Halogenation – A Versatile Tool For Drug Synthesis - The Importance Of Developing Effective And Eco-Friendly Reaction Protocols

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Abstract:

The preoccupation for discovering new therapeutic agents is constant and, in this context, the study of bromine-containing molecules is a research field of great interest. These compounds exhibit a wide range of biological activities and bromination can be considered an effective tool to enhance the potency of bioactive agents. Accordingly, it is very important to find new efficient ways for the synthesis of brominated derivatives. This review focuses on presenting several bromination techniques available at present, emphasizing the advantages and disadvantages associated with each reaction protocol, in search for the most effective ways to insert bromine atoms in different molecules, that are both eco-friendly and efficient. Currently, several bromination strategies are presented in literature, but the modern trend is oriented towards new techniques, that do not affect the environment and reduce to the minimum the consumption of harmful reagents and solvents. Traditional synthetic methods also lack specificity and regioselectivity. Biological halogenation can represent the future for obtaining the desired brominated compounds, the use of enzymes for halogenation being potentially the most effective and environmentally friendly route, but the transfer of this technology to industrial large manufacturing scale was not yet made. An improved understanding of biological halogenation and the progress in the field of enzyme engineering could lead to the development of novel synthetic methods for the creation of new compounds, involving stable and selective biocatalysts.

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1. INTRODUCTION

The preoccupation for discovering new therapeutic agents is actual and, in this context, the study of bromine-containing molecules is a research field of great interest. These compounds exhibit a wide range of biological activities: antibacterial, antifungal, antiparasitic, antiviral, antitumor, antioxidant, anti-inflammatory, many studies also showing that bromination can be an effective tool to enhance the potency of bioactive agents [1-9].

This review focuses on presenting several bromination techniques available at present, emphasizing the advantages and disadvantages associated with each reaction protocol, in search for the most effective ways to insert bromine atoms in different molecules.

The first part of the paper presents several synthetic bromination procedures (bromination with liquid bromine, oxidative bromination, bromination using quaternary ammonium tribromides and bromination using other bromine donating reagents), while the second part focuses on biotechnological halogenation using biocatalysts.

The modern trend is oriented towards new techniques, that are in accordance with the main desiderata of green chemistry: prevention of waste formation, design of less hazardous chemical syntheses, use of biocatalysts, use of safer solvents [10]. Traditional synthetic methods of inserting halogens into complex molecules are often difficult due to the lack of specificity and regioselectivity. Biotechnological halogenation can represent the perfect future solution for obtaining the desired brominated compounds, the use of enzymes for halogenation being potentially the most effective and environmentally friendly route, but, this technology is not applicable yet at industrial level, due to the large manufacturing quantities needed. An improved understanding of biological halogenation and the progress in the field of enzyme engineering could lead to the development of novel synthetic methods for the creation of new compounds, involving robust and selective biocatalysts [11]. Two classes of halogenases are

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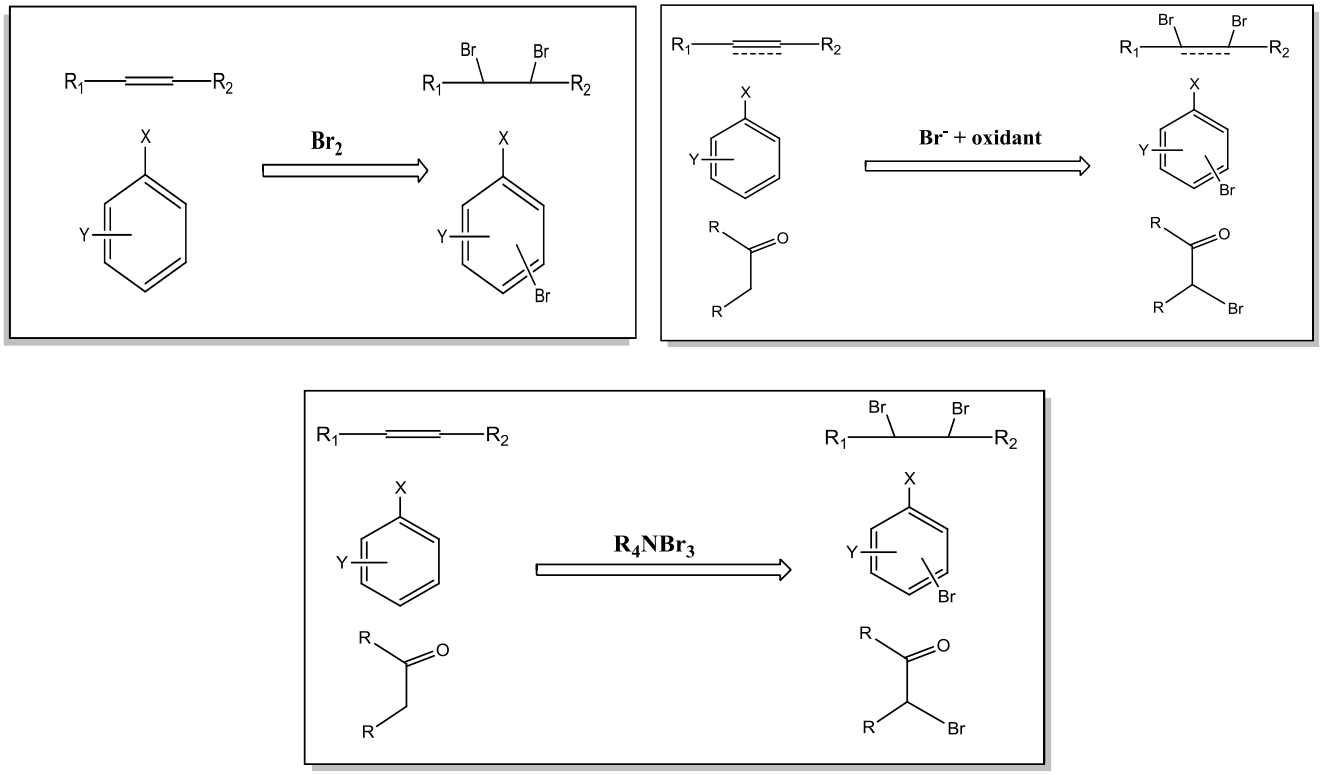

Fig. (1). Bromination protocols – examples.

described in the current review- haloperoxidases and flavin-dependent halogenases, these two groups being extensively studied by scientists so far.

2. SYNTHESIS OF BROMINATED DERIVATIVES – REACTION PROTOCOLS

Taking into account the facts that bromine-containing molecules possess a wide range of important biological actions and that bromination can be considered a very important tool in the synthesis of versatile precursors for many organic transformations into functionalized derivatives, it is very important to find new efficient ways for the synthesis of brominated derivatives.

Currently, several synthetic bromination procedures are available (Fig. **1**).

