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LC Enantioseparations of Halogenated Compounds on Polysaccharide-based Chiral Stationary Phases: Role of Halogen Substituents in Molecular Recognition

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Review

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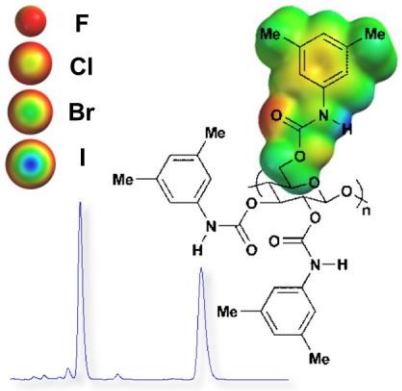

Enantioseparations of Halogenated Compounds on Polysaccharide-based Chiral Stationary Phases

ABSTRACT – Halogenated chiral molecules have become important in several fields of science, industry and society as drugs, natural compounds, agrochemicals, environmental pollutants, synthetic products, and chiral supports. Meanwhile, the perception of the halogen moiety in organic compounds and its role in recognition processes changed. Indeed, the recognition of halogen bond as an intermolecular interaction occurring when the halogen acts as a Lewis acid had a strong impact, particularly in crystal engineering and medicinal chemistry. Due to this renewed interest in the potentialities of chiral organohalogens, here we focus on selected recent applications dealing with enantioseparations of halogenated compounds on polysaccharide-based chiral stationary phases (CSPs), widely used in liquid chromatography (LC). In particular, recently, the first case of halogen bonding-driven high-performance LC (HPLC) enantioseparation was reported on a cellulose-based CSP. Along with enantioseparations performed under conventional HPLC, representative applications using supercritical fluid chromatography (SFC) are reported.

INTRODUCTION

Halogenated chiral compounds, containing fluorine, chlorine, bromine and iodine as substituents have become important in several fields of science, industry and society as drugs, natural compounds, agrochemicals, environmental pollutants, synthetic products, and chiral supports (Fig. 1).

Pharmaceutical industry produces currently several halogen-containing chiral drugs where halogen atoms play an important role in enhancing their biological activity.[1,2] In particular, halogens are able to tune the stereoelectronic properties of an organic molecular system. In this regard, F and CF_3 are widespread and important drug components in lead optimization by bioisosteric replacements.[3]

So far, more or less 5000 halogenated compounds have been identified in nature and most of them are produced by marine sponges, algae and bacteria.[1,4] A large number of these halogenated natural molecules are chiral.

In organic synthesis, halogenated compounds are popular as versatile reagents and intermediates. Halogens are more electronegative than carbon and, consequently, the C-halogen bond is polarized ($C^{\delta+}$). Apart from the fluorine derivatives which possess their own specific chemistry,[5] elimination and nucleophilic substitution are fundamental organic reactions engaging chloro-, bromo- and iododerivatives that are strictly related to the electrophilic character of halosubstituted carbon atoms. Moreover, an electrophilic halosubstituted synthon can be switched into a nucleophilic one ($C^{\delta-}$) by converting the C-halogen to a C-metal bond through metal-halogen exchange or metal insertion reactions. In this context, the discovery and development of elegant methodologies based on the use of transition metals make the cross coupling reaction between nucleophilic organometallic species and electrophilic organohalides one of the most straightforward methods for C-C bond formation.[6]

Halogen substituents can participate in intermolecular interactions and control molecular recognition processes.[7] Due to high electronegativity values, insertion of halogen atoms on a molecule can be used as a convenient strategy to tune the electronic properties by electronwithdrawing effect and,

consequently, to change or modulate the interaction capability of close sites. Moreover, halogen atoms are usually introduced on lead compounds in order to exploit their steric effects, through the ability of these atoms to occupy the full binding site of molecular targets.[2] In this regard, the incorporation of a halogen can become important for blocking metabolism. Nevertheless, it is worth noting that, in the last decades, the perception of the halogen moiety in organic compounds and its role in recognition processes changed. Indeed, for a long time, halogen atoms were merely considered as Lewis bases. In this case, the halogen atom is considered to act as an electron density donor site toward electron-deficient partners. Although oxygen and nitrogen are very important hydrogen bond acceptors (HBAs) involved in chemical and biological processes, interactions involving halogens as HBAs have also been reported.[8] Later, halogen bond (XB) has been recognized as an intermolecular interaction occurring when the halogen acts as electron density acceptor (Lewis acid) and tends to interact with electron donor partners.[9,10] Fluorine only forms XB in special cases, such as in F_2 and FCN,[9] because its electrostatic potential remains negative all around the atom, whereas heavier halogens (Cl, Br, I) prefer to form linear interactions with oxygen atoms and aromatic π systems due to the presence of the electropositive σ-hole. As a result, iodine is the strongest XB donor because the size of the σ-hole increases with the polarizability of the halogen (Cl < Br < I). This type of interaction has been observed in many different molecular contexts and it had a strong impact, particularly in crystal engineering[7] and medicinal chemistry.[11] In this last field, halogen bonds with carbonyl oxygen were found to improve the binding of several ligands to their target protein.[12] In general, halogen-carbonyl oxygen or halogen-π interactions are the most frequently observed in protein-ligand structures.

