BIOSYNTHESIS OF HALOGENATED METABOLITES BY BACTERIA

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ABSTRACT

Halogenated metabolites, originally thought to be infrequent in nature, are actually nothing unusual at all, and are produced by many different organisms, including bacteria. Whereas marine bacteria usually produce brominated compounds, terrestrial bacteria preferentially synthesize chlorometabolites, but fluoro- and iodometabolites can also be found. Haloperoxidases, enzymes capable of catalyzing the formation of carbon halogen bonds in the presence of hydrogen peroxide and halide ions (Cl^-, Br^- and I^-) have been isolated and characterized from different bacteria. These enzymes turned out to be very unspecific and are obviously not the type of halogenating enzymes responsible for the formation of halometabolites in bacteria. A yet-unknown type of halogenating enzyme having both substrate and regio-specificity must be involved in the biosynthesis of halogenated compounds.

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INTRODUCTION

For a long time the production of halogenated metabolites by living organisms was regarded as something unusual. Until 1960, only 29 naturally produced halogenated compounds were known (82), and in 1968, organohalogens of natural origin were still thought to be present infrequently (34). However, since then over 2000 halometabolites, containing one or several halogen atoms, have been isolated from natural sources (39). These halogenated compounds of natural origin are produced by many different organisms (76).

The most abundant naturally synthesized organohalogens are bromine-containing metabolites, preferentially produced by organisms living in marine environments, such as marine invertebrates and marine algae, because of the relatively high bromine concentration in sea water compared to soil (76). The Tyrian purple dye (Figure 1) is produced by several *Murex* species (molluscs) (30). Very simple molecules like bromoform are synthesized by marine algae (76). In terrestrial organisms, mostly chlorinated metabolites are found. Many chlorinated compounds have been isolated from fungi. Some, like griseofulvin (Figure 1) (78), produced by several *Penicillium* species, have antibiotic activity. Others, like chloromethane, are synthesized in huge amounts and are considered responsible for the destruction of the ozone layer (44) or, like drosophilin A (Figure 1) (100), are very difficult to degrade. Chlorometabolites from higher plants include indole derivatives like 4-chloroindole-3-acetic acid (27).

Iodinated compounds occur less frequently, although they too are produced by many different organisms. The thyroid hormone tyroxine (Figure 1) is synthesized by mammals (43). Iodinated compounds are also produced by a number of different marine algae (92).

Fluorinated metabolites are very rare, although the amount of fluoride available to living organisms is higher than that of iodide and bromide (34). Fluoroacetate, one of the most toxic compounds produced by organisms, is synthesized by a few higher plants (77) and is further metabolized to fluorocitrate by the producing plants (81).

STRUCTURAL DIVERSITY OF BACTERIAL HALOMETABOLITES

The first halometabolite isolated from bacteria was the antibiotic chloramphenicol (Figure 2) produced by *Streptomyces venezuelae* (26). In the following
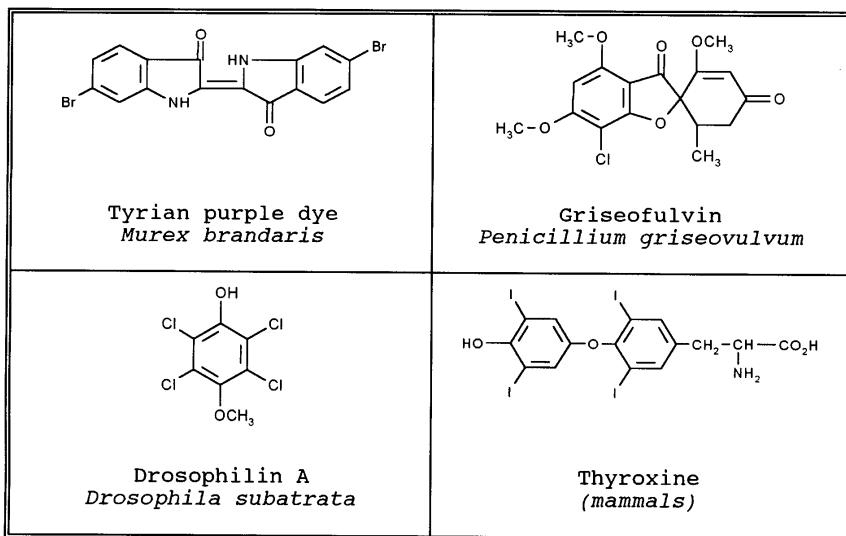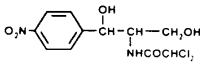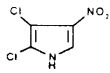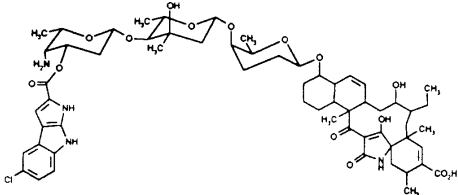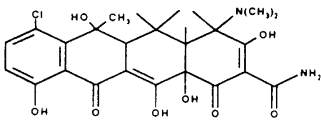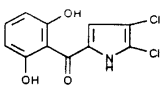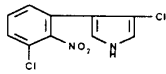
Figure 1   Some examples of halometabolites produced by eukaryotic organisms.

