.5 g of Silicaspheres were washed with 2x20 ml of distilled diethyl ether and dried for 1 h at 100°C. 2.5 ml of 3-aminopropyltriethoxysilane was dissolved in doubly distilled water; the pH of this solution was adjusted to 5 with some acetic acid and the volume was increased to 25 ml with water. 12 ml of this solution was added to 2.5 g of washed Silicaspheres; the mixture was degassed at a water pump, gently swirled at 80°C for 1 h and incubated overnight at room temperature. After decanting the liquid the spheres were washed 5 times with doubly distilled water.

b) 5 ml of 3-aminopropyltriethoxysilane dissolved in 25 ml of distilled toluene was added to 5 g of washed Silicaspheres. The mixture was gently

swirled for 1 h at 90°C and for an additional hour at room temperature. The toluene was decanted, the spheres were washed with 2x40 ml of distilled toluene and dried for 1 h at 110°C.

The aminopropyl content of the spheres was determined by elemental analysis. Prior to analysis the spheres were dried for 2 h at 110°C, stored in vacuo if necessary, and crushed in an agate mortar prior to analysis. The results are expressed in mmol carbon per gram of support (referred to as "K") or as the number of aminopropylgroups per nm^2 (M), which are calculated by means of the following formulas [16]:

$$K \ (mmol/g) = \%C \cdot \frac{1}{12 \cdot Z} \cdot 10$$

$$M \ (propylaminogroups/nm^2) = \frac{K}{(1000-K.MW).SA} \cdot N \cdot 10^{-24}$$

%C = percentage carbon as found by elemental analysis
Z = number of carbon atoms per group (3)
MW = molecular weight of the C_3H_8-NH-Si-O group (102)
SA = specific surface area (87 m^2/g for the Shell Silicaspheres type S 970h 1.6; 350 m^2/g for type S 970b 1.5)
N = Avogadro's number ($6.02 \cdot 10^{23}$)

The results for the S 970h 1.6 spheres were:

blank	0.6 %C				
method a)	1.2 %C	=	0.3 mmol carbon/g	=	2.4 aminopropylgroups/nm^2
method b)	2.8 %C	=	0.8 mmol carbon/g	=	5.8 aminopropylgroups/nm^2

Comparable results were obtained with the S 970b 1.5 spheres. Conducting the reaction in an organic solvent like toluene is a significant improvement compared to the first method, as was already found by Weetall [14]. 5.8 aminopropylgroups per nm^2 is about the highest attainable value [17]. Only spheres modified in this way were used in the immobilization procedures.

Determination of enzyme activity

The activity of free CPO was determined as described before [18] using MCD as a substrate. For immobilized enzyme the procedure was as follows. An appropriate amount of immobilized enzyme was added to 25 ml of 0.1 M phosphate buffer pH 2.7 containing 20 mM of KCl in a 250 ml erlenmeyer. The reaction was started by the addition of 60 µl of 100 mM hydrogen peroxide. 2.7 ml aliquots were taken with suitable time intervals and added to 300 µl of 1.0 M trichloroacetic acid to stop the reaction. The absorption at 270 nm was recorded and the enzyme activity was calculated using $\Delta\varepsilon$= 12,200 $l.mol^{-1}.cm^{-1}$. Generally, the efficiency of the immobilization process is preferably determined by the enzyme activity than by determining the protein content of the immobilized material, because the former method is much more sensitive (down to 0.4 µg enzyme/ml). The yield of the immobilization procedure is defined as the ratio of the total activity of the immobilized enzyme (expressed in Units, U) to the total activity of the initially added enzyme. One Unit is the amount of enzyme that catalyses the conversion of 1 µmol of MCD in 1 min. The recovery is defined as the sum of the enzyme activities in the washing liquids and the immobilized material, divided by the total activity of the initially added enzyme. The recovery indicates how mild the immobilization conditions are.

1) Adsorption to a neutral support

Hydroxyapatite (HAP) was taken for these immobilization studies. The support was pretreated according to [19]: 0.5 g of HAP was mixed with ± 5 ml of 10 mM potassium phosphate buffer pH 6.0 and gently swirled avoiding mechanical stirring. The slurry was allowed to settle for 10 min and then decanted. These steps were repeated twice. The swollen material was transferred to a small column and the bed was allowed to settle overnight. 0.5 ml of CPO (93 µg/ml) was diluted to 10 ml with 9.5 ml of 1 mM phosphate buffer pH 6.0 and pumped through the column at a rate of 25 ml/h at 4°C.

2) Adsorption to charged supports

a) Adsorption to DEAE-Cellulose. 100 mg of DEAE-Cellulose was dissolved in 1 mM potassium phosphate buffer pH 6.0 and placed inside a small column (0.5 cm ID). After removal of the fines 0.4-0.8 mg of CPO dissolved in 5 ml of potassium phosphate buffer pH 6.0 was pumped through the column at a flow rate of 25 ml/h. The immobilization was performed at room temperature.

b) Adsorption to 3-aminopropyl-CPG [20]. 4.5 ml of doubly distilled water and 0.5 ml of CPO (93 µg/ml) were added to 100 mg of 3-aminopropyl-CPG in a 10

ml round-bottomed flask. The pH of this solution was 5.2. The mixture was gently swirled overnight after which the glass beads were decanted.

c) Adsorption to a negatively charged support by means of FPLC. Fast Protein Liquid Chromatography (FPLC) consists of the binding of an enzyme to a column filled with a well-defined adsorbent at elevated pressure. The enzyme molecules are eluted by applying gradients of salts, substrates, inhibitors, etc. This method is very well suited for the rapid determination of the strength of binding between the enzyme and the adsorbent under various conditions.

40 µg of CPO was diluted with 5 mM phosphate buffer pH 2.7 containing 1 mM KCl and injected into a Mono-S FPLC column. Mono-S is an ion exchanger containing sulphonic acid groups and is therefore negatively charged, even at pH 2.7. The initial eluent was 1 mM KCl/5 mM potassium phosphate pH 2.7 and the flow rate was 1.5 ml/h. After 3 min a linear salt gradient was started: 1-1000 mM KCl/5 mM potassium phosphate pH 2.7 or 1 mM KCl/5-100 mM potassium phosphate pH 2.7. Protein was detected by its UV-absorbance at 280 nm.

3) Covalent binding to supports, containing an alkylamino group

a) Covalent binding to 3-aminopropyl-CPG by means of amide links ([21], modified). To 100 mg of support in a 10 ml round-bottomed flask was added 4.5 ml of doubly distilled water and 50 mg of coupling agent (EDC). The pH was adjusted to 4.0-4.5 with dilute hydrochloric acid. 0.5 ml of CPO (93 µg/ml) was added and the mixture was gently swirled overnight after which the glass beads were decanted. The pH of the solution decreased somewhat during the reaction and was readjusted with dilute sodium hydroxide.

b) Covalent binding to 3-aminopropyl-silicaspheres by means of Schiff base formation ([22,23], modified). 300 µl of 100 mM potassium phosphate buffer pH 2.7 containing 20 mM KCl, 100 µl of 0.7 mM sodium periodate (70 nmol) dissolved in the same buffer, and 274 µg of CPO (6.5 nmol) were placed in a 10 ml flask and incubated for 6 h at 4°C in the dark. 0.5 ml of this oxidized enzyme solution was introduced to 100 mg of aminopropyl-silicaspheres; the pH was adjusted to 4.5 with 100 mM potassium phosphate buffer pH 6.0. The mixture was gently swirled overnight at 4°C in the dark after which the glass beads were decanted.

The stability of the Schiff base. This experiment was performed with two different concentrations of periodate. A. 430 µl of potassium phosphate buffer pH 2.7 containing 20 mM KCl, 20 µl of 0.7 mM sodium periodate dissolved in the same buffer, and 203 µg of CPO were placed in a 10 ml flask. B. 350 µl of potassium phosphate buffer pH 2.7 containing 20 mM KCl, 100 µl of 0.7 mM sodium

periodate dissolved in the same buffer, and 203 µg of CPO were placed in a 10 ml flask. These solutions were incubated overnight at 4°C in the dark. The pH of the solution was adjusted to 4.5 with 1 ml of 100 mM potassium phosphate buffer pH 6.0. 306 mg of 3-aminopropyl-silicaspheres was added to solution A and 298 mg to B. After stirring for 24 h at 4°C in the dark the supernatants were decanted and their enzyme activities determined. The immobilized material was divided into three portions and to each was added 1 ml of one of the following solutions: doubly distilled water; 100 mM potassium phosphate buffer pH 4.5; 100 mM potassium phosphate buffer pH 2.7 containing 20 mM KCl. After 1 h incubation the enzyme activity in the supernatants was determined. Another 1 ml of the same liquid was added to the immobilized CPO and the enzyme activity of the supernatant was measured again after 4 days of incubation.

4) Entrapment in calcium alginate

To a cooled and deaerated solution of 0.10 g of sodium alginate (2% w/v) in 5.0 ml of 200 mM potassium phosphate buffer pH 6.0 was added 20 U of CPO in 1.0 ml of the same buffer. The mixture was injected dropwise into 0.1 M potassium phosphate buffer pH 2.7 containing 20 mM KCl and 50 mM $CaCl_2$, giving off-white calcium alginate spheres. The spheres were allowed to harden for 3h after which they were isolated by filtration on a Büchner funnel and washed with a little buffer pH 2.7.

5) Entrapment/covalent binding in an acrylic polymer

Synthesis of poly(acrylamide-co-*N*-acryloxysuccinimide) (PAN) [13]. 0.13 g (0.8 mmol) of AIBN, 2.57 g (15 mmol) of *N*-acryloxysuccinimide and 22.91 g (322 mmol) of acrylamide were dissolved in 250 ml of CaH_2-dried THF, in a 1 l three-necked round-bottomed flask. The solution was stirred for 1 h at 20°C under an atmosphere of dry nitrogen. The stirring was stopped, the temperature was raised to 50°C and a white precipitate formed slowly. After 24 h the precipitate was resuspended in 125 ml of dry THF, filtered under a nitrogen atmosphere, washed with 3x100 ml of dry THF and dried in a vacuum exsiccator, first at room temperature, later at 50°C. The yield was 26.7 g (105%), indicating that not all the THF could be removed. The adsorbed THF did not influence the immobilization procedure.

Active ester content of the PAN. One of the important parameters in this immobilization procedure is the *N*-hydroxysuccinimide ester content, in the following referred to as the active ester content. This *N*-hydroxysuccinimide group is replaced by nucleophilic groups of the enzyme, leading to covalently

bound enzyme, or it is replaced by the amino groups of the cross-linking agent, giving a cross-linked polymer. The active ester content was determined as follows. 50 mg of PAN was dissolved in 5.00 ml of doubly distilled water; a 50 µl aliquot of this solution was added to 2900 µl of 0.1 M HEPES buffer pH 7.5 and the UV-absorption at 259 nm was recorded. 50 µl of 1 M ethylamine was added and after 2 h A_{259} was recorded again. The active ester content was determined from the obtained data using $\Delta\varepsilon$(*N*-hydroxysuccinimide) = 8600 $M^{-1}.cm^{-1}$. Each determination was carried out in duplicate. The active ester content is expressed in µmol *N*-hydroxysuccinimide released per gram of dry PAN and is rounded to the nearest 50; thus, PAN-500 has an active ester content of 500±25.