In regard to this renewed interest in the chemistry of organohalogens, the access to pure enantiomers is a permanent need both for industry and academic research. Separation of enantiomers and asymmetric synthesis represent the most important methods for producing enantiopure molecules. Depending on the research aims, liquid chromatography (LC) on chiral stationary phases (CSPs) can represent the most

convenient and straightforward method for resolving racemic mixtures of chiral compounds. LC, in fact, appears as a flexible technique because several parameters can be tuned in order to optimize the enantioseparation, which are mobile phase composition and additives, flow rate and temperature. In the last years, several applications were focused on the enantioseparation of pharmaceuticals and other bioactive compounds since biological activity differences can occur between the enantiomers of a chiral compound. Overall, chromatographic techniques are widely used to purify small amounts of target enantiomers at early development stages or to study enantioselective metabolism of a bioactive molecule. Moreover, in some instances, the availability of pure enantiomers offers the possibility to explain chemical routes that depend on the stereochemical composition of the chiral reagent (kinetic resolutions, non-linear stereochemical effects, hetero- or homocoupling reactions). Today, polysaccharide-based selectors become the most used for the LC enantioseparation of several chiral targets because of their exceptional resolving power.

In this paper, an overview of the LC enantioseparations of halogenated compounds on polysaccharide-based CSPs is presented by discussing representative recent applications concerning halogenated drugs, natural and non-natural synthetic compounds, agrochemicals, and environmental pollutants. Along with enantioseparations performed by conventional high-performance LC (HPLC) under normal-phase (NP), polar organic (PO) and reversed-phase (RP) elution conditions, applications in supercritical fluid chromatography (SFC) are enclosed. Indeed, today SFC[13] has become an important "green" technology for preparative enantioseparations exploiting carbon dioxide as the major component of the mobile phase.

A view on the role of halogen substituents on the molecular recognition mechanisms is provided.

POLYSACCHARIDE-BASED CSPs: A BRIEF DESCRIPTION

In the last decades, polysaccharide-based CSPs have been privileged for HPLC enantioseparations and the market makes currently available different chiral columns containing selectors based on cellulose and amylose derivatives (aromatic carbamates and benzoates) (Tables 1-3). In particular, the

immobilized version of these CSPs allowed to expand the range of solvents that can be used from the classic *n*-hexane (*n*-hex), *n*-heptane, methanol (MeOH), ethanol (EtOH), isopropanol (IPA), acetonitrile (ACN) and water to mobile phases (MPs) containing non-standard mid-polar solvents such as dichloromethane (DCM), methyl *t*-butyl ether (MTBE), tetrahydrofuran (THF) and ethyl acetate (EtOAc).

The first reported enantioseparation on cellulose dates from 1951[14] and since 1960s various derivatives of polysaccharides[15,16] were applied to enantioseparations but with a limited resolution ability.[17] Nevertheless, starting from 1980s, several polysaccharide carbamates and benzoates were selected, optimized and commercialized by means of an elegant work of molecular engineering.[18-24]

Indeed, native polysaccharides were not practically useful CSPs in LC due to their low enantioselectivities and mechanical properties. Starting from these results, the high versatility of the derivatized polysaccharide-based selectors was achieved just by designing a modular polymeric system (Fig. 3) where molecular, conformational and supramolecular chirality cooperate to determine the separation outcome.[25] Thus, the selectivity of the polymeric selector could be tuned by modifying the overall structure at three different levels:

a) a polysaccharide backbone. In fact, conformational chirality depends on the peculiar helical twist generated by the D-glucose residues with β-1,4 linkage in cellulose or α-1,4 linkage in the amylose polymeric chain;

b) a polar layer containing groups able to exert HBs and dipole–dipole interactions (carbamate -O–CO-NH-, benzoate -O–CO-), located inside the polymer chain;