years, other chlorinated metabolites with antibiotic activity were isolated from bacterial cultures. During these years, halogenated bacterial compounds were always connected with antibiotic activity, and the impression was formed that halogenated metabolites occur very infrequently in nature. Until 1960, only 29 microbial halometabolites were known with eight of bacterial origin (82). All of these halometabolites contained chlorine; however, in some cases the corresponding bromo-analogues could also be obtained by substituting chloride in the culture medium by bromide (1, 23, 28, 93, 105).
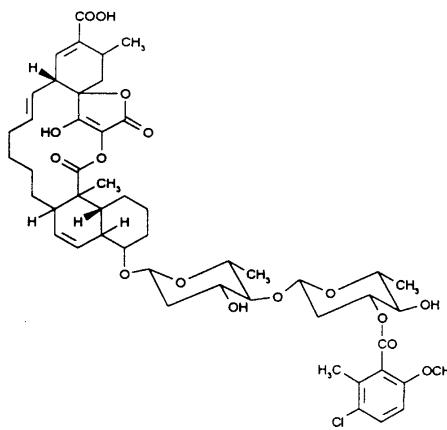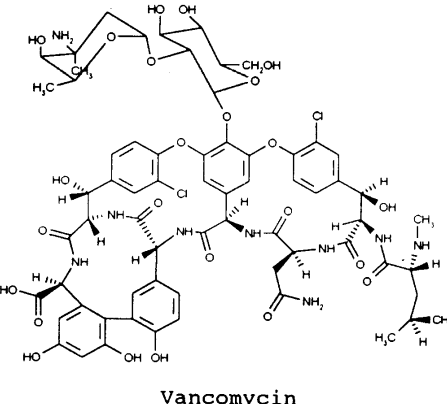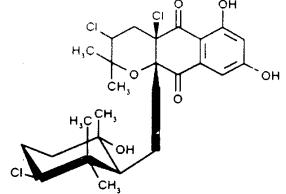
The structural variety of bacterial halometabolites is very broad, although there are no reports on the isolation of halogenated one-carbon compounds from bacteria (39). Other halogenated metabolites with very simple chemical structures are either not produced by bacteria or have not been detected yet. The large majority of bacterial halometabolites are aromatic compounds. There are only very few halometabolites consisting of a single chlorinated pyrrole ring, like pyrrolomycin A (Figure 2) (51), or of a single phenyl ring with small substituents like 3-chloroanthranilic acid (87).

In most aromatic halometabolites, the phenyl ring is either condensed to a heterocyclic ring, as in 3-chloroindole (63), pyrroindomycin B (Figure 2) (22), or rebeccamycin (12), or is condensed to other ring systems, as in the case of

Structure Compound	Producer	Ref.
Chloramphenicol	*Streptomyces* *venezuelae*	26
Pyrrolomycin A (I)	*Actinosporangium* *vitaminophilum sp.*	51
Pyrroindomycin B	*Streptomyces* *rugosporus*	22
Chlortetracycline	*Streptomyces* *aureofaciens*	25
Pyoluteorin	*Pseudomonas* *aeruginosa*	97
Pyrrolnitrin	*Pseudomonas* *fluorescens* *Myxococcus fulvus*	3
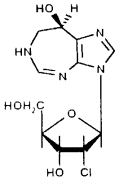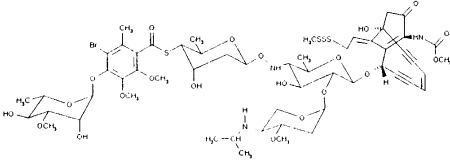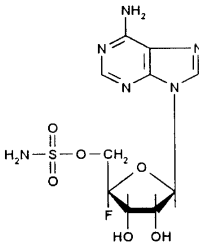
(a)

Figure 2   Examples of structurally different halometabolites produced by various bacteria.

Structure	Organism	Ref.
Chlorothricin	*Streptomyces antibioticus*	52
Vancomycin	*Amycolatopsis orientalis*	115
Napyradiomycin-B4	*Chainia rubra*	90

(b)

Figure 2   (Continued)

2'-Chloropentostatin	*Actinomadura sp.*	99
Calicheamicin β_1^{Br}	*Micromonospora echinospora ssp. calichensis*	60
Nucleocidin	*Streptomyces calvus*	73

(c)

Figure 2   (Continued)

7-chlorotetracycline (Figure 2) (25). The phenyl ring may also be part of an even larger structure, like in vancomycin (Figure 2) (115). Various phenylpyrrole derivatives containing halogen have been isolated: The halogen may be carried only in the phenyl ring (monodechloroaminopyrrolnitrin, Figure 3) (106), only in the pyrrole ring (pyoluteorin, Figure 2) (97), or in both ring systems, like in pyrrolnitrin (Figure 2) (3), pyrrolomycin B (51), or pentachloropseudilin (14).