2.1. Conventional Bromination Using Elemental Bromine

This procedure is simple, but it involves the use liquid bromine as brominating reagent and chlorinated solvents, both toxic and hazardous. Furthermore, bromine is a corrosive liquid reagent that evaporates easily at standard temperature, being harmful to all body tissues, causing burns and damaging the respiratory tract by inhalation. It also requires special attention for storage and transportation [12]. The byproduct of the reaction is HBr, also pollutant and hazardous. Moreover, bromination with liquid bromine leads to only 50% bromine atom efficiency and, in the end, a complex mixture of mono-, di-, tri- and even tetrabrominated organic products is usually obtained.

Lately, several variations of this traditional technique have been developed. The efficiency of several catalysts was tested. For example, using metal oxides and zeolites as co-catalysts promoted an improvement on the reactivity of molecular bromine: ZnO/H-beta-25 was an efficient catalytic system for the regio- and chemoselective bromination of polynitro-stilbenes using molecular bromine. The reactions were conducted under mild condition, in good yields. Zeolites have several advantages: are reusable, highly stable and improve regioselectivity, due to channel morphology and surface properties [13]. Scientific data revealed that zeolites can induce high para-selectivity during the electrophilic bromination of various aromatic substrates (halobenzene, toluene). It is important to keep the pores of the zeolite particles free of absorbed water, because it was observed that selectivity dropped when the water content increased. The pores are the site where selective bromination takes place [14-17]. Para-selectivity in the bromination reaction of toluene using cation exchange Y-type zeolites was improved in the presence of propylene oxide in the medium [18]. Another catalytic system reported to be both efficient and selective in the bromination of substituted aromatic aldehydes was ceric ammonium nitrate and silica gel [19].

The aqueous AlBr3-Br2 and CaBr2−Br2 systems were successfully used to selectively brominate aromatic compounds (anilines and phenols). The brominating reagents had the advantages of being mild, efficient, cost effective and renewable. The methods did not require strong acids and organic solvents [20, 21].

Another negative aspect associated with the conventional procedure that uses elemental bromine is related to the fact that a high percentage of bromine atoms are transferred to the byproduct of the reaction – HBr. One effective solution to

resolve this problem is in situ oxidation of the resulting HBr to reactive bromine species, in the presence of an oxidant. In this way, all bromine atoms are transferred to the bromination products. For example, the bromination of alkoxy-substituted benzene and naphthalene derivatives was achieved using molecular bromine, the residual oxygen in the reaction tube acting as oxidant and 1-butyl-3-methylimidazoilium nitrate ionic liquid being both solvent and catalyst [22].

Graphene oxide is a versatile catalyst that was used to selectively and rapidly brominate aniline derivatives and phenols in an aqueous medium. The physical and thermal stability and reusability of the catalyst were also advantages of this method [23]. The best results were obtained at room temperature using molecular bromine in a protocol promoted by oxidative bromination catalyzed by graphene oxide. The bromine atom economy was 100% and the selectivity for the tribromoanilines and tribromophenols was high. The technique was also effective with N-bromosuccinimide (NBS) as brominating agent [24].

Despite these drawbacks, in some cases, comparative studies (bromine versus other reagents) have shown that reaction with bromine in different solvents was the most effective method. For example, bromination of 7-acetyl-4,6-dimethoxy-3-phenylindole with bromine in acetic acid afforded the 2-bromoindole derivatives in 95% yield, in comparison to the reaction with NBS, when a yield of 68% was obtained [25]. The synthesis of 6-bromo-3-cyanobenzo[b]thiophene (a synthetic intermediate of Raloxifen) was best achieved using bromine and DMF or acetonitrile as solvents. The purpose of using Br2 was to find a balance between efficient conversion and selectivity [26].

Polyvinylpolypyrrolidone – bromine (PVPP-Br2) is an insoluble complex, a non-volatile polymeric reagent, which retains its reactivity even after several months of storage. The complex was involved in the bromination of activated aromatic compounds (phenols and N,N-alkylated amines) with the formation of monobrominated derivatives [27]. Polymer-supported catalysts and reagents have become popular in organic synthesis over the past decades due to their high catalytic activity and stability, low toxicity, recyclability and environmentally-friendly conditions.

Another complex is hexamethylenetetramine-bromine - a non-hygroscopic, homogenous solid, very stable at room temperature, not affected by exposure to light, air or water. It proved to be suitable for the bromination of aromatic rings [28].

Although the current trend in organic synthesis is to replace liquid bromine with other less toxic reagents, the conventional method was not completely forsaken. Some scientists focused their work on overcoming some of the negative aspects associated with this procedure, by developing bromine-containing complexes or several catalytic systems.

2.2. Oxidative Bromination

This method consists in generating the brominating agent in situ starting from harmless reagents and it requires a bromine source, an oxidizing reagent and a catalyst to carry out the transformations. This procedure was inspired by the halogenations that take place in the natural environment (e.g. the bromination of organic substrates in the seabed: bromoperoxidases catalyze the oxidation of bromides in the presence of hydrogen peroxide) [29]. One disadvantage of the protocol is the low atom economy. Bromide salts are used as brominating reagents and the metal elements in bromide salts cannot be transferred to the bromination products [22]. On the other hand, oxidative halogenation can be used in reactions where only one halogen atom is incorporated into the molecule and the residual HBr is regenerated by oxidation [30].

Various oxidative halogenation methods are reported and different oxidants were used for the generation of electrophilic bromine. Some examples of oxidative bromination protocols are presented in Table **1**. Several oxidants can be used: hydrogen peroxide, molecular oxygen (oxidants of choice in the field of green chemistry) or more sophisticated oxidants (Oxone®). The catalysts are usually metal catalysts based on vanadium, molybdenum and tungsten; vanadium catalysts are usually associated with better performances [29, 31].

Oxidative bromination with HBr + H2O2 is a protocol successfully applied to both free-radical bromination and electrophilic aromatic substitution. This system of reagents has several advantages: bromine is generated in situ; a diluted aqueous solution of hydrogen peroxide is a safe and environmentally-friendly oxidizing agent, its only by-product being water; the molar mass of the H2O2 –HBr couple is lower in comparison to other brominating agents and the utilization of bromine atoms is complete, resulting in high atom economy. Moreover, this system is suitable for 'on water' reactions, avoiding the use of pollutant organic solvents. The study was performed for the benzylic bromination of toluene derivatives with an aqueous H2O2 –HBr mixture in comparison to NBS. The results indicated that aqueous H2O2 –HBr was more reactive than NBS during benzylic bromination reactions. The bromination in the benzylic position was selective, the brominated products being formed in high yields without any significant amount of dibrominated derivatives. Furthermore, an aqueous H2O2 –HBr system allowed the benzylic bromination in good yields of toluene derivatives bearing electron withdrawing groups [33].

Regioselective bromination of anilides was achieved with HBr as bromine source and Selectfluor as oxidant. According to Liang et al., Selectfluor was the best oxidant choice for selective bromination of anilides. The reaction proceeded in water, without any additives or metal catalysts. HBr has the disadvantage of being toxic and corrosive, so other bromine sources were also tested (tetrabutylammonium bromide or CuBr2). The yields were good, but the reaction times were longer. The reaction was highly regioselective: para-monobromination occurred exclusively when this position was unoccupied [34].

