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Perspectives on biotechnological halogenation

Part I: Halogenated products and enzymatic halogenation

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ABSTRACT

Chemical halogenation is a well-established technology often accompanied by hazardous chemicals and low yields. Enzymatic halogenation on the other hand is not used by the industry, even though the first halogenating enzymes were discovered in 1956. There is a trend of increased molecular complexity of halogenated compounds which contain multiple covalently substituted halogen atoms. Allmost all of the incorporations of the halogen atoms in active ingredients must proceed with regioselectivity and often also with stereoselectivity. Biological halogenation can provide this specificity and selectivity. But the technology transfer to large scale manufacturing and established industrial methods are yet to be realized. Recently discovered fluorinases and targeted screening of the marine environment should lead to new industrially useful enzymes.

INTRODUCTION

Halogenated compounds, containing F, Cl, Br or I represent not only important pollutants' but are also used in a variety of applications such as solvents, flame retardants, surface modification, antifouling, pesticides and as active ingredients in health care from blood extenders to anticancer drugs. Halogenated compounds have become particularly important in higher value added pharmaceutical and agrochemical products. It is estimated that 20 percent of all pharmaceutical small molecule drugs and ~30 percent of all active compounds in agrochemistry are halogenated. Chemical halogenation is a well-established technology. But procedures are typically characterised by hazardous or even highly toxic chemicals and poor atom economy. It seems obvious to periodically assess how biological halogenation can offer an advantage over chemical halogenation. The marine biosphere is a promising source for halogenating organisms and enzymes. This review is timely as several large marine biodiversity programmes have been launched for bio prospecting for novel halogenases.

HALOGENS IN NATURE AND IN MARKETED PRODUCTS

Halogens are extracted from natural sources in the ionic forms with metals (CaF_2, Na_3AlF_6, NaCl, NaBr, $NaIO_3$) and most terrestrial halogens are found in insoluble form, hindering uptake by living organisms. Halogens have very different reactivity and in case of iodine it usually requires some form of activation (Table 1). Fluorine and chlorine are gases, whereas bromine is liquid and iodine solid at room temperature. The reactivity decreases with increasing atomic number and decreasing electronegativity from fluorine to iodide. The heaviest halogen, astatine, is a radioactive element formed from radioactive decay and not used for chemical halogenation.

Halogenation was once considered as a rare event in nature. However, since their first discovery 100 years ago, almost 5000 halogenated compounds have been identified so far, and most of them are derived from the marine environment. Chlorometabolites (51 percent) and bromometabolites (45 percent) are predominant among the halometabolites, while organoiodines (2 percent) and organofluorines (2 percent) are much less common. Notably, in certain organisms, such as brown algae, it was recently found that iodination is more frequently used as a metabolite. Independent of which halogen containing compounds are screened, it seems that marine organisms are among the most promising sources of halogenated secondary metabolites and the corresponding enzymatic toolbox.

The interest in peptides is increasing with growth rates of close to 10 percent over the last 10 to 15 years. Peptides are also a good example of the increasing complexity of small molecule pharmaceuticals as the diversity achieved by the variability of the amino acids can be further increased by glycosylation, sulfatation, methylation of halogenation. There is a general trend of increasing structural, chiral and functional complexity for small molecule active ingredients. This complexity is pushing organic chemical synthesis to its limits. However, there is already evidence that nature can assist. For example Cadel-Six et al. (1) have reported peptide halogenating activities in the fresh-water organism Cyanobacteria. Considering that only a few percent of observed organisms can be grown under standard laboratory conditions, we often ignore nature's large unexplored diversity as a potential enzyme toolbox. Metagenomics offers a unique solution for identification and isolation of enzymes from such microorganisms. This methodology will allow the discovery of new enzymes which would allow new reactions to be carried out in radically different ways. The number of these so called "as-yet-uncultured" organisms is much higher in the marine environment and this environment is known to have a rich source of halogenated compounds. In order to utilise this

		Atomic Weight	Electron configuration	Reactivity	Electronegativity	Occurrence in natural products
Fluorine	F	18.99	2-7	↓	3.98	very rare
Chlorine	Cl	35.45	2-8-7		3.16	frequent
Bromine	Br	79.91	2-8-18-7		2.96	frequent
Iodine	I	126.90	2-8-18-18-7		2.66	rare
Astatine	At	No stable isotopes	2-8-18-32-18-7 decreasing		2.20	-

Table 1. Position of the halogens in the periodic table and their characteristics.

treasure trove of potential novel halogenating enzymes we need to preserve and explore these often fragile habitats.
Thus particular emphasis should be placed on sourcing samples from the marine biosphere to fill the biocatalytic and halogenation toolbox. Projects have recently began to explore marine biodiversity of the oceans which cover 70 percent of the earth's surface. It is likely that this will lead to new ways of applying enzymatic halogenation to selected products where a high degree of selectivity (chemo-, regio-, enantio-) is needed. Two examples of on-going marine programmes are the "Gordon & Betty Moore Foundation" and the "Global Ocean Sampling".