Some macrolide antibiotics like chlorothricin (Figure 2) (52), oligoglycoside antibiotics (avilamycin A) (71), polyether antibiotics (17), and β-lactam antibiotics (16) containing a chlorinated phenyl moiety are produced by many different bacteria. In a number of glycopeptide antibiotics, several chlorinated phenolic rings can be found, like in vancomycin (Figure 2) (115) or
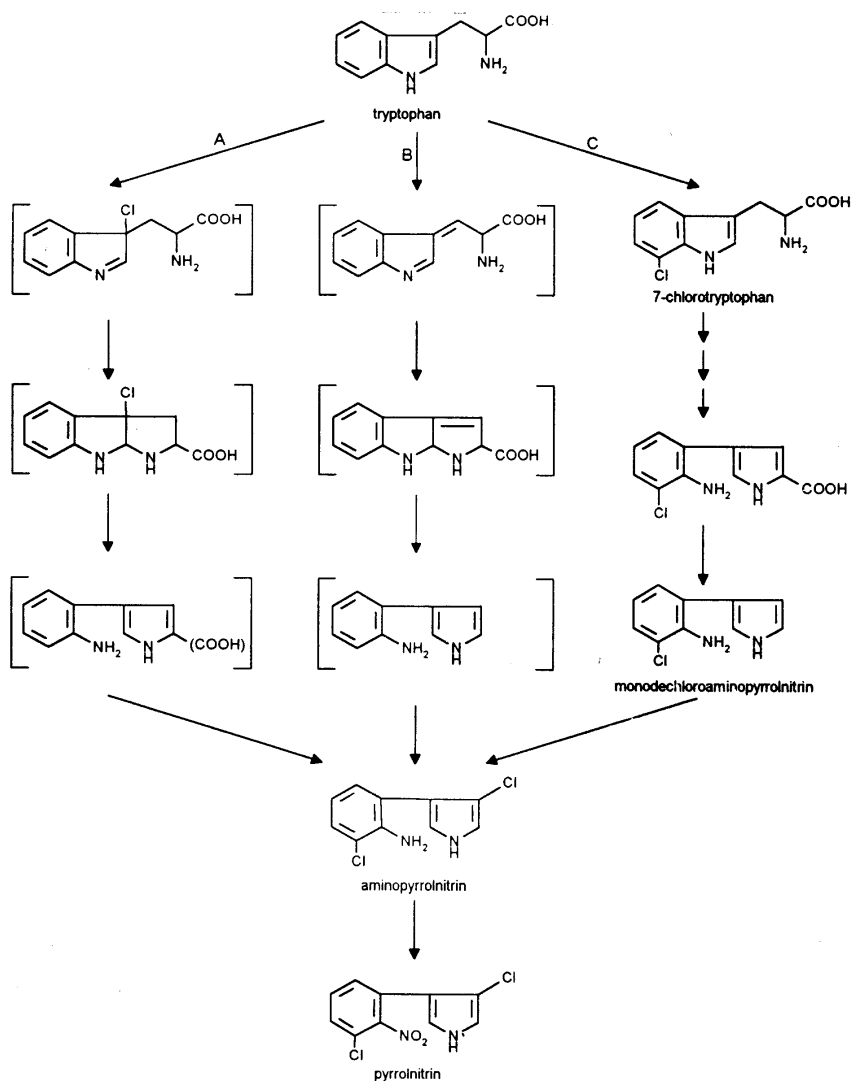
Figure 3   Hypothetical pathways for pyrrolnitrin biosynthesis according to (*A*) Gorman & Lively (37), (*B*) Floss et al (33), and (*C*) van Pée et al (106). The compounds in brackets have not been isolated.

chloropolysporin C (96). In all cases the chlorinated phenyl ring is either a phenol or an aniline derivative.

Chloramphenicol (Figure 2) and indisocin (48) are examples of halometabolites that, although containing a phenyl or indole ring, are not chlorinated in the aromatic moiety but in the aliphatic part of the molecule. 4-Chlorothreonine (119), 4-amino-3-chloro-2-pentenedioic acid (15), and armentomycin (2) are chlorinated aliphatic nonprotein amino acids.

Napyradiomycin B4 (Figure 2) is a very unusual chlorinated metabolite. It contains three chlorine atoms at three asymmetric carbon atoms but none at the phenolic ring (90). 2′-Chloropentostatin (Figure 2) is one of the very few metabolites with a halogenated sugar moiety (99).

Pentabromopseudilin, the bromo-analogue of pentachloropseudilin, is produced by a marine bacterium (94). In the marine environment, bacteria produce bromohalogens instead of chlorohalogens. Some soil bacteria that normally produce chlorinated halometabolites synthesize the corresponding bromo-analogues only when the culture medium is supplemented with bromide instead of with chloride. Under such conditions the bromo-analogues of 7-chlorotetracycline (23), chloramphenicol (93), pyrrolomycin B (28), and pyrrolnitrin (105) were isolated. In contrast to this, caliceamicines (Figure 2) are bromometabolites of which no chloro-analogues have been found (60).

Only very few fluorinated compounds have been isolated from bacteria. Fluoroacetate, which is also synthesized by a few plants (77), and 4-fluorothreonine are produced by bacteria (89). In nucleocidin (Figure 2) the fluorine atom is incorporated into the sugar part of the molecule (73).

Although several iodometabolites have been isolated from mammals, marine algae, and fungi (76), the only iodometabolites isolated from bacteria (60) are iodinated caliceamicins.

HALOMETABOLITE-PRODUCING BACTERIA AND THE BIOLOGICAL SIGNIFICANCE OF HALOMETABOLITES

Diversity of Producing Bacteria and Biological Significance

The search for halometabolites from bacteria was and is still connected with the search for antibiotics or compounds with other biological activities. Therefore it is not astonishing that most of the organohalogens found in bacteria have been isolated from antibiotic-producing *Actinomycetes* species. Many members of the different groups (*Streptomycetes, Actinoplanes, Nocardia*) of this genus of gram-positive bacteria are known to produce halogenated metabolites (Figure 2, 58). Compared to the number of halometabolites synthesized by actinomycetes strains, the number of such compounds produced by other bacteria is rather

low. A few halometabolites are synthesized by pseudomonads (58, 94) and members of other genera. So far no halogenated compound has been isolated from *Archaebacteria*.

It is noteworthy that the same halometabolite can be produced by members of different genera of bacteria. The antifungal antibiotic pyrrolnitrin (Figure 2) for example is synthesized by species from different subgroups of the genus *Pseudomonas* (3, 63, 86) and by different species of the genus *Myxococcus* (36).

Halogenated compounds produced by bacteria are products of secondary metabolism. Their production begins late in the logarithmic phase of growth or in the stationary phase. Fluoroacetate and fluorothreonine production in *Streptomyces cattleya* starts after four days of growth, when growth has already decreased. In work by Reid et al, the maximum of fluoroacetate and fluorothreonine production was reached after 16 days of growth (85). Synthesis of the antifungal antibiotic pyrrolnitrin by *Pseudomonas cepacia* already begins after 24 hours of growth, but the concentration of pyrrolnitrin in the culture broth still increases until 168 hours of fermentation (86).