Another combination adopted for the oxidative bromination of various compounds was a system formed from KBr (the bromine source), an aqueous solution of H2O2, in the presence of a mineral acid and vanadium catalysts (V2O5 or

Table 1. Examples of Oxidative Bromination Protocols.

Bromine source	Oxidant Catalyst / Reaction Medium	Applications	Reference
HBr	H_2O_2	α-bromination of ketones, N-cinnamoyl-aminoacid amides; benzylic bromination	[32], [33]
	Selectfluor®	regioselective bromination of anilides, naphtol	[34]
KBr	H_2O_2 boric acid / V_2O_5	bromination of aromatic compounds (phenols, aniline derivatives)	[30], [35]
	P_2O_5	bromination of alcohols	[36]
	HNO_3 acetic anhydride	bromination of activated arenes (1,2,3-trimethoxy-5-methylbenzene)	[37]
	$KBrO_3$ poly(vinylpyridine) supported bromate	o-, p-bromination of methoxyarenes, anilines and phenols	[38]
	$NaBO_3$	bromination of benzene and derivatives and unprotected amines	[39]
	Nonanebis (peroxoic acid) acetic acid	bromination of substituted and unsubstituted aminoanthracene-9,10-diones	[40]
NaBr	$NaBrO_3$ acidic medium	monobromination of aromatic heterocyclic compounds, bromination of alkenes and alkynes, ketones α-carbon bromination and benzylic bromination of toluene derivatives	[41-44]
	$NaIO_4$ sulphuric acid	bromination of deactivated aromatic compounds	[45]
NH_4Br	Oxone®	α-bromination of ketones, hydroxyl-bromination and dibromination of olefins, bromination of aromatics	[46-48]
LiBr	Ceric ammonium nitrate	bromination of activated aromatic compounds	[49]
$ZnBr_2$	$Pb(CH_3COO)_4$	bromination of alkenes and alkynes	[50]
TBAB (Tetrabutylammonium bromide)	H_2O_2 NH_4VO_3	bromination of alkenes, alkynes and aromatic compounds	[29], [51]
1-butylpyridinium bromide	H_2O_2	bromination of 2-amino-pyridines	[52]
Tertbutylbromide	Graphite oxide	bromination of phenol	[53]

ammonium vanadate). The method has advantages (e.g. low risk reagents) and disadvantages (e.g. high costs, low productivity compared to the use of bromine) [30]. Boric acid was also used as a recyclable catalyst (via the peroxoborate formed in the reaction of boric acid and H2O2) for the regioselective bromination of aromatic compounds with very good results [35].

Another oxidant that was used in combination with KBr is phosphorus pentoxide. P2O5 is a commercially available, inexpensive reagent. The byproduct formed during the reaction (phosphorus oxoacid) is water soluble, these facts making the procedure easy and convenient [36].

The bromination of activated arenes (1,2,3-trimethoxy-5-methylbenzene) with KBr and HNO3 in acetic anhydride was accomplished. Several environmentally benign aspects were reported for this protocol: small quantities of molecular bromine and nitric acid were present throughout the whole reaction, the solvent was acceptable even for large-scale use, the oxidant was used only in a stoichiometric quantity and the reaction was performed at room temperature [37].

A simple and efficient method for selective bromination of activated aromatic compounds using potassium bromide in the presence of poly(4- vinylpyridine)- supported bromate in nonaqueous solution was reported, with good selectivity between ortho and para positions of methoxy-arenes, anilines and phenols. Poly(vinylpyridine)- supported bromate is a polymeric oxidizing reagent, stable, which can be easily regenerated [38, 54] . Aromatic compounds were also

brominated with KBr in a carbon tetrachloride – water mixture, by ultrasound irradiation. H2O and CCl4 are sonolyzed to generate radical species that oxidize Br- to produce brominating agents, which attack the aromatic ring. The reactivity of the brominating agents produced ultrasonically is similar to that of Br2 [55].

Sodium perborate is another oxidant available, a safe and convenient alternative to hydrogen peroxide that can be successfully used during oxidative brominations of various aromatic compounds. It proved to be effective for the bromination of benzene (and derivatives) and unprotected aromatic amines [39].

Nonanebis(peroxoic acid) is a solid, stable peracid that was used as oxidant for the bromination of aminoanthracene-9,10-dione, the source of bromine being KBr. Acetic acid was found to be the most effective solvent, neutralizing the KOH formed during the oxidation reaction of KBr to Br+, in the presence of nonanebis(peroxoic acid). The bromonium ion thus formed reacted with the amine to give the brominated product. The bromination reaction proceeded under mild conditions with high yield and purity [40].

Mixtures of NaBr and NaBrO3 in different ratios were successfully used for the selective monobromination of aromatic heterocyclic compounds, stereoselective bromination of alkenes and alkynes and for regioselective bromine substitution at the α-carbon of ketones and at the benzylic position of toluene derivatives. The 5:1 mole ratio of bromide to bromate was used for the preparation of dibromoderivatives from alkenes/alkynes, while the 2:1 bromide/bromate couple was suitable for substitution reactions, with high bromine atom efficiency. The reactions were conducted under mild aqueous conditions, the reactive species (HOBr) forming in situ, due to acid activation of the bromide/bromate couple. The method had the advantage of using solid reagents, stable, non-hazardous and inexpensive to prepare. It also afforded superior atom economy in comparison to other methods and the reagents were salt-based and could be easily eliminated in the aqueous waste stream [41-44].

The bromination system formed of NaBr as bromine source and NaIO4 as oxidant (in sulphuric acid medium) was effective for the oxidative bromination of deactivated aromatic compounds. NaIO4 was a good choice, being an easily accessible, cheap and non-toxic oxidant [45].

Oxone® is a potassium triple salt (2KHSO5, KHSO4, K2SO4). Potassium peroxymonosulphate is the oxidizing agent. This reagents system was successfully used for the bromination of various substrates: aromatic compounds, ketones, β-keto esters. The use of Oxone® as oxidant has several advantages in comparison to hydrogen peroxide: it has a higher onset of decomposition and liberates less energy, the reaction being performed at lower temperatures; it is also a solid reagent, more easily handled [46-48, 56].

The efficiency of vanadium catalysts was also assessed during oxidative bromination reactions of a variety of substrates. The two main vanadium catalysts used during oxidative halogenations with H2O2 were NH4VO3 and V2O5. The bromine source was usually KBr or a more complex bromine salt, for example tetrabutylammonium bromide (TBAB). Vanadium pentoxide was used very effectively for the selective bromination of aromatic compounds with tetrabutylammonium bromide, in the presence of hydrogen peroxide. The method proved to be highly selective, the reaction conditions were mild and the yields were good [51].

Mendoza et al. obtained better yields when they performed the bromination of a series of aromatics, alkenes and alkynes, using the ammonium metavanadate/hydrogen peroxide (NH4VO3/ H2O2) reagents system and tetrabutylammonium bromide (TBAB) as the source of bromine, in mild conditions at room temperature. TBAB was the best choice, being a crystalline powder, easy to handle and maintaining the desired stoichiometry with the substrate, in contrast with AlBr3, which allowed a quick and selective para-bromination, but it was difficult to handle due to its high volatility. The best yield was achieved when the reaction was carried out in a biphasic system (water – diethyl ether) and the pH control proved to be very important as well; the formation of vanadium species that promoted bromination (oxomonoperoxovanadium) increased with high acidity, but an excess of acidity also promoted polybromination of aromatic compounds.