CPO immobilization in cross-linked PAN. Several parameters were studied in the immobilization process, such as the percentage of PAN in solution, the amount of cross-linking agent (triethylenetetramine, TET), the gelation time and the nature of the gel. A typical experiment was conducted as follows. 225 mg of PAN-500 is dissolved in 1 ml of 0.3 M HEPES buffer pH 6.0 and placed in a small flat-bottomed tube to enable magnetic stirring; a small graduated cylinder proved to be very convenient. When the polymer had dissolved completely 113 µg of CPO (0.5 mg/g PAN) were added and the mixture was stirred for 30 min. 3.3 mg (22.5 µmol) of TET were added and after 6 min the stirring bar started to "jump" because of the increased viscosity of the gelating solution. The gel was allowed to harden for 30 min at room temperature and subsequently for 24 h at 4°C. The hardened gel was pressed through a piece of copper gauze to obtain small particles and washed with 10 ml of 20 mM NH_4Cl in 0.1 M HEPES pH 6.0 to hydrolyze the remaining active esters. The particles were filtered and suspended in 10 ml of 0.3 M HEPES pH 6.0. The enzymatic activity of the washing liquids and the immobilized material was recorded.

The following experiment was executed to determine if the enzyme could leak out of the cross-linked polymer. 15 ml of potassium phosphate buffer pH 2.7 was added to 0.75 g of immobilized material suspended in 12.25 ml of 0.3 M HEPES buffer pH 6.0, in a 50 ml erlenmeyer. The suspension was stirred magnetically for 6 h. Stirring was stopped every 90 min, an aliquot of the supernatant was taken and examined for enzyme activity.

7.3 RESULTS AND DISCUSSION

7.3.1 Adsorption to a neutral support

Hydroxyapatite (HAP) is a well-known support widely used for the binding and purification of proteins and nucleic acids. When chloroperoxidase diluted in 1 mM phosphate buffer pH 6.0 was pumped through a column of HAP all the enzyme molecules were bound (high yield). They also proved to be very active (high recovery, see Table 7.1), indicating that HAP is a very attractive support for this enzyme. However, HAP has only a limited stability at pH 2.7, the optimal pH for the enzyme. 50% of the support material dissolves within 72 h on equilibration with 10 mM H_3PO_4/KOH pH 2.7/20 mM KCl at room temperature and within 24 h if the concentration of the buffer is increased by a factor ten. HAP is much more stable at 4-6 °C and it proved possible to convert barbituric acid to 5-chloro- and 5,5-dichlorobarbituric acid with this system during 70 h

Table 7.1 Stability of CPO immobilized on HAP, with respect to low pH and high ionic strength.[a]

washing liquid	enzyme activity in washing liquid[b]	yield[c]	recovery[d]
10 mM H_3PO_4/KOH pH 2.7	1	73	74
10 mM H_3PO_4/KOH pH 2.7 + 10 mM KCl	1	88	89
100 mM H_3PO_4/KOH pH 2.7 + 20 mM KCl	31	ND[e]	ND

[a] Different batches of immobilized enzyme were washed with the indicated liquids and the enzyme activities in the immobilized material and in the washing liquids were subsequently determined.

[b] As percentage of the total activity of the initially added enzyme.

[c] The yield is defined as the ratio of the total activity of the immobilized enzyme to the total activity of the initially added enzyme.

[d] The recovery is defined as the sum of the enzyme activities in the washing liquids and the immobilized material, devided by the total activity of the initially added enzyme. In other words, the figures in the third column are the sum of those in the first and the second column.

[e] ND = not determined

atic halogenation. Minimizing the ionic strength by using 1 mM phosphate buffer pH 3.0/2 mM KCl resulted in partial denaturation of the enzyme.

b) Adsorption to 3-Aminopropyl-Controlled-Pore Glass (Amino-CPG).

CPG is a very attractive and widely used support for enzymes since it has a number of advantages [25]:

1) great mechanical strength
2) resistance towards organic solvents, low pH and microbial attack
3) possibility to recycle
4) easy use
5) excellent flow properties

The surface of CPG is composed of silanol groups which can be easily modified by trialkoxysilanes. This is usually accomplished by reacting the support with the silane derivative in an aqueous medium. However, conducting the reaction in toluene increases the degree of silanization considerably, presumably because the hydrolysis of the silane reagent by water is minimized. CPG modified with 3-aminopropyltriethoxysilane by this procedure was used for the immobilization of CPO. A test experiment with bovine serum albumin showed that 7 mg of protein could be bound to 100 mg of modified CPG. When CPO was mixed with the support overnight all enzyme activity was bound to the CPG, but the enzyme readily detached on washing with buffers of pH 2.7 (see Table 7.3). An explanation for these results may be that positively charged supports attract chloride ions from the solution leading to high local chloride concentrations. CPO is known to be inhibited by high concentrations of halide ions at low pH [26]. Another reason may be that at pH 2.7 the enzyme is repelled by the support. Binding of the enzyme to the negatively charged support (the Mono-S FPLC column of Pharmacia, which contains sulphonic acid groups) at low pH neither gave good results, as the enzyme already eluted with the void volume when 5 mM H_3PO_4/KOH pH 2.7 containing 1 mM KCl was used as eluent. Thus, ionic binding is not a favorable method for the immobilization of CPO. In two US patents however CPO immobilized on DEAE-Cellulose is applied to the synthesis of halohydrins [27,28]. The immobilization procedure seems to be efficient and was carried out at pH 4.4. The reactions were also carried out at pH 4.4 using 0.42 M potassium bromide. High concentrations of halide are known to shift the optimal pH for haloperoxidases towards higher values, so the enzyme might be rather active under these conditions. However, since we are interested in the

CPO-mediated conversions of heterocyclic compounds which are very water-soluble low ionic strength is needed for a good isolation process (see Ch. 2 and 4). For this reason we continued to search for immobilization procedures which lead to systems that are more stable at low pH.

Table 7.3 Stability of CPO adsorbed on 3-aminopropyl-CPG, with respect to low pH and high ionic strength.[a]

washing liquid	enzyme activity in washing liquid [b]	yield [b]	recovery [b]
doubly distilled water	0	100	100
10 mM H_3PO_4/KOH pH 2.7 + 10 mM KCl	20	ND [c]	ND
100 mM H_3PO_4/KOH pH 2.7 + 20 mM KCl	50	3	59

[a] Different batches of immobilized enzyme were washed with the indicated liquids and the enzyme activities in the immobilized material and in the washing liquids were subsequently determined.

[b] See Table 7.1 for explanation.

[c] ND = not determined.

7.3.3 Covalent binding to supports, containing an alkylamino group

a) Covalent binding to aminopropyl-Controlled-Pore Glass (CPG) by means of amide bond formation.

Enzymes can be covalently bound to amino-supports via their carboxylic acid residues using a carbodiimide such as EDC as coupling agent [21,29]. The results of this approach are shown in Table 7.4. CPO was bound to 3-aminopropyl-CPG in rather low yield (7-13%) but the linkage was stable in buffers of high ionic strength and low pH. Further adjustment of the concentration of the enzyme or the coupling reagent did not improve the yield. It should be noted that the recoveries are not 100%, so part of the enzyme is inactivated. Whether this denaturated enzyme is free or immobilized could not be determined because enzyme concentrations were too low for determination. The fact that a higher concentration of coupling reagent leads to decreased enzyme activity (see Table 7.4) indicates that the coupling reagent is involved in the inactivation procedure, presumably by reacting at the active site of the enzyme.

on a semi-preparative scale without decrease in bed volume. The fact however that the enzyme only reacted very slowly at this temperature (64 µmol barbituric acid/h = 128 µmol MCD/h = 0.4% of the activity of free enzyme at 25°C) made this procedure unfavorable for large-scale conversions. This method is very attractive for the application of the halide-independent oxidation reaction (the "classical" peroxidase reaction) of CPO, which is carried out at pH 4.5, as was shown by Neidleman for the production of iodine on an industrial scale [24].

7.3.2 Ionic binding to charged supports

a) Adsorption to DEAE-Cellulose.

The bonding between oppositely charged molecules is usually stronger than between neutral molecules, so immobilization of CPO on an ion exchange resin could be useful. DEAE-Cellulose was chosen because it has been found previously

Table 7.2 Stability of CPO immobilized on DEAE-Cellulose, with respect to low pH and high ionic strength.[a]

washing liquid	enzyme activity in washing liquid [b]	protein content of washing liquid [c]
10 mM H_3PO_4/KOH pH 6.0	0	0
10 mM H_3PO_4/KOH pH 2.7	34	41
10 mM H_3PO_4/KOH pH 2.7 + 20 mM KCl	85	84
1 mM H_3PO_4/KOH pH 3.0 + 2 mM KCl	28	66

[a] Different batches of immobilized enzyme were washed with the indicated liquids and the enzyme activities and the protein content of the washing liquids were subsequently determined.

[b] As percentage of the total activity of the initially added enzyme.

[c] As percentage of the total initially added amount of protein.

that CPO binds strongly to this support [18]. When CPO in phosphate buffer pH 6.0 was pumped through a small column of DEAE-Cellulose all the enzyme was bound to the column material but on washing with phosphate buffer of low pH the enzyme was readily released from the ion exchanger (see Table 7.2). This effect was increased by the addition of chloride ion, a substrate necessary for enzym-

Table 7.4 Characteristics of the immobilization of CPO on 3-aminopropyl-CPG by amide linkages.[a]

immobilization temperature (°C)	added amount of EDC (mg)	enzyme activity in supernatant[b]	yield[c]	recovery[c]
4-6	50	19	7	26
21-23	20	22	13	35
21-23	50	38	9	47

[a] The formation of the covalent bond was achieved by means of the carbodiimide coupling reagent EDC.

[b] As percentage of the total activity of the initially added enzyme.

[c] See Table 7.1 for explanation

b) Covalent binding of CPO to aminopropyl-silicaspheres by means of Schiff base formation.

The reaction of periodic acid with glycoproteins giving free sugars and aldehydes is well established [30]. These aldehyde moieties have successfully been used for the covalent binding of glycoenzymes to aminated supports giving Schiff bases [11,22,31,32]. Since CPO is a glycoprotein containing 25-30 wt.% of arabinose and glucosamine this method seemed useful for the immobilization of this enzyme. The effect of periodate on the enzyme activity was investigated prior to the experiments. It appeared that periodate inhibits the CPO-mediated chlorination of MCD. When CPO was oxidized by periodate at pH 2.7 and the excess of periodate was removed the activity of the recovered enzyme had increased by 47%. An explanation may be that the enzyme becomes more flexible when large parts of its sugar chains are oxidatively removed, facilitating the approach of substrates. The same phenomenon was observed in the case of invertase [22]. The origin of the periodate-mediated inhibition of the chlorination reaction cannot be explained in this way; possibly the oxidizing agent intervenes with an enzymatic intermediate during catalysis [33].

We chose 3-aminopropylated Shell Silicaspheres as solid support for the experiments, because they have the same advantages as CPG but their properties are better characterized. The procedure described above gave a 9% yield of immobilized enzyme. However, 76% of the added oxidized enzyme remained in the supernatant, so the immobilization efficiency is rather low. Enzyme recovery was 85%, indicating that the immobilization conditions are mild.

Table 7.5 Stability of CPO immobilized on 3-aminopropylsilica by means of Schiff bases, with respect to low pH and high ionic strength.[a]

immobilization method[b]	washing liquid	enzyme activity in washing liquid[c] incubation time: 1 h	4 d
A	doubly distilled water	0	0
	100 mM KH_2PO_4 pH 4.5	ND[d]	8.7
	100 mM H_3PO_4/KOH pH 2.7 + 20 mM KCl	6.8	10.8
B	doubly distilled water	0.3	0
	100 mM KH_2PO_4 pH 4.5	7.7	9.1
	100 mM H_3PO_4/KOH pH 2.7 + 20 mM KCl	4.7	11.7

[a] Different batches of immobilized enzyme were incubated with the indicated liquids for the indicated time intervals, and the enzyme activities in the washing liquids were subsequently determined.