As with secondary metabolites in general, it is still not clear why these metabolites are produced. In bacteria, halometabolites are usually synthesized in concentrations hardly detectable. Therefore it is difficult to imagine that production of these compounds could provide an advantage for the producing bacteria by inhibiting the growth of organisms competing for nutrients. However, pyoluteorin- and pyrrolnitrin-producing strains of *Pseudomonas fluorescens* have been used as biocontrol agents against soil borne plant pathogens (13, 59). In these cases, where there are large numbers of producing bacteria, the amount of halometabolite produced is obviously high enough to have an inhibitory effect on soil borne pathogens, although the inhibitory effect is usually not due to the halometabolites alone but is due to a number of different metabolites produced by these strains (47, 75).

Influence of Halide Ions

As already mentioned, many of the bacterial halometabolites have biological activities, like antibacterial, antifungal, and antitumor activities. These activities not only are due to the general chemical structure of these compounds but also depend on the type, number, and position of the halogen atoms. The antibiotic activities of the bromo-analogues are either very similar or less than the activities of the normally produced chlorometabolites. Bromotetracycline has essentially the same level of activity as 7-chlorotetracycline (82), whereas chloramphenicol (93) and pyrrolnitrin (105) have higher antibiotic activities than their bromo-analogues. The difference of antibiotic activity between 7-chlorotetracyline and its dehalo-analogue tetracycline is very low, whereas chloramphenicol has a 2–15-times higher activity than its dechloro-analogue

corynecin I (95). Mono- and didechlorovancomycin have about 70 and 50% of the activity of vancomycin, respectively (45).

A general rule does not seem to exist concerning the influence of the number and position of the halogen atoms. However, it seems that the major halometabolite produced under normal conditions is the most potent one.

BIOSYNTHESIS OF HALOMETABOLITES

Only limited knowledge is available about the biosynthetic pathways of halometabolites, especially about the halogenation step. In most cases it is not known at what stage of the biosynthesis halogenation occurs, as the pathways are often very complicated. In halogenated peptide antibiotics, it is not known whether halogenation takes place at the amino acid level and the halogenated amino acids are then incorporated into the peptides or whether halogenation occurs after formation of the peptide bonds.

7-Chlorotetracycline Biosynthesis

The biosynthetic pathways of a few of the halogenated antibiotics that have been detected early in the search for antibiotics have been thoroughly investigated (66, 68). McCormick and his group have isolated many of the intermediates of 7-chlorotetracycline biosynthesis in *Streptomyces aureofaciens* (Figure 4) and prepared a number of mutants, some of which were blocked in the chlorination step. These mutants only produced tetracycline, the dehalo-analogue of 7-chlorotetracycline (69). From a mutant blocked in the chlorination step, 4-hydroxy-6-methylpretetramid (Figure 4) was isolated (70); however, the actual substrate for the chlorinating enzyme is 4-ketoanhydrotetracycline (69). Unfortunately this compound is unstable and cannot be isolated in quantities sufficient for in vitro studies with cell-free extracts. Cross-feeding experiments showed that the keto group in position 4 was crucial for the chlorination to occur. After transamination to 4-aminoanhydrotetracycline (Figure 4), chlorination in position 7 was not possible anymore (68). Obviously the chlorinating enzyme has a substrate specificity. As no tetracycline derivative with a chlorine atom at other than position 7 has ever been isolated, this chlorinating enzyme must also have a regio-specificity.

Investigations on the inhibition of the chlorination step in tetracycline biosynthesis showed that this step is completely inhibited by bromide ions (38). With some strains, this is due to the production of bromotetracycline instead of chlorotetracycline (23). Inhibition of the chlorination step by compounds like 2-thiouracil or 2-mercaptobenzthiazol that act as chelating agents inhibiting copper containing oxidases and the elimination of inhibition by an excess of Cu^{2+}-ions resulted in the hypothesis that the chlorinating enzyme could be a

4-hydroxy-6-methylpretetramid

4-ketoanhydrotetracycline

4-ketodedimethylanhydro-
7-chlorotetracycline

7-chloro-4-aminodedimethyl-
aminoanhydrotetracycline

7-chlorotetracycline
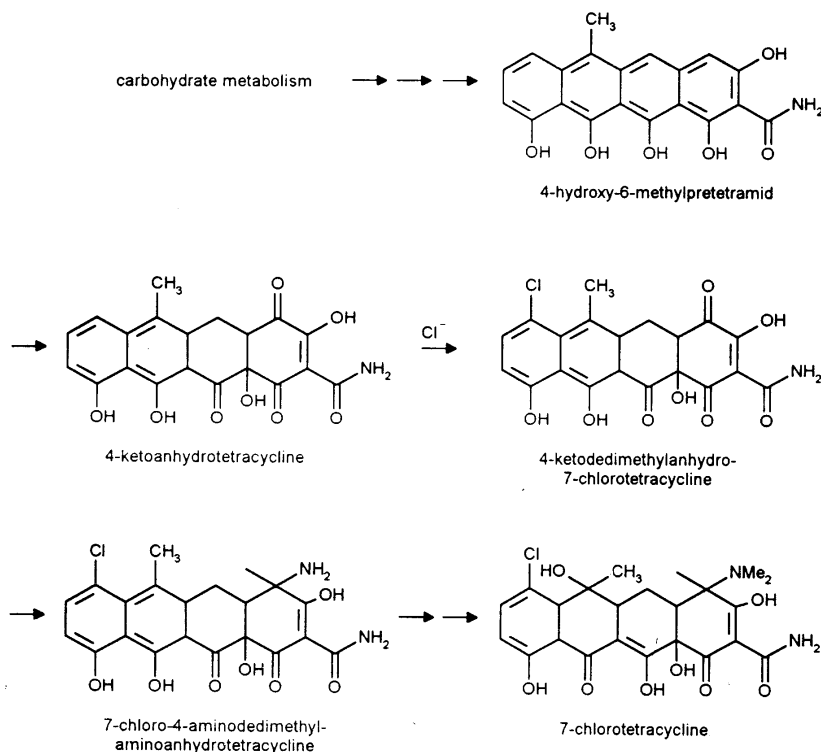
Figure 4   Hypothetical pathway for 7-chlorotetracycline biosynthesis (68).