Vanadium catalysts have also been investigated for the oxidative bromination of alkenes and alkynes. In the case of alkenes and alkynes bromination with TBAB and NH4VO3/ H2O2 /HClO4, the bromination selectivity observed for alkenes was low (bromohydrins, bromoketones or dibrominated compounds were formed), but the bromination of alkynes led mainly to the formation of the corresponding dibrominated alkenes [29].

Other vanadium-based catalysts suitable for oxidative bromination were: salts of synthetic amavidine compounds (amavidine is a natural octacoordinated vanadium (IV) complex, with peroxidase activity) or Schiff base complexes of vanadium (V). The synthetic amavidine compounds catalyzed the bromination of cyclohexane and benzene in acidic conditions, while vanadium (V)–Schiff base complexes were good catalysts for the oxidative bromination of substituted 4-penten-1-ol into β-brominated cyclic ethers. The vanadium (V)–Schiff base complexes can be covalently attached to a polymer support (polystyrene-bound vanadium complex) or encapsulated in zeolite-Y, to achieve higher stability, easier recycling and separation of the catalysts [30]. Various mechanisms of action have been proposed for the brominating agent, the nature of the halogenating species still remaining a controversial issue.

Graphite and graphene oxides were also used to catalyze the bromination of phenol with tertbutylbromide. The mechanism proposed for the reaction involved the electrophilic attack of the electron deficient species Br2Brδ+ on the molecule of phenol, to form p-bromophenol. The Br2Brδ+ cation is formed during the interaction between the formed Br2 with Br- adsorbed on the carbonyl group of the graphite oxides [53].

Oxidative bromination is probably one of the most popular alternative techniques for inserting bromine atoms into organic

Table 2. Bromination using quaternary ammonium tribromides (QATB)

Quaternary ammonium tribromide	Applications	Reference
Pyridinium tribromide	-bromination of double bonds, -bromination of 2,6,9-trisubstituted purines; -α-bromination of carbonyl compounds.	[61-63]
1-butyl-3-methylpyridinium tribromide	bromination of various anilines and phenols	[64]
Tetrabutylammonium tribromide	-bromination of activated aromatics (aniline and phenol derivatives), polycyclic hydrocarbons (anthracene and phenanthrene), -bromination of imidazole, chalcones	[29], [65]
Cetyltrimethyl ammonium tribromide	-bromination of alkenes -bromination of α,β-unsaturated carbonyl compounds	[57]
Tetramethylammonium tribromide	-bromination of aromatic compounds -bromination/desilicobromination of silylated monofluoroalkenes	[66], [67]
Benzyltriphenylphosphonium tribromide	-mild and selective reagent for the bromination of phenol derivatives -bromination of anilines	[68], [69]
Benzyltrimethylammonium tribromide	-electrophilic bromination of aromatic compounds: phenols, aromatic amines, aromatic ethers, arenes -side-chain bromination od aromatic compounds (arenes, acetophenones) -bromo-addition to unsaturated bonds	[70]
Cetylpyridinium tribromide	-bromination of activated aromatic compounds (phenols and aniline derivatives)	[71]
Tetrapropylammonium tribromide	-bromination of aniline and derivatives, phenols, -bromination of double bonded systems	[72]
1-butyl-3-methylimidazolium tribromide	-2-furfural solvent-free bromination -bromination of alkylbenzenes	[73], [74]
1,2-Dipyridinium ditribromide-ethane	-α-bromination of arylacenones -bromination of activated aromatic compounds	[59]
1-Benzyl-4-aza-1-azonia-bicyclo[2.2.2]octane Tribromide	-bromination of phenols under mild conditions -bromination of aniline derivatives	[75], [76]
Quinolinium Tribromide	regioselective monobromination of aromatic amines	[77]
N-Octylquinolinium tribromide	bromination of phenols, aromatic amines, alkenes and alkynes	[78]
1,4-bis(3-methylimidazolium)butane ditribromide	-selective bromination of anilines or phenols -α-bromination of alkanones under mild conditions	[79]

compounds. It has several advantages: it is suitable for a variety of substrates, the bromine sources are easily accessible and the oxidants are very diverse, from simple molecules like hydrogen peroxide, to more sophisticated reagents, like Oxone®.

2.3. Bromination using Quaternary Ammonium Tribromides (QATB)

Quaternary ammonium tribromides (QATBs) have gained enormous attention in recent years as versatile bromine alternatives. They are able to brominate a variety of organic substrates under rather mild conditions (Table **2**). The main advantages of OATBs are that they are crystalline, easy to handle and maintain the desired stoichiometry. Most tribromides are stable at temperatures of up to 200oC. Some compounds also have the ability to work under solvent-free conditions [57, 58].

There are many examples of QATBs currently used in chemical syntheses: pyridinium tribromide, 1-butyl-3-methylpyridinium tribromide, tetrabutylammonium tribromide, cetyltrimethyl ammonium tribromide, N-octylquinolinium tribromide. 1,2-Dipyridinium ditribromide-ethane and 1,4-Bis(3-methylimidazolium-1-yl)butane ditribromide have several extra-advantages: they require only 0,5 equivalents for complete bromination, they have a remarkable reactivity in comparison to other tribromide reagents, they are very efficient bromination reagents under solvent free condition and are recyclable [59, 60].

It is true that QATBs are appreciated because of their many advantages presented in the paragraph above, but it is important to emphasize the fact that most of the traditional methods used for their preparation involve elemental bromine; therefore, developing environmentally friendly protocols for the synthesis of QATB is essential.

2.4. Bromination Using Other Bromine Donating Reagents

N-bromosuccinimide (NBS) is a convenient source of bromine for both substitution and addition reactions. It is available and easy to handle. The reaction by-product (succinimide) can be easily removed. NBS is a highly selective free radical brominating agent. NBS is a good reagent for allylic brominations, because it provides a constant but very low concentration of bromine in the reaction medium, conditions that do not favor the addition of bromine to the double bond.

This technique has the disadvantages of low atom economy (the bromine carrier is the large molecular fragment of N-succinimide) and of the expensive brominating reagent. Efficiency may be increased by ultrasound or microwave irradiation. NBS was successfully used for the bromination of quinoxaline ring, benzylic alcohols, thiophenes and oligothiophenes, indole derivatives, allylic bromination, α-bromination of carbonyl compounds, 8-bromination of purine nucleosides and 5-bromination of pyrimidine nucleosides. It is a reagent of choice for the bromination of polyfunctional aromatic compounds. NBS provided selective monobromination of indomethacin at the benzene ring or at the methyl group in position 2, depending on the reaction conditions [80-85].