c) a hydrophobic layer containing substituted aromatic rings (Ar), located outside the polymer chain and able to exert π– π interactions. After attempts with other atoms or groups such as MeO, Et, F, Br, CF_3 and NO_2, Me and Cl were selected as aromatic substituents and privileged as the best stereoelectronic modulators inside the polymeric system.[18] These aromatic side chains are characterized

by distinctive steric and electronic properties which are the key of the different selectivity of the corresponding CSPs (Table 4).[26] Indeed, the electronic properties of the polar layer and its ability to exert HBs can be tuned by changing type and position of both alkyl- and chlorosubstituents onto the terminal aromatic ring. As a result, the recognition ability is different for the chloro substituted CSPs (Table 3) compared to the completely alkylated one (Tables 1 and 2).

Thus, a multitude of interactions can potentially occur into the groove where both backbone and side chains contribute to form chiral cavities but, as matter of fact, only some of them act to recognize the enantiomers of a given chiral analyte depending on its particular structure, size and shape. In this context, halogens can tune the electronic properties of analytes bearing halosubstituents by electronwithdrawing effect and, consequently, the interaction capability of close sites can change. Moreover, halogens are often involved in repulsive interactions due to their size. In general, the presence of halogens onto a molecule tends to increase its hydrophobic nature. Recently, the first case of halogen bonding-driven HPLC enantioseparation has been reported on a cellulose-based CSP.[27]

DRUGS

The majority of halogenated drugs contain fluorine, followed by chlorine, while those with bromine are quite rare. Only a few iodine-containing drugs are known, mainly because C–I bonds are highly polarizable and, consequently, the iodinated compounds tend to be relatively unstable.[2] Despite the least abundant natural organohalides, fluorinated compounds are widespread and important drug component. Fluorine is the most electronegative element, thus fluorosubstituents prefer to orient toward electropositive regions of receptor sites. On this basis, fluorine can enhance binding efficacy and selectivity in bioactive compounds. Fluorine is an isosteric substitution for hydrogen and isoelectronic with -OH, so a fluorinated ligand can bind the same site as a nonfluorinated derivative, but with a different impact on the receptor-ligand affinity and selectivity. Moreover, the size of the trifluoromethyl group is similar to that of the ethyl group but the shapes of the two groups are very different.[3]

A number of fluorinated chiral compounds have been enantioseparated on polysaccharide-based CSPs.[28-31] In this regard, fluorosubstituents tend to reduce the electron density of close sites containing heteroatoms (N, O) by electronwithdrawing effect causing changes in the interaction capability of the analyte with the carbamate (or benzoate) moiety of the CSP.

In particular, several applications concern the enantioseparations of the fluorinated proton pump inhibitor drugs (PPI) such as pantoprazole and lansoprazole (Fig. 4).

Dixit and co-workers reported the enantioseparation of pantoprazole and lansoprazole on Lux Cellulose-2 (250 × 4.6 mm, 5 μm) using NP eluents.[32] Fluorine as electronegative substituent affects chromatographic behaviour by lessening the basicity of the close sites. Indeed: a) retention of pantoprazole and lansoprazole is shorter than omeprazole and rabeprazole; b) there is no effect of diethylamine (DEA) as basic additive on the enantioseparation of lansoprazole and pantoprazole (Table 5). In this study, baseline enantioseparations was achieved also under PO elution conditions by using pure ACN for lansoprazole (1 ml/min, separation factor (α) = 1.71, resolution (R_s) = 1.81) and pure IPA for pantoprazole (0.7 ml/min, α = 1.18, R_s = 1.71).

Cirilli reported the enantioseparation of pantoprazole and lansoprazole by using two immobilized columns, namely Chiralpak ID-3 and IE-3 (100 × 4.6 mm, 3 μm), under PO elution conditions (Table 6). Moreover, the authors highlighted that the retention properties of the PPIs can be modulated in a dual way (hydrophilic interaction liquid chromatography (HILIC)/RPLC) by means of suitable use of water content in pure ACN or ACN-rich organic mixture.[33]

The enantioseparation of lansoprazole was studied also on Chiralpak IA, IB, IC, ID, IE, and IF using CO_2-based eluents containing non-standard polar modifiers.[34] As reported by DaSilva and co-workers, lansoprazole was baseline resolved on all six columns using mixtures of non-conventional polar modifiers such as DCM, methyl THF, MTBE with MeOH.