copper-containing enzyme (50). However, only very little is known about halogenating enzymes from chlorotetracycline-producing strains (57, 80, 83, 107, 112), and none of these enzymes is a copper enzyme. This lack of knowledge might be overcome in the near future, for Dairi et al (18) succeeded in cloning and sequencing the gene for the chlorinating enzyme of 7-chlorotetracycline biosynthesis from *S. aureofaciens* NRRL3203. However, the cloned gene has no sequence homology to any of the known halogenating enzymes.

Chloramphenicol Biosynthesis

Although much is known about the biosynthetic pathway of chloramphenicol in *S. venezuelae* [most of the intermediates are isolated and characterized (Figure 5)], little is known about the chlorination step (108). Chlorination occurs between p-aminophenylserine and dichloroacetyl-p-aminophenylserine (Figure 5). However, there is still some uncertainty about the nature of the substrate for the chlorinating enzyme. It is not clear yet whether halogenation

precedes or succeeds acetylation. The most favored hypothesis is that acetoacetyl-CoA is chlorinated to dichloroacetyl-CoA (LC Vining, personal communication). As in the case of 7-chlorotetracycline, no in vitro studies have been undertaken, in this case because of the lack of knowledge about the substrate structure for the halogenating enzyme.

Doull et al (24) isolated a mutant, blocked in the chlorination step. This mutant produces corynecines, the dechloro-analogues of chloramphenicol. Corynecines are also produced by *S. venezuelae* in the absence of chloride ions (93) and by *Corynebacterium hydrocarboclastus* (74) lacking the chlorinating enzyme. The production of corynecines by *S. venezuelae* in the absence of chloride and by the mutant blocked in the chlorination step could be explained by chlorination preceding acylation and acyltransferase having a very high affinity to dichloroacetyl-CoA; in the absence of this substrate the acetyltransferase would also accept acetyl-CoA, propionyl-CoA, and isopropionyl-CoA
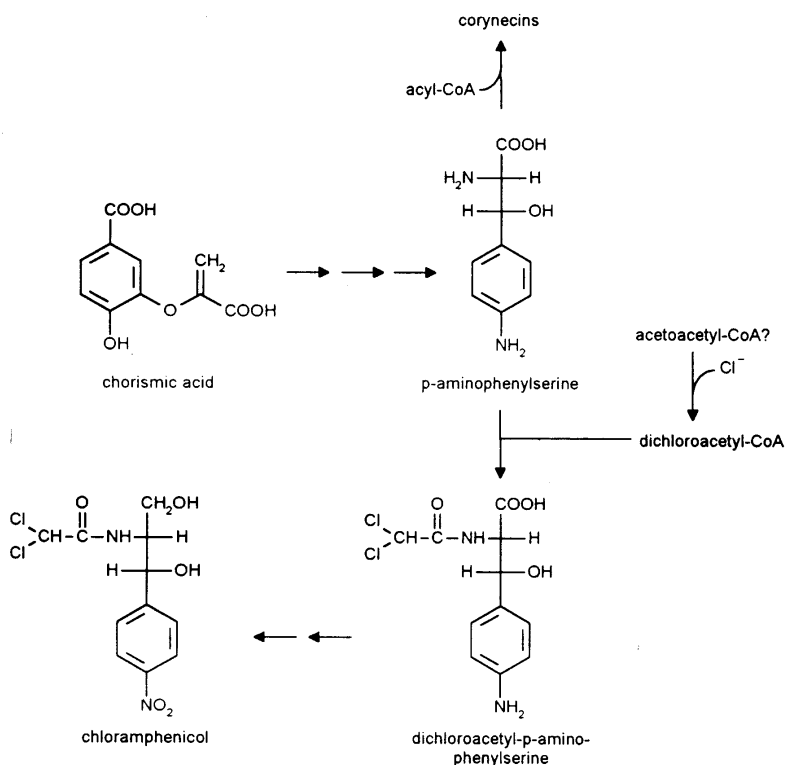
Figure 5   Hypothetical pathway for chloramphenicol biosynthesis (108).

as substrates for the acylation of p-aminophenylserine which would result in the formation of corynecines.

Pyrrolnitrin Biosynthesis

The antifungal antibiotic pyrrolnitrin is produced from tryptophan by several *Pseudomonas* (3, 63, 86) and *Myxococcus* (36) strains. Several pathways were postulated that start from tryptophan (Figure 3) (62, 106). In one of these pathways (Figure 3*A*), the ring rearrangement from the indole ring system to the phenyl pyrrole ring system occurs before the first chlorination step (33). The second hypothetical pathway (Figure 3*B*) involves a transient chlorination step as the starting point for the ring rearrangement, with subsequent loss of the chlorine atom incorporated in the first step. The chlorine atoms contained in pyrrolnitrin are proposed to be incorporated after the ring rearrangement (37).