TBCA (tribromoisocyanuric acid) is a safe brominating agent, less toxic, easily synthesized. It can transfer three bromine atoms to one substrate (high atom-economy). The procedure is efficient, economical, industrially viable and eco-friendly. The by-product (cyanuric acid) is not corrosive and can be used to regenerate the reagent. TBCA is very selective for the benzylic bromination of alkylarenes (no ring bromination being observed). It was also used for the bromination of moderately deactivated arenes, the synthesis of β-bromoethers, β-bromoacetates and bromohydrins in the reaction of tribromoisocyanuric acid with alkenes in the presence of nucleophilic solvents (various alcohols, acetic acid, and H2O in acetone) and for the conversion of alcohols into alkyl bromides using tribromoisocyanuric acid and triphenylphosphine [86-88].

Other bromine donating reagents that were successfully used during bromination reactions are: 1,3-dibromo-5,5-dimethylhydantoin [89, 90], N-bromobenzamide in THF [91], TBBDA (N,N,N,N-tetrabromobenzene-1,3-disulfonamide) and PBBS [poly(N,N-dibromo-N-ethylene-benzene-1,3-disulfonamide)] [92], ZrBr4/diazine mixture [93], barium fluorobromate Ba[BrF4]2 [94-96], hexabromoacetone or ethyltribromoacetate in the presence of triphenylphosphine [97], α,α-dibromo-β-dicarbonyl compounds [98].

The subject of alternative bromination methods has concerned many research groups. Great progress was made in this field and there are many options available that can replace the conventional procedure involving liquid bromine. However, there are scientists questioning the advantages promoted by some of the new bromination techniques. They raise the discussion whether the introduction of some of the new protocols is fully justified. Many new methods introduce other hazardous substances instead of bromine: hydrogen bromide (caustic), aromatic amines – pyridine, quinoline (carcinogenic effects) to produce QATB and the solvent demand is high in many of the protocols. So, it becomes questionable whether the overall hazard potential is significantly reduced [98].

3. BIOTECHNOLOGICAL BROMINATION

It is easy to perceive from the examples presented above that most bromination methods developed so far still present many weak points in terms of selectivity, efficiency, safety and environmental issues. In search of better methods, scientists turned their attention to the natural world, were halogen containing compounds are synthesized with the help of biocatalysts – enzymes. Enzymatic halogenation is potentially the most effective and ecological route.

The evolution of science and technology offers the optimal conditions for the development of biocatalytic processes. The progress in high level expression of enzymes in heterologous systems, the improvement in fermentation technology, the advances in molecular cloning, random and directed evolution of biocatalysts and the development of immobilization techniques are the main areas of advancement that are connected to the rapid development in the field of industrial biocatalysis [99].

The aim of enzyme engineering is to design robust and selective biocatalysts. Two main engineering strategies can be identified: directed evolution and structure-guided protein engineering. Directed evolution involves selecting an enzyme that performs a certain reaction and randomly mutating the polypeptide sequence to produce variants, while structure-guided protein engineering implies a more rational approach for the selection of mutations [100].

There are several examples of enzymes used in the synthesis of pharmaceutical agents: modified recombinant phenylalanine dehydrogenase (the synthesis of Saxagliptin, a dipeptidyl peptidase-IV inhibitor), Lipolase (the synthesis of pregabalin), recombinant Escherichia coli expressing leucine dehydrogenase (the synthesis of (S)-tert-leucine, intermediate in the synthesis of protease inhibitors like Atazanavir, Boceprevir and also Telaprevir), Savinase (for synthesis of Abacavir) [99].

The strategy of biological bromination is based on carbon-halogen bonds formation using enzymes (halogenases), this method being inspired from the natural environment, where bromination of organic substrates occurs in the seabed [29]. Enzymatic halogenation can provide several advantages in comparison to chemical halogenation (it does not require hazardous substances, it can offer a good specificity and selectivity, including stereo-selectivity), but the technology transfer to industrial large manufacturing scale was not yet made, mainly because of the low stability of the enzymes [101].

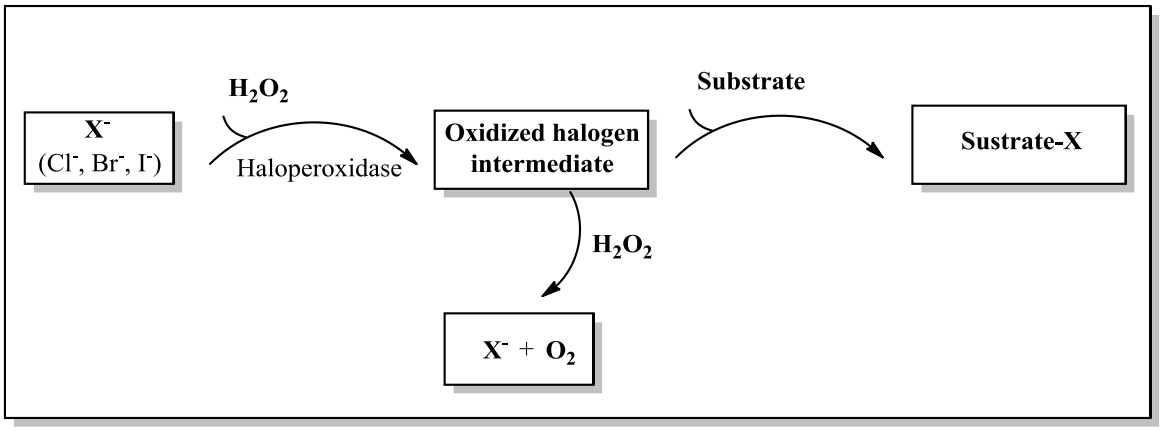

Fig. (2). Halogenation with haloperoxidases – general reactions.

The general strategy for enzymatic halogenation is to generate reactive species – X+ (hypohalite). This implies an oxidation process of the corresponding halide. The oxidant agent is hydrogen peroxide or molecular oxygen.

Halogenases are divided into four main classes: haloperoxidases (heme-dependent and vanadium-dependent), flavin-dependent halogenases, iron (II)/2-(oxo)-glutarate-dependent halogenases and fluorinases. The halogenation mechanisms involved in enzymatic processes catalyzed by halogenases are electrophilic, nucleophilic and radical [102]. This review focuses on the first two classes of halogenases – haloperoxidases and flavin-dependent halogenases.

The first pioneering papers on enzymatic oxidative bromination were puplished more than 50 years ago, when Neidleman and his team used a heme-dependent haloperoxidase (Caldariomyces fumago chloroperoxidase – CPO) to catalyze the bromination of several substrates - steroidal compounds (β-diketones, β-diketolactones), thiazoles and β-ketones. CPO is now the most extensively studied halogenating enzyme and it is currently commercially available [103]. Other commonly studied enzymes are the chloroperoxidase from Curvuluriu inuequalis (a mold) and the bromoperoxidases from Ascophyllum nodusum (a brown alga) and Corallina pilulifera (a red alga). These enzymes have the advantage of being more stable, due to the presence vanadium (V) as a prosthetic group, instead of the heme group [104].