[b] See Materials and Methods.

[c] As percentage of the total activity of the initially added enzyme.

[d] ND = not determined.

The stability of the enzyme-support bond was investigated by incubating the immobilized material with various liquids at 4 °C; the enzymatic activity in the supernatant was determined after 1 h and after 4 days. The results are depicted in Table 7.5 and show that the enzyme remains bound at neutral pH but slowly goes into solution at lower pH values because the formation of Schiff bases is reversible. Although the method has some disadvantages it can be useful, especially when studying the "classical" peroxidase reaction, at pH 4.5, or in cases where some loss of enzyme is acceptable.

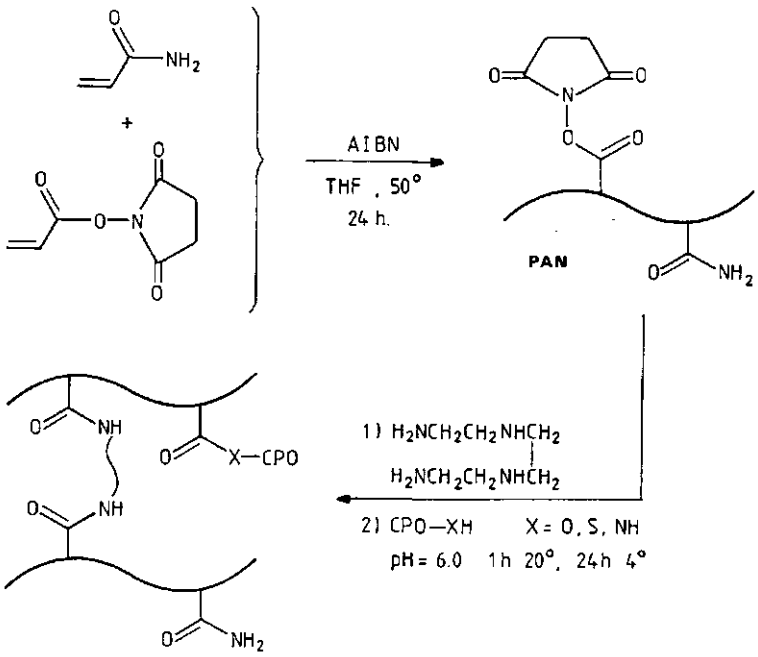
7.3.4 Entrapment in calcium alginate

The entrapment of biocatalysts in polysaccharides such as calcium alginate or κ-carrageenan is a widely employed immobilization procedure. Although usually whole cells are immobilized in this way [12], the method is also applicable to enzymes [34]. The process occurs under very mild conditions and can be

easily scaled up [35]. In contrast to Sepharose and HAP, calcium alginate is resistant towards low pH [36]. Although phosphate ions destabilize the gel this effect is much smaller at low pH because alginate forms gels without calcium ions below pH 3 [36].

Entrapment in calcium alginate consists of dripping a solution of the biocatalyst in buffer containing sodium alginate into a buffered solution of calcium chloride. When the enzyme/alginate drops make contact with the calcium ions a spherical gel is formed. The hardening of the spheres is accomplished in a few hours. Immobilization of CPO in this way, using 2% sodium alginate and 50 mM calcium chloride, gave a mere 1-3% yield: 75-85% of the enzyme had diffused out of the spheres into the calcium chloride solution during the initial stage of the hardening process. Increasing the concentration of alginate or calcium ions led to a faster gelation process but did not not affect significantly the immobilization yield. Apparently the enzyme leaks through the gel into the solution, because the pores are too large [37]. When completely hardened spheres were incubated in 0.1 M potassium phosphate buffer pH 2.7 for 1 h, 40% of the enzyme molecules diffused out of the matrix. This figure did not increase on prolonged incubation, indicating that the rate of leakage is maximal when the gel is freshly formed. An elegant modification to prevent this early leakage seemed to be the immobilization of the enzyme in a dialysis tube. The enzyme and the alginate dissolved in buffer were placed inside the tube and this was immersed in the calcium chloride solution. Calcium ions diffused freely into the tube and effected the gelation of the alginate/enzyme mixture while neither alginate nor enzyme leaked out. After complete gelation the stiff gel was cut into thin slices and the enzyme activity was recorded. This procedure definitely seemed an improvement as the immobilization yield was approximately 14%. This figure may be higher when diffusional limitation of the substrates inside the gel slices is taken into account. However, the enzyme rapidly leaked out of the sliced gel. In 1 h 40% of the enzyme had already gone into solution and after 3-4 h only 45-50% is left, giving a final yield of 7-8%. Several procedures have been advanced for the stabilization of calcium alginate gels, usually by the formation of a branched polymer on the surface of the gel [38-40]. These procedures are conducted at neutral or basic pH or require relatively long reaction times and are therefore unsuitable in the case of CPO.

7.3.5 Entrapment/covalent binding in an acrylic polymer: cross-linked poly-co-acryloxysuccinimide-acrylamide (PAN)



Scheme 7.1 Formation of cross-linked PAN-gels containing immobilized chloroperoxidase [13].

An interesting approach to the immobilization of enzymes was published some years ago by Whitesides *et al.* [13]. Their method is based on a synthetic polymer of acrylamide and *N*-acryloxysuccinimide, abbreviated as PAN. The *N*-oxysuccinimide is a good leaving group and serves a dual function: i) it can be replaced by α,ω-diamines allowing the cross-linking of individual polymer chains; ii) it can be replaced by by nucleophilic groups of the enzyme (see Scheme 7.1). In this way enzymes are immobilized by a combination of entrapment and covalent binding. An advantage above other polymerization reactions is that only a limited amount of heat is produced and the gelation time is short. The method has been applied to a large number of enzymes, including the haloperoxidases lactoperoxidase and horseradish peroxidase, giving immobilization yields of 27 and 14%, respectively [13].

Table 7.6 Dependence of the concentration of poly(acrylamide-co-N-acryloxy-succinimide) (PAN) and the amount of cross-linking agent (TET) on the gelation time and the quality of the formed gel.[a]

%PAN	equivalents of TET	gelation time (min)[b]	gel quality
15			spongy, very sticky
20	0.40	15	firm, somewhat elastic
	0.45	13	firm
	0.50	7	firm
	0.75	5	moderately firm, brittle
	0.90	3	moderately firm, sticky
22.5	0.40	6	firm, brittle
	0.45	3.3	firm, brittle
	0.50	1.3	firm, brittle
	0.75	(3.7) [c]	firm, sticky
	0.90	(2) [c]	firm, somewhat sticky
	1.10	(5) [c]	unstable, sticky
25	0.40	3.3	firm
	0.5	1	firm, brittle
	0.75	(3.2) [c]	firm, sticky
	0.90	(1.3) [c]	firm, elastic, sticky

[a] See Text for details.

[b] The gelation time was taken as the time between the addition of the TET and the moment the stirring bar started to "jump" because of the increasing viscosity of the gelating solution.

[c] These experiments were carried out on a larger scale (5-10 ml PAN solution); therefore mixing of the PAN and the TET was less effective resulting in increased gelation times.

Some investigations were made into the effect of the concentration of PAN and the cross-linking agent on the gelation time and the gel quality prior to the immobilization of CPO by this method. Pollak *et al.* [13] studied a lot of

α,ω-diamines and triethylenetetramine (TET) was proved to be the most effective cross-linking agent, so this compound was used for all our experiments. The results are summarized in Table 7.6; 30% PAN gave very viscous solutions and was not studied further. 22.5% PAN and 0.40 equivalents of TET were chosen for the immobilization experiments with regard to the gel quality, the viscosity of the solutions and the gelation time. The relatively low TET content increases the possibility of covalent attachment of the enzyme to the polymer and thus decreases the leakage of the enzyme from the gel. Sometimes the gel coloured brownish during the hardening step. The origin of the colouration is unknown but no attention was paid to it since it does not affect the gelation time, the gel quality or the enzymatic activity. The gelation was carried out in 0.3 M HEPES buffer pH 6.0 at room temperature because CPO is inactivated above pH 7 and gelation time increased strongly with decreasing pH and lowered temperature.

Table 7.7 Immobilization of CPO in PAN. Effect of the time at which the cross-linking agent (TET) and the enzyme were added.[a]

added at t=0:	added at t=t:	enzyme activity in washing liquid [b]	yield [c]	recovery [c]
TET	2 min CPO	28	36	64
CPO	2 min TET	17	17	34
CPO	5 min TET	15	26 [d]	41
CPO	30 min TET	21	40 [d]	61
CPO	5 h 30 min TET	39	35 [d]	74
CPO	24 h TET	41	32	73

[a] See Materials and Methods for experimental details.

[b] As percentage of the total activity of the initially added enzyme.

[c] See Table 1 for explanation.

[d] These preparations were examined for enzyme leakage.

The immobilization of CPO in PAN was performed by the addition of CPO and subsequently TET to the PAN. The moment of addition of TET is an important parameter. A large interval between the additions of CPO and TET gives the enzyme more opportunity to bind covalently to the polymer, because the amine competes with the enzyme for the active ester groups. The results are shown in

Table 7.7. Adding TET after about 30 min gave the best yields; longer time intervals seemed to give some enzyme inactivation. To our surprise the yield of this procedure is 30-40%, which is considerably higher than with the methods discussed before, and is also higher than found for lactoperoxidase and horseradish peroxidase [13]. Another advantage is that almost no enzyme leaked out of the PAN gel. This result, together with the observation of Pollak [13] that the gel was permeable for dextranes (MW 10^4-10^7) led us to the conclusion that CPO was covalently bound to the polymer rather than entrapped by the cross-linking procedure.

7.3.6 Summary

Several techniques have been applied for the immobilization of the chloroperoxidase from *Caldariomyces fumago*. The five indicated methods were not as applicable to this enzyme as expected from its physical constants and chemical structure. The major problem is not the inability of the enzyme to bind but the low pH at which it is active. Work at low pH combined with the fact that the enzyme needs an ionic substrate (chloride) results in a relatively high ionic strength. This disfavors binding methods which consist of hydrogen bonds or dipolar or ionic interactions. Covalent bonding is possible in principle, if the bond is able to withstand acidic media for some time. For some unknown reason CPO does not couple readily under these conditions. There are few amino groups available in the protein backbone, but there are a lot of aspartic and glutamic acid residues. However, these seem to be quite unreactive. Increasing the amount of coupling reagent leads to inactivation. It should be noticed that only enzyme activities were determined and not enzyme concentrations in all our studies. So the immobilization yields may therefore be rather low because the specific activity of the immobilized enzyme is lowered due to diffusional limitation; the given yields should be regarded as a lower limit.

Another problem is the low molecular weight of the enzyme, preventing its entrapment in gels like calcium alginate. The best procedure appeared to be the method of Whitesides [13], probably because the enzyme comes into close contact with a large amount of reactive ester groups resulting in stable covalent linkages without deterioration of the active site. This method is therefore preferable when performing large-scale halogenation reactions using chloroperoxidase.

Hydroxyapatite is also an attractive support for the enzyme. Barbituric

acid was converted to a mixture of 5-chlorobarbituric acid and 5,5-dichlorobarbituric acid by CPO immobilized on HAP. At this low temperature the support did not dissolve, but the enzyme activity was only 0.4% of the activity at 25°C. Hydroxyapatite has proved to be very convenient for the classical peroxidase reaction, which can be conducted at pH 4.5.