Recently, non-standard MPs were used by Hoveyda for the HPLC enantioseparations of a series of novel F-substituted antagonists to the human neurokinin-3-receptor studied for the treatment of sex-hormone disorders by using four immobilized columns, namely Chiralpak IA, IB, IC and ID (250 x 4.6 mm, 5 μm) (Fig. 5).[35]

The enantioseparation of chlorinated drugs[36-38] is of interest because, along with fluorine, the chlorine atom is also incorporated in several biologically active compounds. Indeed, it is considered to be isosteric and isolipophilic with the methyl group, able to alter the metabolism and effective in inhibiting metabolic oxidation.

Chlorinated benzothiadiazine-1,1-dioxide derivatives proved to be interesting for the treatment of neurodegenerative disorders. In this context, on-column stopped flow multidimensional HPLC and dynamic HPLC were applied to investigate the influence of substituents at the benzothiadiazine 1,1-dioxide derivatives on hydrolysis and enantiomerization rate constants. With this aim, the enantioseparation of a number of analogues of the series **7** (Fig. 6) was performed by Cannazza on Chiralcel OD-RH (150 × 4.6 mm, 5 μm) by using water/ACN 60:40 as mobile phase.[39,40]

The structurally related benzothiazine-1,1-dioxide derivatives **8** are decorated with fluorine or trifluoromethyl as substituents instead of chlorine. These compounds are effective aldose reductase inhibitors and potential drugs for various diabetic complications. The synthesis of enantiomerically enriched esters and acids of the series **8** was reported by Zhu and the enantiomeric purity measured by elution on Chiralpak ID under NP elution conditions.[41] The comparison of the elution times of some derivatives **8** (Table 7) proves that the chromatographic behaviour is affected by the stereoelectronic properties of the fluorine substitution.

Amlodipine is a chlorinated calcium-channel antagonist, commercially available as a racemic mixture. Nevertheless the (*S*)-(-)-isomer is more tolerable and 1000 times more potent than the (*R*)-(+)-isomer. In this case, the development of the pure enantiomers of the drug marketed as racemate (*chiral*

switch) is considered. In this context, Doussou reported an effective LC method for the determination of (*R*)-amlodipine in a pharmaceutical formulation of (*S*)-amlodipine (R_s = 4.1) by using Sepapak-4 (250 × 4.6 mm, cellulose tris(4-chloro-3-methylphenylcarbamate) as chiral column with ACN/0.1% DEA/0.1% formic acid (FA) as MP.[42] Under this elution conditions, the (*R*)-amlodipine was the first eluting peak. Chankvetadze's group studied the enantioseparation of amlodipine on five different CSPs, namely Lux Cellulose-1, Lux Cellulose-2, Lux Cellulose-3, Lux Cellulose-4, and Lux Amylose-2 (250 × 4.6, 3 o 5 μm) with various NP, PO and RP eluents.[43] In this study, the retention of amlodipine was significantly long in *n*-hexane/IPA based eluents. In any case, the enantiomers were resolved by using *n*-hexane/IPA/DEA 90:10:0.1 on Lux Cellulose-4 better than Lux Cellulose-2. These columns proved to be effective for amlodipine enantioseparation also under PO elution mode (Table 8). In the same study, enantioseparation of amlodipine was performed on Lux Cellulose-4 with aqueous MPs. Depending on the water content, the separation system behaved both HILIC-like and RP-like.

In general, RP separations offer several advantages for bioanalytical applications and chiral LC-MS, namely compatibility with biological matrices, facilitated sample preparation from physiological liquids, and suitable interface with mass spectrometry. It is worth noting that hydrogen bonding interactions are considered very important for enantioselective recognition on the polysaccharide-based CSPs. For this reason, at the beginning the RP elution mode was not privileged. However, later the usefulness of this elution mode was reconsidered.

The studies on the enantioseparation of series of halogenated compounds which differ only by the nature of the halogen atom (F, Cl, Br) are very interesting in order to understand the role of the halogenated substitution on chromatographic behaviour. Pittman reported the enantioseparations of a series of 4-haloaryl-1,4-dihydropyridines using Chiralpak AD-H with *n*-hexane-containing MPs.[44] Caccamese reported the direct HPLC enantioseparation of venlafaxine and eleven analogues, among which the halogenated β-aminoalcohols **9**-**11** (Table 9).[45] Venlafaxine is a second-generation

antidepressant drug marketed as a racemic mixture. As in case of amlodipine, venlafaxine appears a good candidate for chiral switch. Indeed the (*R*)-enantiomer exhibits dual presynaptic inhibition of serotonin and noradrenaline uptake, whereas the (*S*)-enantiomer is a serotonine reuptake inhibitor. The best enantioseparation of these molecules were achieved on Chiralpak AD (250 × 4.6 mm) with EtOH as alcoholic modifier in a hexanic MP made basic by DEA. Moreover taking into account that an acidic additive forms an ion pair with the amine group, and that the complex is reported to have more effective interactions with the chiral groove of the amylose-based CSP, some enantioseparations were performed by using IPA as alcoholic modifier and TFA as additive but poor resolutions were obtained. In general, the nature of the substituent on the phenyl ring seems to play a role on retention and selectivity. As shown in Table 9, bromine onto the aromatic ring have a beneficial effect on selectivity compared to fluorine under basic NP elution conditions.