3.1. Haloperoxidases

Haloperoxidases are a group of enzymes that use halides and hydrogen peroxide (as oxidant). They are divided into two subcategories: heme-dependent and vanadium-dependent. Heme-dependent haloperoxidases require hydrogen peroxide and halides and they usually catalyze the halogenation of aromatic substrates (Fig. **2**). There are still discussions regarding the exact nature of the halogenating species: an enzyme-bound heme-Fe (III)-O-halogen intermediate or free hypohalous acid. Larger substrates probably react with diffusible X+, because they are unable to access heme intermediates. Vanadium-dependent haloperoxidases use vanadate as cofactor, the halogenating agent being hypohalous acid or an enzyme bound V-O-halogen intermediate [11].

Relative electronegativity, nucleophilicity and size of the halides are the main parameters that influence the interactions of halogenases with a certain halogen and their selectivity. Haloperoxidases (both heme and vanadium-dependent) can catalyze chlorination, bromination and iodination as well, but none of these enzymes has the redox power to oxidize fluorine, due to its high electronegativity.

In the catalytic cycle of heme-dependent haloperoxidases, a high-valent ferryl (IV)-oxo heme cation radical species was identified. It reacts with halides to form an iron (III)-hypohalide complex, which attacks different substrates, converting them to their halogenated analogues. The degree of stereo- and regioselectivity for this complex is low.

In vanadium-dependent haloperoxidases, vanadium represents the catalytic factor, the metal acting as a Lewis acid, without changing its oxidation state [105]. This aspect is very important, because it is the reason why vanadium-dependent haloperoxidases do not suffer from oxidative inactivation during turnover [106].

Heme-dependent haloperoxidases are unstable in the presence of H2O2 and their catalytic performance is also low. Under these circumstances, the applicability and preparative value of this enzyme class is reduced. Vanadium-dependent haloperoxidases appear as more practical biocatalysts, being more robust and stable.

Vanadium-dependent haloperoxidases are classified according to the most electronegative halide that they are able to oxidize (chloroperoxidases, bromoperoxidases and iodoperoxidases). The first vanadium haloperoxidase was isolated in 1984 from Ascophyllum nodosum. Since then, several other enzymes were isolated from macroalgae, terrestrial fungi and marine bacteria and characterized [107]. A chloroperoxidase from the fungus Curvularia inaequalis was the first of the vanadium-containing enzyme to be cloned and sequenced [108].

Crystallographic studies on vanadium-dependent haloperoxidases revealed a five-coordinate trigonal bipyramidal vanadium (V) atom, the four oxygen atoms of the vanadate group being organized into a trigonal bipyramid in which three of the oxygen atoms form the equatorial plane and one oxygen occupies an axial position. The other axial position is occupied

Table 3. Brominations using haloperoxidases as biocatalysts

Enzyme	Applications	References
Chloroperoxidase, produced by *Caldariomyces fumago*	bromination of steroids: 16-ketoprogesterone, 16-keto-A-norprogesterone, 15-keto-1-dehydrotestololactone	[111]
	bromination of the thiazole ring of 2-acetoacetamido-4-methylthiazole and 2-acetamidothiazole	[112]
	synthesis of steroidal bromohydrins (the reaction of the enzyme with the isolated double bonds of 9(11)-dehydroprogesterone, pregnenolone and pregnenolone acetate)	[113]
	synthesis of α-bromoketones	[114]
	synthesis of halohydrins from alkenes	[115]
	formation of vicinal dihalogenated products from alkenes and alkynes	[116]
	halogenation of flavanones and flavones	[117]
Non-heme bromoperoxidases from *Corallina pilulifera* and *Streptomyces fumago*	bromination of anisole, 1-methoxynaphthalene and thiophene	[118]
	bromohydrins formation from cyclohexene, styrene, cinnamic acid and cinnamoyl alcohol	
Non-heme bromoperoxidase isolated from *Streptomyces aureofaciens* Tü 24	synthesis of brominated nikkomycin Z derivatives	[119]
Agrocybe aegerita peroxidase (AaP)	bromination of phenols (AaP has strong brominating and weak chlorinating activities)	[120]
Lactoperoxidase (LPO) and chloroperoxidase (CPO)	bromination of tyrosine, thyroglobulin, and bovine serum albumin (BSA)	[121]
VBrPO(*An*I)	bromination of phenols	[122]
	bromination of pyrrole derivatives (Ester-, cyano-, and carboxamide-substituted 1H-pyrroles)	[123]
Vanadium bromoperoxidase from marine red algae (*Plocamium cartilagineum*, *Laurencia pacifica*, *Corallina officinalis*)	bromination and cyclization of terpenes and terpene analogs - (E)-(+)-nerolidol	[124]
Vanadium-dependent chloroperoxidase from *Curvularia inaequalis*	transformation of alkenes into bromo- and chlorohydrins	[125]
Immobilized bromoperoxidase from *Corallina pilulifera* (*Corallinaceae*, *Rhodophyta*)	bromination of uracil	[126]

by the nitrogen of a histidine residue. Several aminoacid-residues were identified around the vanadate center and His496 and Lys353 appeared to be the most important for the enzymatic activity. This fact was emphasized during mutagenesis studies in which these residues were replaced by alanine [109].

Crystallography also allowed a comparison between the active centers (vanadate binding residues) in bromoperoxidases and chloroperoxidases, revealing that there are only a few differences: the Arg 395 and His480 fragments in bromoperoxidases are replaced with tryptophan and phenylalanine in chloroperoxidases [11]. Ohshiro et al. managed to change the halide specificity of vanadium-dependent bromoperoxidase (BPO) from the marine algae Corallina pilulifera by a single amino acid substitution. By replacing Arg 395 with tryptophan or phenylalanine, the enzyme also gained chlorination activity [110].

The observation that all chloroperoxidases also possess bromination and iodination activities raised the question whether halogen specificity is related to the selective halogen binding at the active site of the enzyme or it depends more on the hydrogen bonding capabilities and the presence of charged residues around the active site, the active site cavity providing the correct electrostatic conditions to favor the binding of a certain halogen [11].

The preoccupation for finding the most convenient ways to replace traditional catalysts with biocatalysts (enzymes) is very popular in the scientific world. In the field of biological halogenation, several studies were performed.

In Table **3** are presented several examples reported so far of successful enzymatic brominations using haloperoxidases.

One example is the bromination of phenols with NaBr, hydrogen peroxide and vanadate-dependent bromoperoxidase I [VBrPO(AnI)], enzyme extracted from the browm alga

Ascophyllum nodosum . A comparative study was performed on the bromination of phenols under different conditions: bromoperoxidase-catalyzed reaction and bromination using hypobromous acid, molecular bromine or tetrabutylammonium tribromide as reagents. The results pointed out that molecular bromine, tetrabutylammonium tribromide and the combination of sodium bromide, hydrogen peroxide and VBrPO(AnI) were able to brominate phenol in aqueous tert-butanol under mild acidic conditions, while the reaction with hypobromous acid did not have the expected outcome. Another important aspect that was observed is related to the selectivity of the reaction, the enzymatic oxidation selectively leading to the formation of monobromophenol as the major compound, while bromine and tetrabutylammonium tribromide gave tribromophenol [122].