7.4 REFERENCES AND NOTES

[1] B.P. Sharma, L.F. Bailey and R.A. Messing, Angew. Chem. **94** (1982), 836-852
[2] J. Tramper, in *Solid Phase Biochemistry: Analytical Synthetic Aspects*, (1983), (W.H. Scouten, ed), Wiley, New York, 393-477
[3] H.O. Hultin, Food Technol. **37** (1983), 66
[4] A.M. Klibanov, Science **219** (1983), 722-727
[5] A. Rosevear, J. Chem. Technol. Biotechnol. **34** (1984), 127-150
[6] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100
[7] M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70
[8] M.C.R. Franssen, H.G. van Boven and H.C. van der Plas, J. Heterocyclic Chem., in press
[9] A.S.W. Sae and B.A. Cunningham, Phytochemistry **18** (1979), 1785-1787
[10] D.R. Morris and L.P. Hager, J. Biol. Chem. **241** (1966), 1763-1768
[11] O.R. Zaborski and J. Ogletree, Biochem. Biophys. Res. Commun. **61** (1974), 210-216
[12] W.H. Scouten, *Solid Phase Biochemistry: Analytical Synthetic Aspects* (1983), Wiley, New York, 779 pp.
[13] A. Pollak, H. Blumenfeld, M. Wax, R.L. Baughn and M.W. Whitesides, J. Am. Chem. Soc. **102** (1980), 6324-6336
[14] H.H. Weetall, Meth. Enzymol. **44** (1976), 134-148
[15] M. Lynn, in *Immobilized Enzymes, Antigens, Antibodies, and Peptides; Preparation and Characterization* (1975), (H.H. Weetall, ed), M. Dekker, New York, 1-48
[16] H. Hemetsberger, W. Maasfeld and H. Ricken, Chromatografia **9** (1976), 303-310
[17] K. Unger, Angew. Chem. **84** (1972), 331-343
[18] P.F. Hollenberg and L.P. Hager, Meth. Enzymol. **52** (1978), 521-529
[19] *Materials, Equipment and Systems for Chromatography, Electrophoresis, Affinity Chromatography, Immunochemistry and HPLC* (1980), Bio-Rad Laboratories, Alphen aan de Rijn, The Netherlands, 59-64
[20] *Affinity Chromatography, principles and methods* (1976), Pharmacia Fine Chemicals, Uppsala, 71 pp.
[21] V.G. Janolino and H.E. Swaisgood, Biotechnol. Bioeng. **24** (1982), 1069-1080
[22] M. Marek, O. Valentová and J. Káš, Biotechnol. Bioeng. **26** (1984), 1223-1226
[23] S. Barbarič, B. Kozulič, I. Leuštek, B. Pavlovič, V. Česi and P. Mildner, Proc. Third. Eur. Congr. on Biotechnol., vol. 1 (1984), Verlag Chemie, Weinheim, 307-312
[24] S.L. Neidleman and J. Geigert, US 4,282,324 (1980)
[25] *Corning Controlled Pore Glass*, Pierce Chem. Co., Rockford, Ill., US (1975), 32 pp.
[26] J.A. Thomas, D.R. Morris and L.P. Hager, J. Biol. Chem. **245** (1970), 3135-3142
[27] S.L. Neidleman, W.F. Amon Jr. and J. Geigert, US 4,247,641 (1981)

[28] S.L. Neidleman, W.F. Amon Jr. and J. Geigert, US 4,284,723 (1981)
[29] W.F. Line, A. Kwong and H.H. Weetall, Biochim. Biophys. Acta **242** (1971), 194-202
[30] R.G. Spiro, Meth. Enzymol. **28** (1972), 3-43
[31] H.-Y. Hsiao and G.P. Royer, Arch. Biochem. Biophys. **198** (1979), 379-385
[32] For example, the haloperoxidase horseradish peroxidase has been oxidized by periodate and cross-linked with α,ω-dihydrazino compounds without giving yields [23].
[33] The periodate oxidation is generally stopped by the addition of an excess of ethylene glycol, which is oxidized by periodate to formaldehyde [22]. Therefore the effect of these compounds on CPO was measured. Ethylene glycol was proved to be an inhibitor of the CPO-mediated chlorination of MCD. The effect of the concentration of hydrogen peroxide on the enzyme activity for various ethylene glycol concentrations was plotted in Lineweaver-Burk and Dixon plots. These led to the conclusion that the binding of hydrogen peroxide is inhibited in a competitive manner yielding a K_I of 3.90 M. Formaldehyde inactivates CPO very rapidly, even at low concentrations, possibly by reaction with nucleophilic groups at the enzyme's active site. In addition formaldehyde reacts with MCD, giving unknown products. The conclusion is that the termination of the periodate oxidation reaction by the addition of ethylene glycol is unsuitable for CPO, not because of the nature of the reagent itself but because of the reactivity of its product, formaldehyde. The excess of periodate was therefore not removed and the solution containing the oxidized enzyme was simply added to the support after adjustment of the pH.
[34] M. Kierstan and C. Bucke, Biotechnol. Bioeng. **19** (1977), 387-397
[35] A.C. Hulst, J. Tramper, K. van 't Riet and J.M.M. Westerbeek, Biotechnol. Bioeng. **27** (1985), 870-876
[36] R.L. Whistler and C.L. Smart, *Polysaccharide Chemistry* (1953), New York, 493 pp.
[37] J. Klein, J. Stock and K.D. Vorlop. Eur. J. Appl. Microbiol. Biotechnol. **18** (1983), 86-91
[38] I. Takata, T. Tosa and J. Chibata, J. Solid Phase Biochem. **2** (1977), 225-236
[39] P. Brodelius and K. Nilsson, FEBS Lett. **122** (1980), 312-316
[40] F. Lim and A.M. Sun, Science **210** (1980), 908-910

8 NEW HALOMETABOLITES FROM *CALDARIOMYCES FUMAGO**

8.1 INTRODUCTION

The occurrence of haloorganic compounds in living matter is a rapidly growing field of interest in natural product chemistry. Over 700 halometabolites are known to date. The subject has been reviewed in general [1] as well as for individual classes of organisms [2-7]. Some of the authors concerned also briefly discuss the biosynthesis of these compounds [2,4-7]. The role of the enzymes being responsible for the actual halogenation step has, until recently [8], only been mentioned without further comment. This is remarkable, since the discovery of the chloro-antibiotic caldariomycin (2,2-dichloro-1*S*,3*S*-dihydroxy-cyclopentane) in the culture medium of the mold *Caldariomyces fumago* [9] directly induced the search for and the detection of the halogenating enzyme chloroperoxidase [10,11], now one of the most studied halogenating biocatalysts. This very versatile enzyme has been used in several laboratories for the synthesis of chlorinated or brominated steroids [12], alkenes [13,14], cyclopropanes [15] as well as chlorinated or brominated derivatives of the heterocycles thiazole [16], antipyrine [17] and barbituric acid [18-21]. In these investigations as well as in those from other laboratories [22,23] opposite results were presented regarding the reaction mechanism of the enzyme; in particular the specificity of the halogenation reaction was a matter of debate. Some of the controversy may have been caused by the fact that only a minority of the researchers used cyclopentanedione, the only known natural substrate of the enzyme. It would be interesting to know whether there are other natural substrates for chloroperoxidase, and whether this enzyme could halogenate them in a stereo- or regiospecific manner. Therefore we wanted to have a more detailed picture of the natural products, being formed in the culture medium of *Caldariomyces fumago*.

When Clutterbuck and coworkers [9] extracted the culture medium of *Caldariomyces fumago* with EtOAc, they not only isolated white crystals of caldariomycin, but also noted that: "An oil remained finally from which nothing crystalline has been separated, but which contains considerable amounts of Cl in organic combination". This induced us to reexamine the culture medium using

---

* Adapted from: M.C.R. Franssen, M.A. Posthumus and H.C. van der Plas, Phytochemistry, in press

GC/MS. Halogen-containing compounds are readily recognized by this technique because of the characteristic $^{35}Cl/^{37}Cl$ and $^{79}Br/^{81}Br$ isotope patterns.

In this paper we report on the extraction and characterization by GC/MS of six new chloro-containing metabolites from the culture medium of *Caldariomyces fumago*. The biosyntheses of these compounds and their implications for the reaction mechanism and possible stereospecificity of chloroperoxidase are discussed.

8.2 MATERIALS AND METHODS

General

Chloroperoxidase (CPO) was isolated as described previously [24]. The extraction liquids were distilled prior to use.

Growth of the mold and isolation of the metabolites

Caldariomyces fumago Woron. strain CBS 123.26 was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The mold was grown on Czapek-Dox medium, consisting of 50 g of glucose, 2.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4.7H_2O$, 0.02 g of $FeSO_4.7H_2O$ and 3.3 g of yeast extract per liter of deionized water. The mold was grown for 7 days in a 1 liter culture flask and then transferred to a 20 liter culture flask where it was grown for 9-10 days. Subsequently the cells were centrifugated and the cell-free supernatant was concentrated in an Amicon hollow-fiber apparatus with an $H_{10}P_{10}$-20 cartridge (molecular weight cutoff: 10,000). The 4.5 liter of clear culture medium thus obtained was divided into two equal portions and each portion was extracted with 3x500 ml of petroleum ether 60-80, CH_2Cl_2 and EtOAc, successively. The residue was then extracted continuously with EtOAc for 24 hours. The extracts were dried with $MgSO_4$, concentrated with a rotary evaporator using a 30° water bath and analysed.

GC/MS analysis

2.0 µl extract was injected into a gas chromatograph equipped with a SIL19CB WCOT capillary column, 26 m x 0.22 mm ID, film thickness 0.2 µm. The compounds were detected with a VG 7070F mass spectrometer using electron impact ionization (70 eV, ion source temp. 200°). The initial column temperature (50°) was maintained for 2 min and then raised to 260° at a rate of 6°/min.

Blanks

Two blank experiments were carried out to correct for the presence of contaminating halogenated compounds in the chemicals used.

i) 3 liter of CH_2Cl_2 and 3 liter of EtOAc were evaporated to a small volume on a rotary evaporator using a 30° water bath and analysed by GC/MS. A small amount of tetrachloroethene was detected in the CH_2Cl_2 concentrate; the EtOAc concentrate was free of contaminants.

ii) 4.5 liter of freshly prepared growth medium was extracted and analysed as described above. The petroleum ether fraction only showed solvent peaks. The CH_2Cl_2 fraction contained the following additional compounds:

spectrum no.	525	5-(1-hydroxyethyl)dihydro-2(3*H*)furanone
	802	$C_{10}H_{16}N_2O_2$ (structure unknown)
	839	composition and structure unknown
	851	$C_{11}H_{18}N_2O_2$ (structure unknown)
	863	composition and structure unknown
	1071	$C_{14}H_{16}N_2O_2$ (structure unknown)

The spectra numbers refer to the abscissa of Figure 8.1 and to Table 8.1 (GC/MS analysis of the CH_2Cl_2-extract of the culture medium of *Caldariomyces fumago*). The last four compounds seem to be closely related to the compound with spectrum no. 802, but their structures could not be elucidated. Samples of the EtOAc extract and the EtOAc-continuous extract only contained the compound with spectrum no. 525, which was also found in the CH_2Cl_2-extract. The compounds described here most probably originate from the yeast extract in the growth medium.

Mass spectra

The mass spectra of the six new halometabolites from *Caldariomyces fumago* (**2**-**7**) are tabulated below; only peaks with intensities higher than 5% are given:

2,2-Dichloroethanol (**2**): m/z (rel int.): 31 (100); other important peaks: 29 (3), 43 (4), 48 (3), 49 (4), 79 (3), 83 (2).

1,3-Dichloropropanol-2 (**3**): m/z (rel int.): 27 (6), 43 (42), 49 (7), 57 (9), 79 (100), 81 (32).