Based on the isolation of 7-chloroindole derivatives (65, 87), Salcher et al (88) postulated a pathway in which the first step is the chlorination of tryptophan to 7-chlorotryptophan followed by the rearrangement of the ring system (Figure 3*C*). Feeding experiments with 7-chlorotryptophan showed that this compound was actually incorporated into pyrrolnitrin (106); however, it was still not clear at that time whether 7-chlorotryptophan was a true intermediate. Only recently has 7-chlorotryptophan been isolated from a pyrrolnitrin-producing *P. fluorescens* strain (53). This supports the hypothesis of Salcher et al (88) and van Pée et al (106), in which the first step of pyrrolnitrin biosynthesis is a chlorination step. The ring rearrangement eventually results in the formation of monodechloroaminopyrrolnitrin (Figure 3*C*), which is the substrate for the second chlorination step. Because monodechloroaminopyrrolnitrin can be obtained by fermentation (106), it is available as substrate for in vitro investigations. Wiesner et al (114) isolated an enzyme that catalyzed the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (Figure 3*C*), the next metabolite in pyrrolnitrin biosynthesis but not the chlorination of tryptophan.

Fluoroacetate Biosynthesis

The biosynthesis of fluoroacetate by *S. cattleya* is still not clear. According to Tamura et al (98), it probably proceeds from glycerol via β-hydroxypyruvate to fluoroacetate, where the actual fluorination reaction is the replacement of the β-hydroxyl group of β-hydroxypyruvate by fluoride; however, so far no enzyme catalyzing this reaction has been detected in cell-free extracts.

In contrast to the results of Tamura et al (98), Reid et al (85) found that glycolate is better incorporated into fluoroacetate than glycerol. They speculate that fluoroacetate could be formed by nucleophilic displacement of the phosphate group of phosphoglycolate by fluoride, involving an enzyme system that is known to catalyze the conversion of fluoroacetate to glycolate (85).

monochlorodimedone   2-chloro-1,3-cyclo-
                                 pentanedione
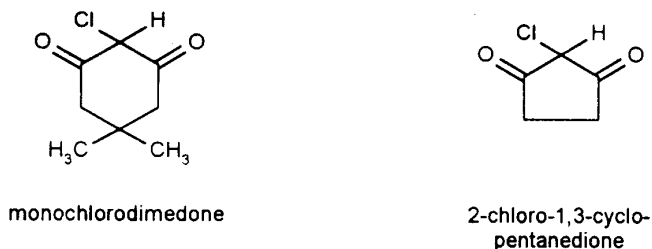
Figure 6 Chemical structures of monochlorodimedone, the substrate used for the search for halogenating enzymes for over 30 years, and of 2-chloro-1,3-cyclopentanedione, a late intermediate in caldariomycin biosynthesis.

The origin of 4-fluorothreonine is still under debate. Whereas Tamura et al (98) speculate that fluoroacetate is further metabolized to 4-fluorothreonine by *S. cattleya*, the results obtained by Reid et al (85) suggest that this is not the case.

HALOGENATING ENZYMES

Heme-Containing Haloperoxidases

The first chlorinating enzyme was isolated from the fungus *Caldariomyces fumago* by Morris & Hager (72) in 1966. As this enzyme catalyzes the chlorination of organic substrates in the presence of chloride ions and hydrogen peroxide, it was named chloroperoxidase. In the following years a number of bromoperoxidases, i.e. enzymes catalyzing the bromination but not the chlorination of organic substrates in the presence of hydrogen peroxide, were isolated from different algae and from sea urchin eggs (4, 21, 67).

These bromoperoxidases and the fungal chloroperoxidase all contain protoporphyrin IX as the prosthetic group, and all catalyze the chlorination or bromination of monochlorodimedone (Figure 6) (76). Monochlorodimedone is a synthetic compound (41) that is not produced by any of the organisms from which haloperoxidases have been isolated; thus it is not the natural substrate of halogenating enzymes. It has only some structural resemblance to 2-chloro-1,3-cyclopentanedione, a late intermediate in the biosynthesis of caldariomycin (Figure 6) (7); however, it was used for the search for halogenating enzymes for over 30 years.

By using monochlorodimedone as the organic substrate, the first bacterial bromoperoxidase was isolated from the chloramphenicol-producer *Streptomyces phaeochromogenes* in 1985 (104). The isolation and characterization of bacterial bromoperoxidases from *Streptomyces* and *Pseudomonas* strains showed that they also contained protoporphyrin IX as the prosthetic group and

had catalytic properties similar to the eukaryotic heme-containing haloperoxidases (76, 101). However, none of these heme-containing bacterial haloperoxidases was able to catalyze the chlorination of organic compounds.

The reaction mechanism of chloroperoxidase from *C. fumago* was thoroughly investigated, and it can be assumed that the other heme-containing haloperoxidases follow the same mechanism. In the first step the enzyme reacts with hydrogen peroxide with the formation of compound I, the hydroperoxide of the enzyme. Compound I then reacts with chloride, bromide, or iodide (X), resulting in the formation of compound EOX . There is still some debate as to whether EOX is the actual halogenating agent or whether it decomposes, leading to the formation of an X^+-species that does not exist freely in water but will form HOX, X_2, or X_3^-. The lack of substrate and regio-specificity suggests that most substrates are halogenated outside the active site by one of the decomposition products of compound EOX (35).

Vanadium-Containing Nonheme Haloperoxidases

A different type of haloperoxidases was first found in the brown alga *Ascophyllum nodosum* (110). This bromoperoxidase did not contain heme but was vanadium-dependent instead. Similar vanadium chloro- and bromoperoxidases were later isolated from other algae (20, 56), a lichen (84), and fungi (91).

The reaction mechanism of vanadium haloperoxidases has almost been completely resolved. The vanadium haloperoxidases also catalyze the formation of HOX, which results in unspecific halogenation of organic compounds that are susceptible to electrophilic attack (35).