A vanadium bromoperoxidase able to catalyze the bromination and cyclization of terpenes and terpene analogs was isolated from marine red algae (Plocamium cartilagineum, Laurencia pacifica, Corallina officinalis) and cloned. This enzyme was used in a study where the bromination of the sesquiterpene (E)-(+)-nerolidol was performed under both enzymatic and nonenzymatic conditions. Vanadium bromoperoxidase catalyzed the bromination of (E)-(+)-nerolidol, producing single diastereomers of α-, β-, and γ-snyderol (bromo-alcohols) and a mixture of diastereomers of (+)-3β-bromo-8-epicaparrapi oxide. In parallel, nonenzymatic reactions were carried out with aqueous bromine or with TBCO (2,4,4,6-tetrabromocyclohexa-2,5-dienone). The reaction with aqueous bromine produced only minimal quantities of the brominated cyclized products, while with TBCO in nitromethane produced an equal mixture of each diastereomeric product, emphasizing the diastereoselectivity of the enzymatic process [124].

In another study, the vanadium-dependent chloroperoxidase from Curvularia inaequalis was used to catalyze the synthesis of bromo- and chlorohydrins of various alkenes. The results emphasized the synthetic potential of the enzyme, but also raised a series of problems that need to be considered in order to make the reaction practical and efficient. One of the issues refers to finding the appropriate in situ H2O2 generating system [125].

The main challenge when working with haloperoxidase enzymes is their low operational stability (they are easily inactivated in the presence of H2O2 or organic solvents). Several solutions were proposed for resolving this issue: maintaining a low H2O2 concentration during the reaction (continuous addition of peroxide or in situ generation of H2O2), replacing H2O2 with tert-butylhydroperoxide, adding polymers, antioxidants or co-solvents. Immobilization of haloperoxidases on solid supports is a way to increase the stability of the enzyme and to facilitate its recovery [30]. A bromoperoxidase extracted from Corallina pilulifera (Corallinaceae, Rhodophyta) was immobilized on DEAE-Cellulofine and entrapped in the soft gels using photo-cross-linkable resin prepolymer (ENT-2000). The immobilized enzyme was tested for the transformation of uracil in 5-bromouracil, presenting a high activity and a half-life of 45 days [126].

The information collected on natural vanadium-dependent-haloperoxidases and their potential as selective biocatalysts in halogenation reactions stimulated research on the coordination chemistry of vanadium, in search of synthetic vanadium complexes that mimic the catalytic activities of natural enzymes. Maurya obtained oxidovanadium (IV) and dioxidovanadium (V) complexes and investigated their catalytic activity in the oxidative bromination reaction of salicylaldehyde. In order to improve their stability and recycle ability, the complexes were also immobilized on polymer support (chloromethylated polystyrene cross-linked with 5% divinylbenzene). The complexes presented a good catalytic potential, the bromination of salicylaldehyde leading to 5-bromo-salicylaldehyde and 3,5-dibromo-salicylaldehyde. The conversion was better in the case of polymer-supported complexes and also the selectivity towards the formation of 5-bromo-salycilaldehyde was improved [127].

Another direction of research is the synthesis of oxovanadium (IV/V) complexes with Schiff bases derived from different types of amino acids and peptides. Saha et al. obtained an oxovanadium (IV) complex with a Schiff base derived from salicylaldehyde and L-valine, which was able to efficiently catalyze in vitro bromination of olefinic alcohols, mimicking vanadium-haloperoxidases activity [128].

Beside vanadium, other metals that were used to obtain complexes acting as artificial models for haloperoxidases were molybdenum and uranium, the catalytic activity of these complexes being similar to that exhibited by vanadium based haloperoxidases [129].

Wang et al. investigated the catalytic activity of CuO nanoparticles and they established that these nanoparticles are very good haloperoxidase-mimics, catalyzing the oxidation of both chlorine and bromine ions. Nanoenzymes have several advantages: easier preparation, higher stability, they maintain their catalytic properties even under harsh conditions, they can be easily transported and stored [130].

The intense research directed towards the field of reactions catalyzed by both haloperoxidase and haloperoxidase-mimics suggests that these catalysts have the potential to replace many of the traditional bromination reagents, wherever the use of hazardous chemicals should be avoided for toxicological reasons.

3.2. Flavin -dependent halogenases

Flavin-dependent halogenases are a class of halogenases that possess in the active site a flavin-adenine-dinucleotide (FAD) moiety. They can be divided into two subcategories: flavin-dependent halogenases that catalyze halogenation of free small-molecule substrates and flavin-dependent halogenases that react with substrates tethered to a thiolation domain in a nonribosomal polypeptide synthetase system [11].

These enzymes require FADH2, molecular oxygen and halides to perform the halogenation of various organic substrates (Fig. **3**) and they also contain an FAD reductase domain, necessary to regenerate the biocatalysts [106]. Bmp5 is a phenol brominase isolated from marine bacteria Pseudoalteromonas

and Marinomonas that does not require the presence of a flavin reductase for in vitro activity. It is also unique because it catalyzes a two-step reaction, in which the initial bromination of the aromatic ring of 4-hydroxybenzoate is followed by subsequent decarboxylative-bromination [131].

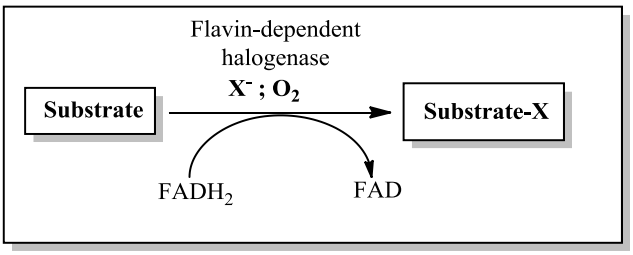

Fig. (3). General reaction catalyzed by flavin-dependent halogenase.

While haloperoxidases are usually non-selective, due to the halogenating agent, the reactions catalyzed by flavin-dependent halogenases are highly substrate specific and regioselective. Flavin-dependent halogenases also use hypohalous acid as a halogenating agent, but scientific data supports the idea that the generated hypohalous acid is not freely diffusible in the solution, being restricted, instead, to a channel that directs it towards the substrate [105]. The halogenation is selective due to the formation of a haloamine on a catalytic lysine in the enzyme active site. The reactivity of the haloamine is attenuated, being able to perform the regioselective halogenation of the substrate. In an experiment, Na36Cl was used as the chlorine source and the data suggested the formation of a covalent adduct between the protein and chlorine [102]. The lysine residue is present in the active site of all known flavin-dependent halogenases, being essential in catalysis and site-selectivity, its mutation to alanine abolishing the halogenation activity [132].

Flavin-dependent halogenases are able to catalyze chlorination and bromination reactions, but not iodination and fluorination. This fact could be explained taking into account the high electronegativity of fluorine and the large size of iodine, which affects binding at the active site [11].

The crystal structures of some bacterial L-tryptophan flavin-dependent halogenases (tryptophan-7-chlorinase PrnA, tryptophan-7-chlorinase RebH, tryptophan-5-chlorinase PyrH) were elucidated. Fungal halogenases were also discovered, the first being Rdc2 from Pochonia chlamydosporia [133].