1,1,3-Trichloropropanol-2 (**4**): m/z (rel int.): 27 (10), 29 (6), 43 (30), 49 (19), 51 (6), 77 (7), 79 (100; calcd. for $C_2H_4{}^{35}ClO$ m/z = 78.9951, found 78.9954), 81 (31), 113 (17; calcd. for $C_2H_3{}^{35}Cl_2O$ m/z = 112.9560, found 112.9559), 115 (10; calcd. for $C_2H_3{}^{35}Cl{}^{37}ClO$ m/z = 114.9530, found 114.9525).

1,1,3,3-Tetrachloropropanol-2 (**5**): m/z (rel int.): 49 (32), 51 (12), 61 (6),

77 (38), 78 (6), 79 (12), 83 (18; calcd. for $CH^{35}Cl_2O$ m/z = 82.9455, found 82.9457), 85 (13), 113 (100; calcd. for $C_2H_3{}^{35}Cl_2O$ m/z = 112.9560, found 112.9560), 115 (65), 117 (10).

2-(3-Chloro-4-hydroxyphenyl)ethanol (**6**): m/z (rel int.): 51 (14), 77 (22), 105 (5), 107 (7), 141 (100; calcd. for $C_7H_6{}^{35}ClO$ m/z = 141.0106, found 141.0092), 142 (10), 143 (30), 172 (23; calcd. for $C_8H_9{}^{35}ClO_2$ m/z = 172.0291, found 172.0272), 174 (9).

2-(3,5-Dichloro-4-hydroxyphenyl)ethanol (**7**): m/z (rel. int.): 31 (7), 51 (7), 75 (17), 111 (11), 141 (10), 175 (100; calcd. for $C_7H_5{}^{35}Cl_2O$ m/z = 174.9716, found 174.9705), 176 (12), 177 (63), 178 (8), 179 (11), 206 (25; calcd. for $C_8H_8{}^{35}Cl_2O_2$ m/z = 205.9900, found 205.9910), 208 (15).

Chlorination of 2-(4-hydroxyphenyl)ethanol (**9**)

A. By chloroperoxidase (CPO): to a solution of 138 mg (1 mmol) of 2-(4-hydroxyphenyl)ethanol in 0.1 M H_3PO_4/KOH pH 2.7 containing 20 mM of KCl was added H_2O_2 in eight portions with 45 min-intervals, and 300 µg of chloroperoxidase in three portions with 135 min-intervals. The mixture was stirred for 1 day; the small amount of orange precipitate that formed was discarded. The mixture was extracted with three portions of CH_2Cl_2; the extract was dried on magnesium sulphate and the solvent was evaporated using a rotary evaporator. The residue consisted of 2-(3-chloro-4-hydroxyphenyl)ethanol together with some of the 3,5-dichloro compound, as was proved by NMR (90 MHz, $CDCl_3/d_6$-DMSO).

B. By HOCl: to a similar solution as under *A* was very slowly added 186 µg (2.5 mmol) of NaOCl. The reaction mixture was stirred for 6 h at room temperature and extracted with CH_2Cl_2. The extract was dried with $MgSO_4$, evaporated and analysed. The product proved to be 2-(3-chloro-4-hydroxyphenyl)ethanol together with a small amount of the 3,5-dichloro compound, as was evidenced by NMR.

8.3 RESULTS AND DISCUSSION

Caldariomyces fumago Woron. was grown as described in the Materials and Methods section and the filtered culture medium was extracted successively with petroleum ether 60-80, CH_2Cl_2 and EtOAc, in order to separate the polar from the less polar compounds. The extracts were analysed by GC/MS. The petroleum ether fraction was practically devoid of any mold compounds and was not analysed further. Gas chromatography of the CH_2Cl_2 extract showed a multitude of peaks (see below), whereas the EtOAc fraction and the EtOAc continuous extract

showed 2,3-butanediol, caldariomycin (**1**, see Scheme 8.1), 2-(4-hydroxyphenyl)-ethanol (**9**, see Scheme 8.2) and an unhalogenated compound of unknown structure [25] as major constituents. We concentrated ourselves on the CH_2Cl_2 extract because of the large number of compounds present therein (see chromatogram in Figure 8.1). Usable mass spectra were obtained from about 80 individual compounds, and the most abundant ones are tabulated in Table 8.1. The CH_2Cl_2 extract appeared to contain a considerable number of small, relatively simple unhalogenated molecules as well as twelve chlorinated organic compounds, among which caldariomycin (**1**). Five of the eleven new chlorometabolites of *Caldariomyces fumago* were only present in very small amounts and could not be analysed further. Six compounds, however, gave clean mass spectra and comparison with literature data and authentic samples allowed complete identification of these molecules.

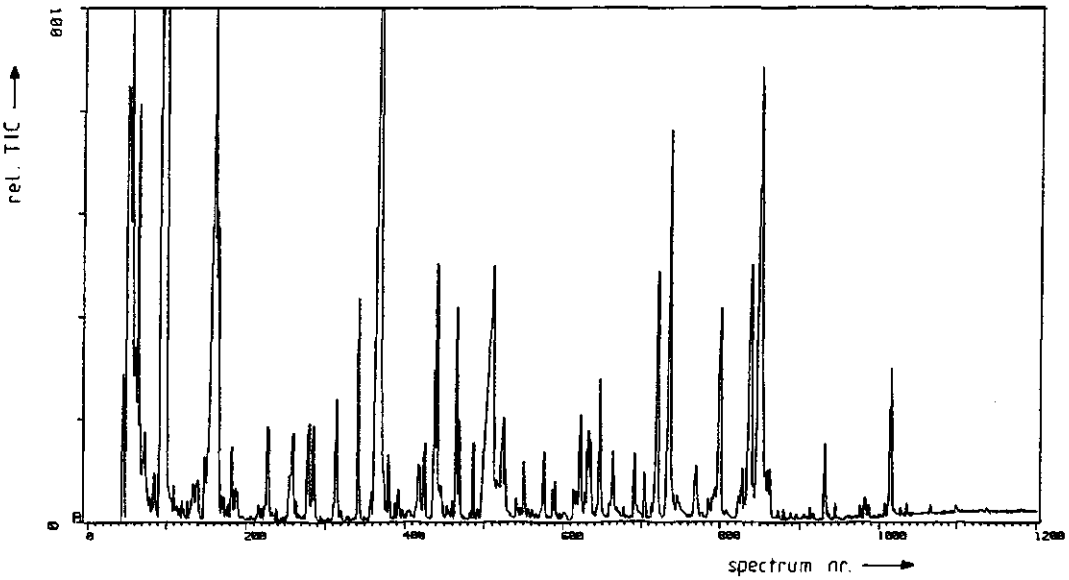


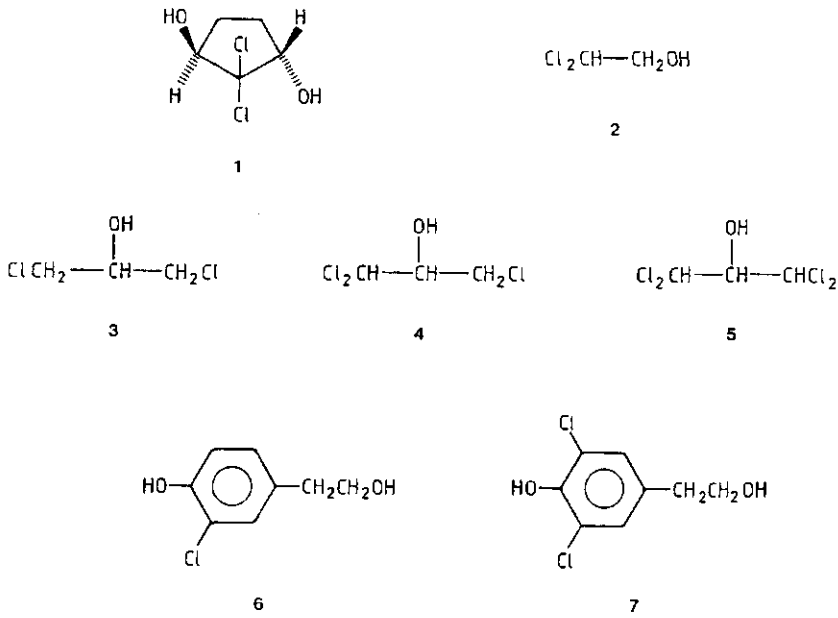
Figure 8.1 Gas chromatogram (rel. total ion current vs. spectrum number) of the CH_2Cl_2-extract of the culture medium of Caldariomyces fumago. *Trace reconstructed from the spectra accumulated during GC/MS analysis. See Experimental for GC-conditions.*

Table 8.1 *Major constituents of the CH_2Cl_2-extract of the culture medium of the mold* Caldariomyces fumago.[a]

Spectrum no.		total ion current (arbitrary units)	compound no.	compound name or molecular formula or most intense peak in mass spectrum (rel. int.)
62		1187		diethoxyethane
65		6374		isobutanol
94	o	3776		tetrachloroethene
96		9730		3-methyl-1-butanol
101		3610		3-hydroxy-2-butanone
149		1326		3-ethoxy-1-propanol
151	x	539	2	2,2-dichloroethanol
160		5197		2,3-butanediol
166		3797		4-hydroxy-4-methylpentan-2-one
228	x	1226	3	1,3-dichloropropanol-2
261		1578		butyrolacton
279		1265		2-pentanone
343	x	2827	4	1,1,3-trichloropropanol-2
370		9695	8	phenylethanol
381		702		unknown, 44 (100), 59 (12)
426		1314		unknown, 30 (100), 73 (29)
439		1845		unknown, 43 (100), 87 (9)
442	x	3061	5	1,1,3,3-tetrachloropropanol-2
466		2606		unknown, 101 (100), 43 (59)
469	x	1313	1	caldariomycin (2,2-dichloro-1*S*-3*S*-dihydroxycyclopentane)
488		936		unknown, isomer of 466
512		3111		phenylacetic acid
525	x	1386		5-(1-hydroxyethyl)dihydro-2(3*H*)-furanone
591	x	498		unknown
619	x	298		unknown
623	x	1285	6	2-(3-chloro-4-hydroxyphenyl)ethanol
633		1094	9	2-(4-hydroxyphenyl)ethanol
706	x	511		unknown
722		3004		$C_{10}H_{10}O_3$, structure unknown
737	x	4686	7	2-(3,5-dichloro-4-hydroxyphenyl) ethanol
802	o	2550		$C_{10}H_{16}N_2O_2$, structure unknown
839	o	2783		structurally related to 802
851	o	5543		$C_{11}H_{18}N_2O_2$, structure unknown
854	x	1342		unknown, contains one chlorine atom
863	o	628		structurally related to 802
933	x	1394		unknown, contains two chlorine atoms
1071	o	1870		$C_{14}H_{16}N_2O_2$, structure unknown

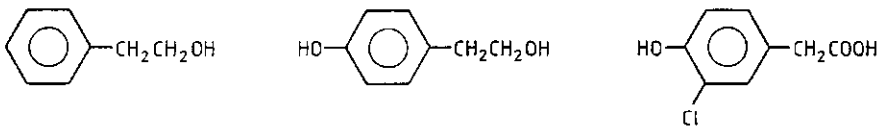
[a] In the second column the total ion current of the spectrum of the compound is mentioned as an indication of the relative concentration of the compound in the extract. Elemental compositions are obtained by means of accurate mass measurement; identifications are based on mass spectra, unless noted otherwise in the text.
x = chlorine-containing compound;
o = compound derived from solvent or growth medium itself (see Section Blanks, Experimental).