Bacterial Nonheme Haloperoxidases

In bacteria, vanadium haloperoxidases have not been detected yet. However, a different type of nonheme haloperoxidase was isolated from the pyrrolnitrin-producer *Pseudomonas pyrrocinia* (CPO-P) that catalyzed the chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin (Figure 3*C*). This bacterial nonheme haloperoxidase did not contain a prosthetic group or any metal ions (114). Other members of this type of haloperoxidase were isolated from other chlorometabolite-producing bacteria (57, 107, 112, 120).

Surprisingly, nonheme haloperoxidases were also found in *Streptomyces lividans* and *Serratia marcescens*, bacteria from which no chlorometabolites have ever been isolated (6, 11). Investigations of the substrate specificity of bacterial nonheme haloperoxidases showed that the enzyme from the 7-chlorotetracycline-producer *S. aureofaciens* Tü24 (CPO-T) catalyzed the chlorination of pyrrolnitrin in the same way as does CPO-P, the enzyme from the pyrrolnitrin-producer *P. pyrrocinia* (9). Enzymatic chlorination of indole using the chloroperoxidases from *S. aureofaciens* Tü24 (CPO-T), *S. lividans*

(CPO-L), and *P. pyrrocinia* (CPO-P) yielded 3-chlorindole (5, 8) and not 7-chloroindole as reported by Wiesner et al (113). 3-Chloroindole is also obtained when indole is chlorinated using hypochlorite (10), showing that enzymatic chlorination using bacterial nonheme haloperoxidases is not more specific than chemical halogenation.

Cloning and sequencing of the genes for bacterial nonheme haloperoxidases (6, 80, 83, 116), sequence comparison (6, 79), and detailed investigations of the three-dimensional structure (46) and of the reaction mechanism (103) showed that these enzymes contained a catalytical triad consisting of the amino acids aspartate, histidine, and serine. Pelletier et al (79) demonstrated that this catalytical triad was necessary for halogenating activity and thus showed that an earlier hypothesis involving a methionine residue (40) was wrong. According to recent results, the first step in the halogenation catalyzed by bacterial nonheme haloperoxidases is the formation of an acetate ester at the serine residue of the catalytical triad. This ester, however, is not hydrolyzed by water but by hydrogen peroxide, resulting in the formation of peracetic acid (Figure 7) (103). As a strong oxidizing agent, peracetic acid is able to oxidize bromide, chloride, and aromatic amino to nitro groups.

Bacterial Haloperoxidases and the Formation of Halometabolites

A heme-containing bromoperoxidase with a very high catalase activity and some properties typical for classical catalases, and thus called a bromoperoxidase-catalase, was isolated and characterized from the chloramphenicol-
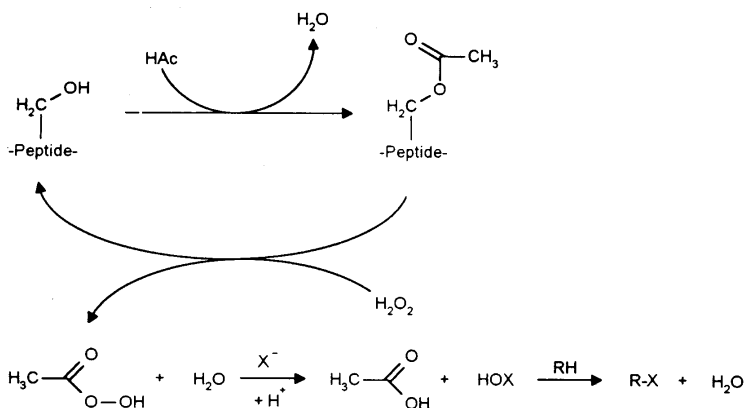

Figure 7   Hypothetical reaction mechanism of bacterial nonheme haloperoxidases (103).

producer *S. venezuelae* (55). This enzyme was only present in an inactive form in a mutant blocked in the chlorination step, which suggested its involvement in chloramphenicol biosynthesis. However, when the cloned gene was inactivated and exchanged against the functional chromosomal gene in *S. venezuelae*, it was found that this gene replacement had no effect on chloramphenicol production. This clearly demonstrated that the bromoperoxidase-catalase was not involved in chloramphenicol biosynthesis (29).

The lack of substrate specificity observed with bacterial nonheme haloperoxidases indicated that these enzymes were not likely to be involved in the biosynthesis of halometabolites like 7-chlorotetracycline, chloramphenicol, or pyrrolnitrin. In the case of 7-chlorotetracycline, in vitro studies were not possible because the natural substrate, 4-ketoanhydrotetracycline (Figure 4), was not available. However, tryptophan and monodechloroaminopyrrolnitrin (Figure 3*C*), the natural substrates for the chlorinating enzymes involved in pyrrolnitrin biosynthesis, were available. Whereas monodechloroaminopyrrolnitrin was shown to be chlorinated by CPO-P to aminopyrrolnitrin, which is the next metabolite in the biosynthetic pathway of pyrrolnitrin (114), tryptophan was not chlorinated by this enzyme. CPO-P also catalyzed the oxidation of aminopyrrolnitrin to pyrrolnitrin in vitro, which strongly suggested that this enzyme could be involved in pyrrolnitrin biosynthesis (54). However, the observation that bacterial nonheme haloperoxidases catalyze the formation of peracetic acid explains why CPO-P not only catalyzed halogenation reactions but also the oxidation of amino to nitro groups (54). Thus halogenation and oxidation of amino groups to nitro groups catalyzed by bacterial nonheme haloperoxidases are unspecific. Such an enzyme is not likely to be involved in the specific chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin and the oxidation of aminopyrrolnitrin to pyrrolnitrin (Figure 3) in vivo, for the chlorination and oxidation of other cell components would also occur. The unusually high K_m-values of bacterial nonheme haloperoxidases for hydrogen peroxide (1.6–30 mM) and bromide (11–69 mM) also make it very unlikely that these enzymes could be involved in the biosynthesis of halometabolites (11).