One challenge associated with the utilization of flavin-dependent halogenases as biocatalysts is to assure sufficient flavin reductase for the production of FADH2. Two strategies were proposed to resolve this issue: the immobilization of the halogenase together with the flavin reductase in an aggregate or the genetic fusion of the two enzymes.

Flavin-dependent tryptophan halogenase RebH was successfully used for the bromination of tryptophan. The enzyme was immobilized together with other enzymes required for cofactor regeneration (a flavin reductase from Pseudomonas fluorescens BL915 and an alcohol dehydrogenase from Rhadococcus sp.) in a cross-linked enzyme aggregate. The advantages associated with this method were the stability of the aggregates (they could be reused up to 10 times and they could be stored for up to 4 months at 4ºC) and the low contamination with chloride ions that usually appears due to the unwanted chlorination [134]. Andorfer et al. obtained functional flavin-dependent halogenase (RebH and RebH variants) - flavin reductase fusions, which were used successfully for the chlorination of tryptophan and antranilic acid [135].

Another direction of research in this field is directed towards the investigation of several halogenases' ability to catalyze reactions on non-native substrates. Homology modelling can provide a guide to find the best potential substrates. There are studies confirming the activity of Rdc2 (a fungal halogenase that specifically chlorinates monocillin II in its natural environment) on non-native phenol-containing substrates. Dihydroresorcylide is halogenated (chlorinated and brominated) in the presence of Rdc2 to generate mono- and dihalogenated analogues [133]. The same research group also tested the ability of Rdc2 to chlorinate isoquinoline substrates (4-hydroxyisoquinoline and 6-hydroxyisoquinoline). The results indicated that chlorination took place at the position ortho to the hydroxyl group [136].

In Table **4** are presented examples of successful enzymatic brominations using flavin-dependent halogenases reported in literature so far.

The selectivity of RebH-catalyzed chlorination reactions was compared with that of the small-molecule chlorinating agent N-chlorosuccinimide. In many situations, the chlorination of several substrates using RebH as catalyst provided a single isomer, while, with N-chlorosuccinimide, the chlorination took place at several positions [132].

Targeted mutagenesis of several flavin-dependent halogenases has been used in the attempt to elucidate their mechanism of action or for the purpose of altering their catalytic properties. An excellent review on the subject was recently published [132].

Substrate walking approach is a form of stepwise enzyme adaptation, where a slightly different substrate was employed in each iteration of the screening compared to the preceding iteration. In this way, a series of substrates are sequential modified, linking the natural substrate through to the desired substrate.

Enzyme engineering was used to induce shifts in the site-selectivity of the halogenation reactions or to modify the substrate selectivity of natural wild enzymes. In this regard, the mutation of the phenylalanine residue in the structure of PrmA changed the site of halogenation from C7 to both C5 and C7 [100, 138]. Several RebH variants with altered selectivity were also obtained. One example is RebH variant 4V, an enzyme that is able to react with biologically active compounds that are significantly larger than L-tryptophan, its natural substrate. 3-SS is another variant, which provides high conversion of tricyclic tryptoline derivatives [139]. Another RebH mutant variant (Y455W) is capable to selectively halogenate tryptamine over tryptophan [132, 140].

Table 4. Brominations using haloperoxidases as biocatalysts

Enzyme	Applications	References
Rdc2 from *Pochonia chlamydosporia*	halogenation of non-native phenol-containing substrates (dihydroresorcylide)	[133]
	halogenation of isoquinoline substrates (4-hydroxyisoquinoline and 6-hydroxyisoquinoline)	[136]
Tryptophan halogenase RebH	halogenation of tryptophan and antranilic acid biosynthesis of Rebeccamycin halogenation of substituted naphthalenes	[100], [134], [135], [137]
Tryptophan halogenase RebH variant 4V	halogenation of pindolol, carazolol, carvedilol, yohimbine, evodiamine	[139]
Tryptophan halogenase RebH variant SS	conversion of tricyclic tryptoline derivatives	[139]
RebH variant Y455W	selective halogenation of tryptamine over tryptophan	[132], [140]

One very interesting application of flavin-dependent halogenases is to expand the diversity of natural halogenated substances, via incorporation of halogenases into different organisms, natural producers of halogenated compounds (bacteria, yeast, plants), generating new biosynthetic routes and new halogenated metabolites (introduction of tryptophan 7-halogenase PrnA into Streptomyces coeruleorubidus, integration of bacterial RebH and PyrH in the plant host Catharanthus roseus). Fräbel et al. managed to obtain new halogenated indigoid precursors in a plant-based system, by incorporating tryptophan (Trp) halogenases (PyrH from Streptomyces rugosporus, halogenating tryptophan at C5, SttH from Streptomyces toxitricini, generating 6-chlorotryptophan, as well as RebH, Trp 7-halogenase from Lechevalieria aerocolonigenes). They also established that substrate supplementation with bromide can direct the plant's metabolism towards the production of bromo-substituted indican [141].

Fraley et al. characterized other two flavin-dependent halogenases – MalA and MalA' - involved in the halogenation of malbrancheamide, an indole alkaloid; MalA' represents a new class of zinc-binding flavin-dependent halogenases. This research team also achieved to generate two unnatural bromo-chloro-malbrancheamide analogues through MalA-mediated chemoenzymatic synthesis [142].

All the scientific knowledge acquired until now reveals the exciting potential of FDHs as site-selective and environmentally-friendly halogenation catalysts [143]. So far, most of the experiments performed were focused on using flavin-dependent halogenases in chlorination reactions, but the information could be successfully applied also for bromination reactions.

The future of the industrial enzymatic halogenation implementation appears promising, providing a biotechnological alternative to chemical synthesis. Great progress was made towards solving some of the limitations associated with biocatalysts' usage (e.g. utilization of enzyme aggregates to increase stability; design of mutant enzymes), but there are still obstacles that need to be overcome (e.g. low stability of enzymes in organic solvents).

4. CONCLUSIONS

In conclusion, the field of bromine-containing substances constitutes a research area of great interest due to the importance of these derivatives as bioactive agents. Many natural or synthetic compounds have been studied so far and very interesting biological properties have been revealed. The insertion of bromine atoms in molecules usually enhances their bioactive potential, bromination becoming an important tool for the design of new drug candidates. In this context, there is a constant preoccupation for the development of new bromination techniques, eco-friendly and efficient. This review presents different strategies for the insertion of bromine atoms, stating both their advantages and disadvantages. When choosing the best technique, a researcher must consider several aspects: efficiency, selectivity, low toxicity of the reagents, mild reaction conditions.

The important progress registered in the field of biocatalysts' development determined a great expansion of the repertory of halogenating biocatalysts. In this context, enzymatic halogenation is becoming a viable alternative to achieve efficient and highly selective halogenation reactions, with a great impact on medicinal chemistry and drug-development. Enzyme engineering allowed the development of more stable and robust site-selective biocatalysts, which become suitable for industrial applicability.

CONFLICT OF INTEREST

There is no conflict of interest.

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