The six new halometabolites can be divided into two groups: chloroethanols/propanols and chlorohydroxyphenylethanols. The first group consists of 2,2-dichloroethanol (**2**, see Scheme 8.1; designated under spectrum no. 151 in Table 8.1), 1,3-dichloropropanol-2 (**3**; spectrum no. 228), 1,1,3-trichloropropanol-2 (**4**; spectrum no. 343) and 1,1,3,3-tetrachloropropanol-2 (**5**; spectrum no. 442). The structure of **2** was assigned by comparison of its mass spectrum with literature data [26]. The structure of **3** was established by comparison of its mass spectrum with authentic material; further support was obtained by the fact that **3** in the CH_2Cl_2 extract coeluted with authentic **3** on GLC-analysis. The fragmentation patterns of **3**, **4** and **5** are very similar: the molecular ion peak is not visible, but the spectra show characteristic peaks at m/e 79 and 113, corresponding to the α-cleavage products. These fragments subsequently lose an HCl molecule (signals at m/e 43 and 77, respectively). The compounds **3**, **4** and **5** also showed a good correlation between their retention times and their molecular weights, as was found earlier for a series of halogenated isopropanols [27]. The mass spectrum of **5** is in good agreement with that published before [27].



Scheme 8.1 Halometabolites of Caldariomyces fumago.

The second group consists of 2-(3-chloro-4-hydroxyphenyl)ethanol (**6**; spectrum no. 623) and 2-(3,5-dichloro-4-hydroxyphenyl)ethanol (**7**; spectrum no. 737). Their structures were confirmed by comparing their retention times and mass spectra with those of authentic samples prepared by reaction of 2-(4-hydroxyphenyl)ethanol with HOCl. These chlorometabolites have not been described previously in the literature. However, some analogues are known. 2-phenylethanol (spectrum no. 370, phenetol, **8**, see Scheme 8.2) and 2-(4-hydroxyphenyl)ethanol (spectrum no. 633, tyrosol, **9**) are well-known compounds occurring in many molds, and *Marasmius palmivorus* produces 3-chloro-4-hydroxyphenylacetic acid (**10**) [5]. These metabolites are derived from the shikimic acid route, as is caldariomycin [28] (see Scheme 8.3). **6** and **7** are probably synthesized via the shikimic acid pathway, but another route can certainly not be ruled out. For instance, *Candida sp.* and various pathogenic fungi produce tyrosol (**9**) as the decomposition product of tyrosine [5] (see Scheme 8.3). We found large amounts of tyrosol in the culture medium of *Caldariomyces fumago*, so this may well be a precursor for the chlorinated tyrosols **6** and **7**. To test this hypothesis we incubated tyrosol with the pure *Caldariomyces* halogenating enzyme, chloroperoxidase, and found that it was readily converted to 2-(3-chloro-4-hydroxyphenyl)ethanol (**6**). Remarkably, 2-(3,5-dichloro-4-hydroxyphenyl)ethanol (**7**) was formed in only small amounts, even after prolonged incubation.

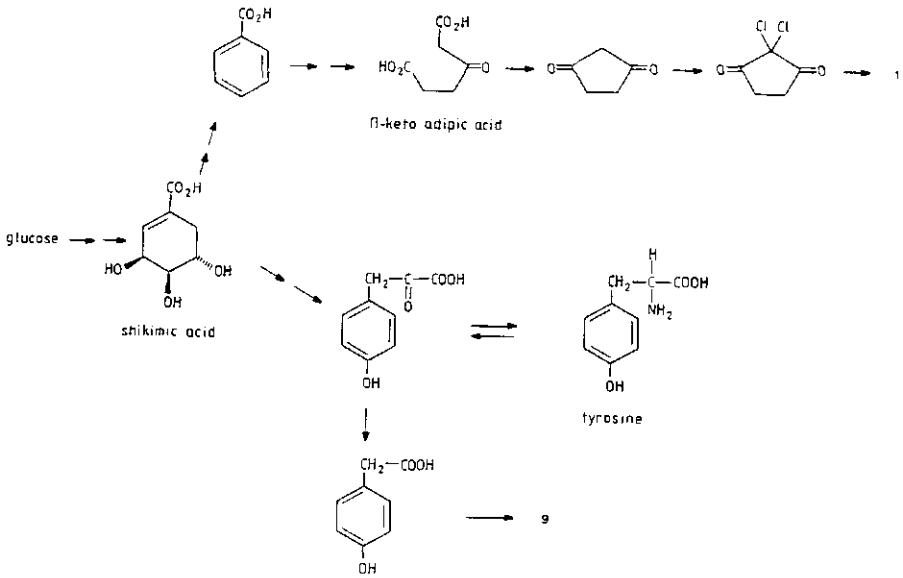


8 9 10

*Scheme 8.2 Compounds, related to the chlorohydroxyphenylethanols **6** and **7**. **8** and **9** are found in several microorganism as well as in the culture medium of* Caldariomyces fumago*; **10** is a metabolite of the mold* Marasmius palmivorus.

Since it is well-known that tyrosine is a substrate for chloroperoxidase [29], four possibilities for the formation of the chlorine-carbon bonds ultimately leading to **6** and **7** can be advanced: i) chlorination of tyrosine residues

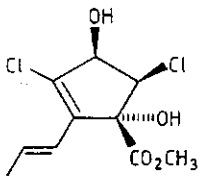
in proteins [30]; ii) chlorination of free tyrosine, related or not to protein synthesis or degradation; iii) chlorination of tyrosol; iv) chlorination of intermediates in the shikimic acid route, as found for caldariomycin (**1**). Since **6** can be formed from tyrosol by chloroperoxidase, as already noted above, all four possible biosynthesis routes may be valid for **6**. However in the case of **7** route iii) is not likely to occur because **6** proved to be a poor substrate for the enzyme. More information is needed before the biosynthesis of **6** and **7** is known with certainty.



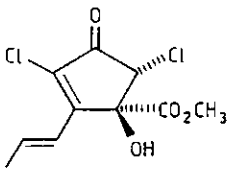
Scheme 8.3 The biosynthetic pathway leading from shikimic acid to caldariomycin (1) [28], *tyrosine and tyrosol (9)* [5].

The origin of the dichloroethanol and chloropropanols (**2-5**) is even more intriguing. Compounds of this kind are, up to now, only known from marine organisms. 1,3-Dichloropropanol-2 (**3**) has been found in antartic krill [31], and Woolard et al. [27] detected a large number of haloacetones and haloisopropanols in the CH_2Cl_2 extract of vacuum-dried *Asparagopsis taxiformis* (Rhodophyta, Bonnemaisoniaceae), including our compound **5**. The halometabolites **2** and **4** have not been found before. The authors did not study the biosynthesis of these compounds, but since neither ethanol nor acetaldehyde, propanol-2 or

acetone are substrates for chloroperoxidase, we feel that the metabolites **2-5** must be derived from larger molecules. Perhaps these larger precursors are molecules like compound **11** (see Scheme 8.4), from *Periconia macrospinosa*, and cryptosporiopsin (**12**, from *Phialophora asteris, Octospora carbonigena* and many *Cryptosporiopsis* species). These mold metabolites contain the 1,3-dichloropropanol-2 and 1,3-dichloroacetone moiety respectively, and possibly these compounds are the precursors of the compounds **2-5** or the corresponding chloroacetone derivatives [32]. The hypothesis described above is speculative especially since it does not explain the occurrence of tri- and tetrachloroisopropanols.



11



12

Scheme 8.4 *Mold metabolites, possibly related to the chlorinated isopropanols found in* Caldariomyces fumago. *11 is a metabolite from* Periconia macrospinosa; *cryptosporiopsin (12) is found in* Phialophora asteris *(the - isomer),* Octospora carbonigena *and many* Cryptosporiopsis *species (the + isomer).*

The biological function of the halometabolites is unclear so far, but there is some evidence that these compounds play a role in the defence mechanism of the organisms [8]. It is interesting to mention that chloroisopropanols are bactericides [33] and that 2-phenylethanol (**8**), the major constituent of the CH_2Cl_2-extract of *Caldariomyces fumago*, shows antimicrobial activity [34,35].

One or more halogenation steps are involved somewhere in the biosynthesis route for the formation of **1-7**. The enzyme responsible for these conversions is chloroperoxidase, an extracellular enzyme produced in relatively large amounts by *Caldariomyces fumago*. Whether this enzyme can act in a stereospecific manner is still not established [19,36-38]. Studying the stereochemistry of the natural substrates and products of the enzyme possibly can throw light on this

matter. Five of the halometabolites found here are achiral; **4** has a chiral central C-atom, but since it was not possible to isolate this compound its stereochemistry could not be studied. The key step in the biosynthesis of caldariomycin is the conversion of cyclopentane-1,3-dione into its 2,2-dichloro derivative, again a reaction with no need for stereochemical control. We conclude from the structures of the compounds found in this study that chloroperoxidase has no need to be a stereospecific chlorinating agent. One should, however, bear in mind that the growth medium of *Caldariomyces* probably contains besides the small and volatile halometabolites larger non-volatile halogenated compounds which cannot be detected by GC/MS. Until these molecules are fully characterized we have to refrain from a statement indicating that chloroperoxidase can catalyze halogenation reactions in a stereospecifical manner.

8.4 REFERENCES AND NOTES

[1] J.F. Siuda and J.F. DeBernardis, Lloydia **36** (1973), 107-143
[2] W.B. Turner: *Fungal Metabolites*, Academic Press, New York (1971)
[3] W. Fenical, J. Phycol. **11** (1975), 245-259
[4] D.J. Faulkner, Tetrahedron **33** (1977), 1421-1443
[5] W.B. Turner and D.C. Aldridge: *Fungal Metabolites II*, Academic Press, New York (1983)
[6] D.J. Faulkner, Nat. Prod. Rep. **1** (1984), 251-260
[7] K.C. Engvild, Phytochemistry **25** (1986), 781-791
[8] S.L. Neidleman and J. Geigert: *Biohalogenation: Principles, Basic Roles and Applications*, Ellis Horwood Ltd., Chichester (1986), 1-203
[9] J.R. Clutterbuck Jr., S.L. Mukhopadhyay, A.E. Oxford and H. Raistrick, Biochem. J. **34** (1940), 664-677
[10] P.D. Shaw and L.P. Hager, J. Am. Chem. Soc. **81** (1959), 1011-1012
[11] P.D. Shaw and L.P. Hager, J. Biol. Chem. **236** (1961), 1626-1630
[12] S.L. Neidleman, P.A. Diassi, B. Junta, R.M. Palmere and S.C. Pan, Tetrahedron Lett. (1966), 5337-5342
[13] J. Geigert, S.L. Neidleman, D.J. Dalietos and S.K. DeWitt, Appl. Environ. Microbiol. **45** (1983), 366-374
[14] H. Yamada, N. Itoh and Y. Izumi, J. Biol. Chem. **260** (1985), 11962-11969
[15] J. Geigert, S.L. Neidleman and D.J. Dalietos, J. Biol. Chem. **258** (1983), 2273-2277
[16] S.L. Neidleman, A.I. Cohen and L. Dean, Biotechnol. Bioeng. **11** (1969), 1227-1232
[17] P.L. Ashley and B.W. Griffin, Arch. Biochem. Biophys. **210** (1981), 167-178
[18] M.C.R. Franssen and H.C. van der Plas, Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100
[19] M.C.R. Franssen and H.C. van der Plas, Bioorg. Chem. **15** (1987), 59-70
[20] C. Laane, W. Pronk, M.C.R. Franssen and C. Veeger, Enzyme Microb. Technol. **6** (1984), 165-168
[21] C. Laane, A. Weyland and M.C.R. Franssen, Enzyme Microb. Technol. **8** (1986), 345-348
[22] A.M. Lambeir and H.B. Dunford, J. Biol. Chem. **258** (1983), 13558-13563
[23] B.W. Griffin, Biochem. Biophys. Res. Commun. **116** (1983), 873-879
[24] P.F. Hollenberg and L.P. Hager, Meth. Enzymol. **52** (1978), 521-529