Final proof that a nonheme haloperoxidase is not involved in pyrrolnitrin biosynthesis was obtained by a gene replacement experiment (53). The cloned nonheme haloperoxidase gene (*cpoF*) from a pyrrolnitrin-producing *P. fluorescens* strain was inactivated and used to replace the chromosomal haloperoxidase gene. The resulting chloroperoxidase-negative mutant still produced pyrrolnitrin, which proved that nonheme chloroperoxidases are not involved in pyrrolnitrin biosynthesis and probably not in the formation of any other halometabolites produced by bacteria.

By complementation of a mutant blocked in the chlorination step, Dairi et al identified and cloned the gene for the chlorinating enzyme in 7-chlorotetracycline biosynthesis (18, 19). Comparison of the amino acid sequences of the halogenating enzyme obtained by Dairi et al (18) with the sequences of bacterial nonheme haloperoxidases from 7-chlorotetracycline-producing *S. aureofaciens* strains revealed no sequence homology at all. This leaves no doubt that the halogenating enzyme in 7-chlorotetracycline biosynthesis, like the one in pyrrolnitrin biosynthesis, is not a nonheme haloperoxidase.

The results of the gene replacement experiments with the heme-containing bromoperoxidase-catalase gene from *S. venezuelae* (29) and the nonheme chloroperoxidase gene from *P. fluorescens* (53) clearly show that these enzymes are not involved in the biosynthesis of halometabolites produced by these strains. Sequence comparison of the cloned gene of the halogenating enzyme of 7-chlorotetracycline biosynthesis with those of known heme-containing and nonheme-haloperoxidases reveal no homology, showing that neither heme-containing nor nonheme haloperoxidases are involved in the biosynthesis of chloramphenicol, 7-chlorotetracycline, and pyrrolnitrin. The lack of substrate specificity and regio-specificity of all haloperoxidases makes them unlikely candidates for enzymes catalyzing specific halogenation reactions in bacterial cells. However, this raises the question about the nature of the enzymes catalyzing halogenation reactions in the biosynthesis of bacterial halometabolites.

One of the most crucial steps towards the detection of the halogenating enzymes involved in the formation of halometabolites in bacteria will be the use of natural substrates and not of a substrate like monochlorodimedone (Figure 6) that has no structural similarity to the subtrates halogenated by these enzymes in vivo. Using monochlorodimedone means selection for unspecific and therefore wrong halogenating enzymes.

Another type of halogenating enzyme involved in the formation of methyl halides are S-adenosyl methionine transferases (117). The mechanism of the halogenation reaction catalyzed by these enzymes is totally different from that of haloperoxidases because the halide is not oxidized but is incorporated as halide anion. There are no reports on the production of methyl halides by bacteria or on halogenating activities of bacterial S-adenosyl methionine transferases.

HALOGENATION AND DEHALOGENATION

There has always been the question of whether enzymatic halogenation and dehalogenation reactions are reversible and whether they could be catalyzed by the same enzymes. Whereas there is quite substantial knowledge about bacterial dehalogenases (31, 49), information on bacterial halogenases is rather limited.

Several different dehalogenases have been isolated that have different mechanisms. In most of the dehalogenation reactions catalyzed by these different dehalogeneases, the halogen is substituted by oxygen or a hydroxyl group (31, 49) derived either from water (hydrolases, glutathion transferases, and hydratases) (42, 61, 64) or molecular oxygen (oxygenases) (32). In the few cases where the actual substrates for the halogenating enzymes in vivo are known, the halogen introduced into the organic subtrate by a halogenating enzyme replaces a hydrogen atom and not an oxygen atom (7, 53, 69, 106). The only dehalogenating enzymes substituting halogen for a hydrogen atom are enzymes catalyzing reductive dehalogenation reactions (111, 118). However, in these reactions no hydrogen peroxide is produced, whereas haloperoxidases, the only type of halogenating enzymes from bacteria known to date, need hydrogen peroxide for the catalysis of halogenation reactions (35).

The elucidation of the three-dimensional structure of bromoperoxidase A2 (BPO-A2) from *S. aureofaciens* ATCC 10762 revealed that this enzyme belongs to the α/β hydrolase fold enzymes (46), as does haloalkane dehalogenase from *Xanthobacter autrophicus* (109). This similarity, however, is restricted to the overall structure of these enzymes and does not extend to the reaction mechanism. Haloalkane dehalogenase has a substrate specificity and is a hydrolytic dehalogenase, whereas bromoperoxidase A2 has no substrate specificity with regard to the halogenation reaction.

The halogenating enzymes known to date do not seem to be related to dehalogenases. However, as mentioned earlier, there is strong evidence that haloperoxidases are not the halogenating enzymes involved in the biosynthesis of halometabolites. Halogenases with substrate and regio-specificity that catalyze halogenation reactions via a different reaction mechanism must exist. These halogenases, however, could be more closely related to dehalogenases.

HALOGENATING ENZYMES IN BIOTECHNOLOGY

The use of free halogen in chemical halogenation reactions results in the formation of unwanted by-products, due to unspecific halogenation. These by-products are very often toxic and difficult to degrade, and they can even lead to the formation of polychlorinated dioxins (39). Thus there is considerable interest for more specific ways to achieve the synthesis of organohalogens. Although a lot of effort was put into the search for halogenating enzymes, only haloperoxidases with a lack of specificity have been isolated. Investigations into the usefulness of these haloperoxidases as catalysts for halogenation reactions were disappointing, for enzymatic halogenation using haloperoxidases has no great advantage, if any at all, over chemical halogenation reactions using

free halogen (35, 76, 102). However, other more specific halogenases like the enzymes involved in the biosynthesis of halometabolites could be the enzymes needed for application in biotechnology.

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