HALOGENATED PHARMACEUTICALS AND AGROCHEMICALS

The introduction of a carbon – halogen bond can have a number of effects. The two most important are: a) an increase in thermal and oxidative stability, which means less sensitivity towards oxidation by the liver P450 detoxification and b) an increased biological membrane permeability. Halogenation therefore is a strategy to lessen drug failure in clinical trials and increase drug efficacy (Table 2). However, the disadvantage is that these compounds are more persistent in the environment and recalcitrant to degradation. These halogenated compounds include some blockbusters and well known drugs, a selection of which is listed in Table 3.

Lack of efficacy	.....46%
Animal toxicity	.....17%
Adverse effects in men	.....16%
Miscellaneous	.....7%
Pharmacokinetics	.....7%
Commercial reasons	.....7%.

Table 2. Reasons for failure in drug development. from an analysis of 121 BioNECs between 1964-1985 (2).

Pharmaceuticals		Agrochemicals	
Chloramphenicol	antibiotic	Imidacloprid (Gaucho®)	insecticide
Fluoxetine (Prozac)	depression	Chlorpyrifos (Dursban®)	insecticide
Iododoxorubicin	anticancer	Tebuconazole (Folicur®)	fungicide
Salinosporamide	anticancer	S-Metachlor (Dual Magnum®)	herbicide
Vancomycine	antibiotic	2,4-D (Herbifen®)	herbicide
Ciproflaxin (Cirpobay)	antibiotic	Atrazine (Atranex®)	herbicide
Sitagliptin (Januvia®)	antidiabetic	Fenoxaprop-P-Ethyl (Puma S®)	herbicide

Table 3. A few examples of halogenated products on the market.

One trend with marketed halogenated compounds is that they increasingly contain multiple covalently substituted halogen atoms. For agrochemicals, for example (3), fungicides often have about 2 fluorine atoms; insecticides / acaricides tend to contain 4 or more fluorine atoms and herbicides often contain 3 or more fluorine atoms. By 2008 121 agrochemicals (insecticides, acaricides, fungicides and herbicides) were halogenated. Over the last 10 years (1998-2008) 78.5 percent of agrochemicals were halogenated (Br, I < Cl, Cl/F < F) and these halogenated compounds are among the best-selling agrochemicals.
We screened and analysed the most recent International Drug Directory (4) for marketed products used for medical purposes.
Over 450 halogenated small molecule compounds were identified in 2010. Table 4 shows the distribution of the halogens.
The situation is different in nature, where chlorometabolites and bromometabolites predominate among halometabolites.
Breaking down the proportion of the total that each of these compounds makes we see that approximately 51 percent are organochlorine, 45 percent organobromine, 2 percent organoiodine and 2 percent organofluorine (5).

Chlorinated compounds	.....53%
Fluorinated compounds	.....36%
Brominated compounds	.....6%
Iodated compounds	.....5%

Table 4. Relative distribution of the different halogenated marketed products. About 10 percent of the screened molecules contained two or even more different halogens in the same molecule.

CHEMICAL HALOGENATION

Many methods of chemical halogenation have been developed and improved over the last years but problems such as low yield, lack of selectivity and the use of hazardous materials are still associated with their use. These include free radical halogenation, ketone halogenation, electrophilic halogenation, halogen addition reaction. Molecular halogen can serve as the halogenation species. However, with direct fluorination F_2 also attacks C-C bonds and degrades e.g. aromatic compounds. It is very difficult to regiospecifically fluorinate aromatics by direct fluorination. It is different with Cl_2 or Br_2, where chlorine for example is used to produce intermediates which are used as agrochemicals or pharmaceuticals. The reaction with fluorine, however, is strongly exothermic which often leads to perfluorinated compounds and iodine is not capable of attacking and substituting C-C bonds. Chlorination with elementary chlorine is straightforward but of little selectivity. Besides elementary chlorine chlorocompounds such as phosgene ($O=CCl_2$), *N*-chlorosuccinimide (NCS), phosphopentachloride (PCl_5), *tert*-butylhypochlorite ($(CH_3)_3COCl$, sulfurylchloride (SO_2Cl_2) or tetrachlorocarbon CCl_4 are used. Bromination with elemental bromine is easier and of higher selective due to the lower reactivity. However, the cost with elemental bromine is very high. Therefore, as with chlorine bromoderivates instead of elemental bromine can be used. Examples are bromotrichloromethane BrCCl3, *tert*-butylhypobromit (BrOC(CH3)3) or especially *N*-bromsuccinimide which are frequently used. However, yield issues and the use of hazardous chemicals remain.