[25] The mass spectrum of this compound contains the following peaks: m/z (rel. int.): 27 (6), 29 (6), 31 (12), 39 (16), 41 (31), 51 (7), 53 (11), 55 (9), 65 (6), 69 (25), 81 (7), 83 (6), 97 (100), 98 (7), 109 (12), 110 (6), 111 (15), 128 (52).
[26] *Uncertified Mass Spectral Data* (R.S. Gohlke, ed), Dow Chemical Co., Midland, Michigan (1963), spectrum no. 439.
[27] F.X. Woolard, R.E. Moore and P.P. Roller, Tetrahedron **32** (1976), 2843-2846
[28] J.R. Beckwith, R. Clark and L.P. Hager, J. Biol. Chem. **238** (1963), 3086-3090
[29] L.P. Hager, D.R. Morris, F.S. Brown and H. Eberwein, J. Biol. Chem. **241** (1966), 1769-1777
[30] E.D. Mitchell Jr., R. Loring, K.L. Carraway and B. Cunningham, Proc. Okla. Acad. Sci. **61** (1981), 43-48; Chem. Abstr. **96** (1982), 158187
[31] K. Kubota, K. Yokoyama, T. Yamanishi and S. Akatsuka, Nippon Nogei Kagaku Kaishi **54** (1980), 727731; Chem. Abstr. **94** (1981), 63937
[32] It was recognized already in 1924 that brewer's yeast can convert 1,1-dichloroacetone into 1,1-dichloropropanol-2: H.K. Sen, Biochem. Z. **151** (1924), 51-53
[33] G. Schaefer, W. Rosenthal and K.J. Bock, Ger. 1,095,995 (1958); Chem. Abstr. **56** (1962), 6100h.
[34] The Merck Index, 10th Ed., M. Windholz (ed), Merck & Co., Rahway, N.Y. (1983), 7094
[35] W. Fenical, Tetrahedron Lett. (1974), 4463-4466
[36] T.D. Lee, J. Geigert, D.J. Dalietos and D.S. Hirano, Biochem. Biophys. Res. Commun. **110** (1983), 880-883
[37] K. Ramakrishnan, M.E. Oppenhuizen, S. Saunders and J. Fisher, Biochemistry **22** (1983), 3271-3277
[38] J. Kollonitsch, S. Marburg and L.M. Perkins, J. Am. Chem. Soc. **92** (1970), 4489-4490

9 GENERAL DISCUSSION

Studies on two halogenating enzymes are presented in this thesis: the chloroperoxidase from the mold *Caldariomyces fumago* (CPO) and the bromoperoxidase from the brown alga *Ascophyllum nodosum* (BPO).

CPO is capable of halogenating barbituric acid and some of its derivatives exclusively at the 5-position. In this way, 5-chloro- or 5,5-dichloro-barbituric acid (Chapter 2,3) are obtained in high yields. CPO also catalyzes the smooth halogenation of pyrazoles, 2-aminopyridine and 8-hydroxyquinoline (Chapter 4). However, the mechanism of the halogenation reaction is not clear. Thus far, all observed reaction products and their stereochemistry can be explained by the enzymatic production of hypohalous acid (HOX, X=Cl or Br). This is in contrast to studies on the kinetics of the chlorination reactions with CPO which indicate that a ternary enzyme-hydrogen peroxide-organic substrate complex may be the active halogenating species [1]. Our own kinetic studies do not give a decisive answer: variation in the structure of the organic substrates does not influence the overall reaction rate because the actual halogenation step is not the rate-determining one. It is very difficult to study this step under non-standard assay conditions in the presence of chloride ions because it is extremely fast [2], as is the reaction of HOX with barbituric acid and its derivatives [3]. Therefore, a more detailed picture of the reaction mechanism of CPO must await further investigations. Perhaps the regio- and stereochemistry of the natural products of the enzyme can throw some light on this matter. The halometabolites of *C. fumago* (Chapter 8) do not point in the direction of enzyme-organic substrate interaction.

BPO converts barbituric acid and some of its derivatives smoothly into the corresponding 5-bromo or 5,5-dibromo derivatives. The reaction mechanism of the enzyme is obscure. In contrast to CPO the enzyme contains no heme group, but an essential vanadium (V) in its active centre [4]. This fact alone means that BPO reacts according to a mechanism different from that of CPO. However, meticulous studies on the kinetics of the bromination of monochlorodimedon and various barbituric acids (Chapter 5) showed that BPO produces hypobromous acid (HOBr) as the active halogenating agent. The HOBr is produced in a very low concentration, homogeneously distributed throughout the solution, allowing the bromination reaction to proceed with high yields.

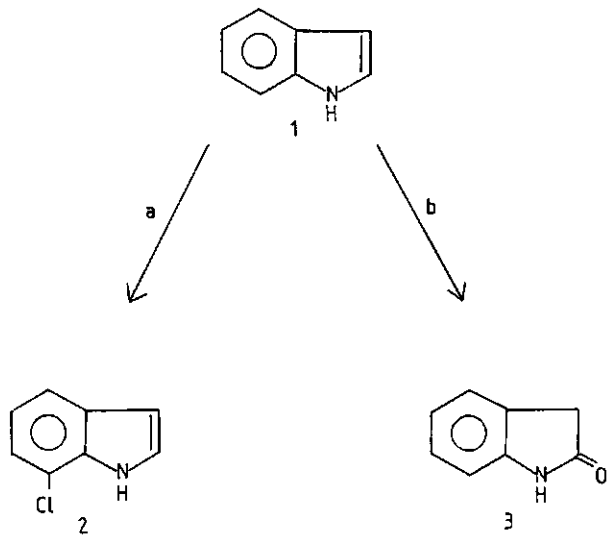
The bromoperoxidase from *A. nodosum* has some advantages compared to the chloroperoxidase from *C. fumago*. CPO is rapidly inactivated by water-miscible

organic solvents [5], although it can convert apolar compounds efficiently when entrapped into reversed micelles (Chapter 6). Enzymatic conversions in reversed micelles are very useful extensions of synthetic organic methodology, but the isolation of the product and especially the removal of the surfactant and the cosurfactant may be a problem. BPO is activated by the addition of ethanol and n-propanol to its reaction medium [6] or its storage medium [7]; so with this enzyme relatively apolar substrates can be halogenated in a much simpler system. There are indications in recent publications that the stability of nonheme haloperoxidases in general is higher than that of the heme-containing ones. De Boer et al. not only found that the BPO from *A. nodosum* is very stable towards organic solvents [8] but is also very resistant towards higher temperatures [6] and higher concentrations of HOBr [8]. The latter fact was also found for the nonheme chloroperoxidase from the mold *Curvularia inaequalis* [9]. Apparently the heme group is a rather vulnerable part of haloperoxidases, leading to an increased interest in the nonheme enzymes [8,10]. Therefore the regio- and stereochemical nature of these enzymes should be studied in more detail. The substituted barbituric acids used in this thesis are very well suited for studies on the kinetics of these halogenation reactions but less suited for stereochemical investigations because the bromine atom at C5 is easily exchanged.

As haloperoxidases are still quite expensive, immobilization of the enzyme is required for the large-scale production of halogenated compounds. Preliminary results on the immobilization of the BPO from *A. nodosum* showed that it can be bound in high yield to CNBr-activated Sepharose and Sepharose substituted with n-octylamine groups [11]. The chloroperoxidase from *C. fumago* posed some problems because of the low pH at which the enzyme is optimally active. However, this problem was solved (Chapter 7) with the use of an acrylamide-*N*-acryloxysuccinimide copolymer. The yield of enzymatic activity after immobilization was about 40%, the support was very stable at low pH and no leakage of enzyme occurred on storage. The stability of this system under turnover conditions has not yet been tested. Promising results have already been obtained for CPO physically immobilized in a hollow fiber reactor composed of cellulose acetate (Chapter 3): the turnover number of the enzyme is at least 1,000,000, compared to 550,000 in water and 90,000 in reversed micelles (Chapter 6). The combination of a bioreactor with an electrochemical cell further enlarges its applications as is shown in Chapter 3 for the production of 5-chlorobarbituric acid.

In summary: the chloroperoxidase from *C. fumago* (CPO) and the bromo-

peroxidase from *A. nodosum* (BPO) are useful enzymes for the synthesis of halogenated compounds because they operate under mild conditions resulting in high yields of product. However, there are some differences between the enzymes. CPO has a higher specific activity and is able to chlorinate, while BPO is more stable, in water and in organic solvents, but is only able to brominate. So far, neither of these enzymes has been shown to react in a regio- or stereospecific manner. Whether there are haloperoxidases that can act in this manner is unknown, but it can certainly not be ruled out. There are lots of known halometabolites that possess one or more chiral carbon atoms with a halogen atom attached to it [10]; this chirality must have been introduced somewhere in their biosynthesis.



Scheme 9.1 *Reactions of indole (1) with the chloroperoxidase from* Pseudomonas pyrrocinia *(route a)* [12] *or from* Caldariomyces fumago *(route b)* [13,14].

Very recently a regiospecific chloroperoxidase was isolated from *Pseudomonas pyrrocinia* [12]. The enzyme was unable to chlorinate monochlorodimedon but converted indole (**1**, see Scheme 9.1) exclusively into 7-chloroindole (**2**) at pH 4.0, whereas the CPO from *C. fumago* gave oxindole (**3**) at pH 2.5-6.0, possibly via 3-chloroindole [13,14]. Thus, the interactions between

the enzymes and 1 must be different. Extensive screening of (micro)organisms for haloperoxidase activity is being carried out at the moment at the Universities of Amsterdam (dr. Wever), Stuttgart (dr. van Pée) and Kyoto (dr. Yamada) and previously at the Cetus Corporation (Emeryville, USA). This work will most probably result in the detection and isolation of (more) regio- or stereospecific halogenating enzymes. Haloperoxidases may therefore become increasingly important in the forthcoming years.

REFERENCES

[1] See Chapter 2. Very recently another study was published which strongly supports this theory [2]
[2] H.B. Dunford, A.M. Lambeir, M.A. Kashem and M. Pickard, Arch. Biochem. Biophys. **252** (1987), 292-302
[3] M.C.R. Franssen and H.J. Kamphuis, unpublished results
[4] E. de Boer, Y. van Kooyk, M.G.M. Tromp, H. Plat and R. Wever, Biochim. Biophys. Acta **869** (1986), 48-53
[5] C.L. Cooney and J. Hueter, Biotechnol. Bioeng. **16** (1974), 1045-1053
[6] R. Wever, H. Plat and E. de Boer, Biochim. Biophys. Acta **830** (1985), 181-186
[7] E. de Boer, H. Plat, M.G.M. Tromp, R. Wever, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer and H.E. Schoemaker, Biotechnol. Bioeng., in press
[8] E. de Boer, H. Plat and R. Wever, in *Biocatalysis in Organic Media* (C. Laane, J. Tramper and M.D. Lilly, eds), Elsevier, Amsterdam, The Netherlands, Stud. Org. Chem. **29** (1987), 317-323
[9] T.E. Liu, T. M'Timkulu, J. Geigert, B. Wolf, S.L. Neidleman, D. Silva and J.C. Hunter-Cevera, Biochem. Biophys. Res. Commun. **142** (1987), 329-333
[10] S.L. Neidleman and J. Geigert, *Biohalogenation: principles, basic roles and applications*, Ellis Horwood Ltd, Chichester (1986), 1-203
[11] M.C.R. Franssen, A. Berg and H.J. Kamphuis, unpublished results
[12] W. Wiesner, K.-H. van Pée and F. Lingens, FEBS Lett. **209** (1986), 321-324
[13] M.D. Corbett and B.R. Chipko, Biochem. J. **183** (1979), 269-276
[14] M.C.R. Franssen and A. Berg, unpublished results

SUMMARY

The subject of this thesis is the use of haloperoxidases in synthetic organic chemistry. Haloperoxidases are enzymes capable of halogenating a variety of organic compounds. They require hydrogen peroxide and halide ions as cosubstrates. The enzymes operate under mild conditions, compared to conventional halogenating agents, resulting in increased yields and purity of products. This thesis presents some new substrates for a chloroperoxidase from the mold *Caldariomyces fumago* (CPO) and a bromoperoxidase from the brown alga *Ascophyllum nodosum* (BPO). The reaction mechanisms of both enzymes and their natural function are discussed. The immobilization of CPO is studied.

CPO catalyzes the smooth halogenation of various heterocyclic compounds. Barbituric acid and some of its derivatives give the corresponding 5-chloro or 5,5-dichloro compounds in very high yields (Ch. 2). 5-Monochlorobarbituric acid is obtained in high yield when the CPO-mediated chlorination reaction is combined with an electrochemical process. The 5,5-dichloro compound is reduced to its 5-monochloro analogue at an electrode, which is simultaneously used for the production of the cosubstrate hydrogen peroxide. The system is very efficient, as shown by the high turnover obtained for CPO (10^6-10^7) (Ch.3). Pyrazoles are also good substrates for the enzyme, giving their 4-chloro derivatives in high yields. CPO further converts 2-aminopyridine into 2-amino-3-chloropyridine and 8-hydroxyquinoline into the corresponding 5,7-dibromo compound (Ch. 4). Kinetic studies indicate that there are two possible reaction routes for CPO: I) the organic substrate binds to an oxidized enzyme intermediate and direct transfer of halogen takes place; II) the enzyme produces hypohalous acid as the active halogenating agent. The available data do not as yet permit a definitive choice of reaction mechanism. (Ch. 2).

BPO smoothly brominates barbituric acid and some of its derivatives giving the corresponding 5-bromo or 5,5-dibromo compounds in good yields. Kinetic measurements show that BPO produces free hypobromous acid which either brominates the organic halogen acceptor or reacts with hydrogen peroxide giving singlet oxygen (Ch. 5).

Relatively apolar substrates like monochlorodimedon and resorcinol are efficiently halogenated by CPO entrapped in reversed micelles. The system is composed of cetyltrimethylammonium halide (CTAX, X=Br or Cl), pentanol, octane and a small amount of aqueous buffer containing the enzyme and hydrogen peroxide. The CTAX serves a dual function: i) as a surfactant, it stabilizes the

reversed micelle, and ii) as a supplier of the halide substrate. The reaction rates obtained are twice as high as in water (Ch. 6).

CPO can be immobilized on various solid supports, but the low stability of the enzyme-support bond and the support itself at the optimal pH of the enzyme is a serious problem. The best way to immobilize CPO seems to be the cross-linking of a mixture of the enzyme and a water-soluble acrylamide-*N*-acryloxy-succinimide copolymer with an α,ω-diamine. In this way CPO is bound by means of both entrapment and covalent bonds, in 40% yield (Ch.7).

Six new halometabolites have been detected by GC/MS analysis of the dichloromethane extract of the culture medium of *Caldariomyces fumago*. The compounds are chlorinated derivatives of ethanol, propanol-2 and 2-(4-hydroxyphenyl)ethanol. Their possible biosynthetic routes and the implications for the reaction mechanism of CPO are discussed (Ch.8).

In short, the two haloperoxidases studied here are biocatalysts which are potentially useful in synthetic organic methodology, because they perform their halogenation reactions in a very smooth and mild way. However, haloperoxidases with greater specificity in their reactions would certainly be of more advantage to the organic chemist.

SAMENVATTING

Het onderwerp van dit proefschrift is het gebruik van haloperoxidasen in de synthetisch-organische chemie. Haloperoxidasen zijn enzymen die in staat zijn een breed scala aan organische verbindingen te halogeneren. Hiervoor hebben ze waterstofperoxide en halide ionen nodig als cosubstraten. In tegenstelling tot chemische halogenerende agentia werken deze enzymen onder zeer milde omstandigheden, hetgeen hoge opbrengsten tot gevolg heeft. In dit proefschrift zijn enkele nieuwe substraten beschreven voor een chloorperoxidase uit de schimmel *Caldariomyces fumago* (CPO) en een broomperoxidase uit *Ascophyllum nodosum* (knotswier) (BPO). De werkingsmechanismen van de enzymen en hun natuurlijke funktie zijn bestudeerd, evenals de immobilisatie van CPO.

Verscheidene heterocyclische verbindingen worden op milde wijze gehalogeneerd door CPO. Barbituurzuur en enkele derivaten daarvan worden in goede opbrengst omgezet in de overeenkomstige 5-chloor of 5,5-dichloorverbindingen (H. 2). Als de CPO-gekatalyseerde chlorering gecombineerd wordt met een elektrochemisch proces wordt uitsluitend 5-monochloorbarbituurzuur verkregen. De 5,5-dichloorverbinding wordt dan aan een elektrode gereduceerd tot het 5-monochloor analogon; de elektrode wordt tevens gebruikt voor de produktie van het cosubstraat waterstofperoxide. Het systeem werkt zeer efficient zoals blijkt uit de hoge turnover getallen die voor CPO verkregen worden (10^6-10^7) (H. 3). Pyrazolen zijn ook goede substraten voor het enzym en leveren de 4-chloorderivaten. Voorts zet CPO 2-aminopyridine om in 2-amino-3-chloorpyridine en 8-hydroxychinoline in de overeenkomstige 5,7-dibroomverbinding (H. 4). Kinetische studies wijzen op twee mogelijke reaktiemechanismen voor CPO: I) het organisch substraat bindt aan een geoxideerde vorm van het enzym en rechtstreekse overdracht van het chlooratoom vindt plaats; II) het enzym produceert HOX, dat als aktief halogenerend agens optreedt. De tot nu toe verkregen gegevens laten niet toe een keuze te maken uit deze twee mechanismen (H. 2).

De bromering van barbituurzuur door BPO verloopt zeer goed. Hierbij ontstaan de overeenkomstige 5-broom of 5,5-dibroomverbindingen in goede opbrengst. Uit kinetische metingen blijkt dat BPO vrij hypobromiet (HOBr) produceert dat het organisch substraat kan bromeren ofwel kan reageren met waterstofperoxide onder vorming van singlet zuurstof (H. 5).

Het insluiten van CPO in omgekeerde micellen biedt de mogelijkheid om relatief apolaire verbindingen als monochloordimedon en resorcinol zeer efficient om te zetten. Het systeem bestaat uit cetyltrimethylammonium halide

(CTAX, X=Br of Cl), pentanol, octaan en een kleine hoeveelheid buffer waarin zich het enzym en waterstofperoxide bevinden. De CTAX heeft een dubbele funktie: i) het stabiliseert de omgekeerde micel vanwege zijn zeepwerking, en ii) het levert het halide substraat. De verkregen reaktiesnelheden zijn ongeveer tweemaal zo hoog als in water (H. 6).

CPO is ook geimmobiliseerd op vaste dragers, maar de labiliteit van de enzym-drager interaktie en de drager zelf bij de optimale pH van het enzym is een groot probleem. Een goede immobilisatiemethode is het cross-linken van een mengsel van het enzym en een wateroplosbaar acrylamide-*N*-acryloxysuccinimide copolymeer met behulp van een α,ω-diamine. Op deze wijze wordt CPO zowel door middel van insluiting als door middel van covalente bindingen gebonden, in 40% opbrengst (H. 7).

GC/MS-analyse van het dichloormethaanextrakt van het kweekmedium van *Caldariomyces fumago* resulteerde in de detektie van zes nieuwe halometabolieten. De verbindingen zijn chloorhoudende derivaten van ethanol, propanol-2 en 2-(4-hydroxyfenyl)ethanol. Hun mogelijke biosyntheseroutes en de implicaties daarvan voor het reaktiemechanisme van CPO worden besproken (H. 8).

Samenvattend, de twee hier bestudeerde haloperoxidasen zijn biokatalysatoren die potentieel nuttig zijn in de synthetisch-organische chemie omdat zij zeer milde en goed werkende halogenerende agentia zijn. Echter, haloperoxidasen die meer specifiek reageren zouden zeker nog van groter nut kunnen zijn voor de organisch chemicus (H. 9).

LIST OF PAPERS

M.C.R. Franssen and H.C. van der Plas
A new enzymatic chlorination of barbituric acid and its 1-methyl and 1,3-dimethyl derivatives.
Recl. Trav. Chim. Pays-Bas **103** (1984), 99-100

C. Laane, W. Pronk, M.C.R. Franssen and C. Veeger
Use of a bioelectrochemical cell for the synthesis of (bio)chemicals.
Enzyme Microb. Technol. 3 (1984), 165-168

C. Laane, A. Weijland and M.C.R. Franssen
Bioelectrosynthesis of halogenated compounds using chloroperoxidase.
Enzyme Microb. Technol. **8** (1986), 345-348

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

M.C.R. Franssen, J.G.J. Weijnen, J.P. Vincken, C. Laane and H.C. van der Plas
Haloperoxidases in reversed micelles: use in organic synthesis and optimisation of the system.
Stud. Org. Chem. **29** (1987), 289-294

E. de Boer, H. Plat, M.G.M. Tromp, R. Wever, M.C.R. Franssen, H.C. van der Plas, E.M. Meijer and H.E. Schoemaker
Vanadium containing bromoperoxidase: an example of an oxidoreductase with high operational stability in aqueous and organic media.
Biotechnol. Bioeng., in press

M.C.R. Franssen, H.G. van Boven and H.C. van der Plas
Enzymatic halogenation of pyrazole and pyridine derivatives.
J. Heterocyclic Chem., in press

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

M.C.R. Franssen, M.A. Posthumus and H.C. van der Plas
New halometabolites from *Caldariomycee fumago*.
Phytochemistry, in press

M.C.R. Franssen, J.G.J. Weijnen, J.P. Vincken, C. Laane and H.C. van der Plas
Chloroperoxidase-catalyzed halogenation of apolar compounds using reversed micelles.
Biocatalysis, submitted

CURRICULUM VITAE

Maurice Charles René Franssen werd geboren op 5 februari 1957 te Venlo. Zijn lagere en middelbare schoolopleiding volgde hij te Venlo en Roermond; het diploma Atheneum-B werd behaald in 1975 aan het Bisschoppelijk College te Roermond. In datzelfde jaar begon hij met de studie scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S2) werd afgelegd in oktober 1978. Gedurende de doktoraalfase werden twee hoofdvakken gekozen: organische chemie (prof. dr. B. Zwanenburg, onderwerp: natuurstofsynthese) en Biochemie (prof. dr. H. Bloemendal, onderwerp: primaire struktuur van eiwitten; prof. dr. J.J.H.H.M. de Pont, onderwerp: enzym-lipid interakties). Het doktoraalexamen werd afgelegd in mei 1982. In augustus van datzelfde jaar werd hij aangesteld als wetenschappelijk medewerker in tijdelijke dienst bij de vakgroep Organische Chemie van de Landbouwhogeschool te Wageningen, alwaar hij het in dit proefschrift beschreven onderzoek heeft verricht. Gedurende het promotie-onderzoek was hij betrokken bij het onderwijs aan studenten in verschillende fasen van hun studie. Sedert januari 1987 is hij in vaste dienst verbonden aan voornoemde vakgroep.