ENZYMATIC HALOGENATION

As described above, chemical halogenation is well established in industry. Alternative approaches based on bio-mimetic halogenation methods are presently not utilized in the industry and chemical procedures are the standard methods used. The first natural halogenated (iodine) metabolite was reported in 1896 (6) already and on the other hand, the first microbial halogenating enzyme but it was not until 1959 that the first halogenating microbial enzyme (fungal chloroperoxidase) was discovered and described (7). In addition, the chloroperoxidase consists of haem complex that uses hydrogen peroxide and chloride ions to catalyse the chlorination of caldariomycin. Years later it was observed that this enzyme could also utilize bromide and iodide as substrate for its halogenating reactions (8).
Haloperoxidases, which lack substrate specificity, were the only halogenating enzyme class known for the next 30 years and believed to be responsible for all biological halogenations. This is somehow reminiscent of the dogma of glycobiology which believed that bacteria and archea lacked the ability to glycosylate proteins. Today we know that they not only can glycosylate but offer much more varied glycosylation patterns than eukaryotes. Similar to glycosylation, the practicality of using biological halogenation is just starting to emerge. Biological halogenation is a very new emerging discipline and is proven by the fact that the first fluorinating enzyme was found less than 10 years ago (9) in *Streptomyces cattleya*, although the first fluorometabolite was reported during world war II in a South-African plant (10, 11). The *S. cattleya* enzyme has also been shown to be capable of chlorinating molecules by changing the halide salts in the medium (12). Most known enzymatic halogenase reactions are oxidative, but more and more different strategies are being discovered in the marine environment.
The role of theses halogenated natural products are mostly unknown but it seems logical that they cover the same areas as other non-halogenated biologically active secondary metabolites.

Enzyme type	Cellular function	Characteristics	References
Haloperoxidase	Many unknown; thyroid peroxidase is responsible for iodination of tyrosine	Can halogenate a wide range of substrates by generating hypohalite	Murphy (2003)
FADH2-dependent halogenase	Biosynthesis of phenyl- and pyrrole-containing antibiotics	Specific halogenation via HOCl	Van Pee and Patallo (2006)
Non-heme, Fe(II), a-ketoglutarate and O2-dependent halogenase	Halogenation of unactivated (aliphatic) carbon centres	Formation of specific carbon-halogen bonds via free radicals	Valliancourt et al. (2005)
β-Mannosidase	Transglucosylation	Glu519Ser mutant forms a transient carbon-fluorine bond	Zechel et al. (2001)
Fluorinase	Biosynthesis of fluorometabolites in *Streptomyces cattleya*	Catalyses nucleophilic displacement of methionine in SAM	O'Hagan et al. (2002), Dong et al. (2004)
Chlorinase	Biosynthesis of salinosporamide in *Salinispora Tropica*	Analogous reaction that is catalysed by fluorinase	Eustaquio et al. (2008)

Table 5. Types of enzymes that catalyse carbon-halogen bonds (taken from Murphy and Grant (2010) (14).

A recent study has even shown that a morphogenic and quorum sensing small molecule containing 2 Cl atoms was identified in *Dictyostelium*, a social amoeba.

Halogenating enzymes can be classified in two categories: (i) highly substrate-specific halogenases requiring O_2 for enzymatic activity and (ii) less specific haloperoxidases (HPO) utilizing hydrogen peroxide (13). Several halogenating enzymes have been described in different organisms. The number of publications in the last 10 years has been increasing rapidly (Table 4). Application of these enzymes in the synthesis of new or modified halogenated compounds is now an area of particular commercial interest (14). Biological halogenation by flavin dependent halogenases is an example how nature can use bleach, a relatively highly active compound in a specific and selective way in an enzyme (15). In contrast to haloperoxidases, flavin dependent halogenases carry out regioselective halogenations. The haloperoxidases produce hypochlorous acid (HOCl) which indiscriminately halogenates their substrates. There is therefore a lack of substrate selectivity and the reaction is not regioselective (15). However, flavin dependent halogenases also produce HOCl. This enzyme is comprised of two modules. One module binds the FAD and the other module binds the substrate. These halogenases differ in two important aspects to haloperoxidases. In the case of tryptophan-5-halogenase the HOCl is formed when the reduced flavin bound to the first module reacts with oxygen to form flavin hydroperoxide. The flavin hydroperoxide then reacts with the chloride ion forming HOCl, which in contrast to the HOCl formed by haloperoxidases, is not released from the enzyme but guided along a tunnel to the bound substrate (16). The tryptophan is bound to the second module in such a manner that the C5 atom is the only atom available for chlorination. In this way the other atoms are protected from indiscriminate halogenation by the HOCl species (17).

The other known halogenase that is capable of regiospecific halogenation is the non-heme enzyme which requires α-ketoglutarate and oxygen as well as the chloride ion to carry out halogenation. Valliancourt et al. describe the role that this enzyme plays in the synthesis of syringomycin E, a phytotoxin produced by *Pseudomonas syringae*. This enzyme is responsible for the monochlorination of the methyl group of the threonine moiety in the 4-position (18).

OUTLOOK

Fluorinated compounds are the second most important group among halolgenated marketed products and there is a huge interest in finding novel fluorinating enzymes, which are stable and capable of making site specific substitutions, due to its large range of market applications and economic importance. Marine habitats are the most promising environments for the discovery of novel halogenases and are expected to yield unique properties as they have evolved in extreme conditions of pressure, temperature, salinity and nutrient availability that are not present in terrestrial habitats. This has led to several large marine biodiversity programmes aimed for bio prospecting of novel halogenases. The second part of this article, "Prospecting for future biohalogenases", talks about the only fluorinase that has been characterized to date, its impact and the future perspective in biological halogenation.

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