Péter reported the enantioseparation of 19 β-lactams on AmyCoat and CelluCoat (150 × 4.6 mm, 5 μm) using NP elution mode.[46] Among them, the halogenated series **13-15** was considered (Table 10). β-lactams showed to be lethal inhibitors of the growth of the cell walls of pathogenic bacteria. Interestingly, the authors noted that at constant MP composition, retention factor increased in the sequence F < Cl < Br. They claimed that the polar interaction between the CSP and the molecule increases when fluorine was substituted by chlorine or bromine resulting in larger retention factor (*k*). Moreover, the larger size of the analyte may contribute to the retention by the increased steric effect (bulkiness).

Recently, Welch and co-workers described chromatographic method development screening systems for the separation of halogen-containing pharmaceuticals from associated isomers or dehalogenation impurities. Four chromatographic method development platforms were used among which chiral SFC on twelve polysaccharide-based columns (150 x 4.6 mm, 3 μm) (mobile phases: 25 mM isobutylamine in ROH/CO_2).[47]

In summary, chiral separations have a key role in drug discovery and development because enantiomers of drugs are often characterized by different pharmacological and toxicological properties. Meanwhile, the full comprehension of the chiral discrimination phenomenon on polysaccharide-based CSPs is a still open issue. Thus, in the last years, a large number of concerning separation (screening and optimizations steps) and chemiometric strategies for enantioseparation of pharmaceuticals on polysaccharide-based CSPs in NP,[48] PO,[49,50] RP,[51] multimodal elution mode[52] and SFC[53,54] has been reported. Several applications focused on marketed halogenated pharmaceuticals (Fig. 7).

NATURAL COMPOUNDS

Since the discovery that halogenation is a real event in nature, almost 5000 halogenated compounds have been identified and most of them are derived from the marine environment.[4] Among the halometabolites, chlorometabolites and bromometabolites (51 and 45 percent, respectively) are predominant, while organoiodines and organofluorines (both 2 percent) are much less common.[1]

The 5,5'-dichloro-1,1'-dimethyl-3,3',4,4'-tetrabromo-2,2'-bipyrrole (DBP-Br_4Cl_2, BC-10) (Fig. 8) is the most frequently described congener of the hexahalogenated 1,1'-dimethyl-2,2'-bipyrroles (HDBPs), a group of marine natural products that have been detected in environmental samples from all over the world. This compound is axially chiral and forms stable atropisomers by hindered rotation about the 2,2' bond. Although natural producers of HDBPs have not been identified, the structurally related hexabromo-2,2'-bipyrrole is known to be produced by the marine bacterium Chromobacterium sp..

The atropisomers of DBP-Br_4Cl_2, BC-10 were separated within 30 min by Vetter on the Nucleocel Delta (250 × 4.6 mm, 5 μm) in the NP mode with a mixture of *n*-hexane/IPA 95:5 as MP.[55] In this case, the hydrophobic nature of the polyhalogenated pattern as well as the symmetric substitutions around the 2,2'-axis reduce the polarity of this compound and a low flow rate (0.3 ml/min) is required in order to achieve baseline separation. In a subsequent study, the same group performed the enantioseparation of the synthetic analogue 2-bromo-3,3',4,4',5,5'-hexachloro-1-methyl-1,2'-bipyrrole on the Nucleocel Delta by using *n*-hexane 100% (0.3 ml/min, elution time 40 min).[56]

Today, enantioselective studies of chiral organohalogen compounds, both natural and xenobiotic, represent an innovative research field in environmental analysis, and may contribute to understand their environmental fate.

AGROCHEMICALS

In the past three decades, a significant increase of halogenated active ingredients in the field of modern crop protection research and development was observed. Interestingly, there has been a remarkable rise in the number of commercial products containing "mixed" halogens. Also in this case, generally, iodine-containing compounds are in the minority, and some of them are "mixed" with other halogens like bromine or chlorine.

Among the frequently used agrochemicals, several are chiral. In most cases, these chiral products are manufactured and employed in racemic form with an evident environmental risk. Indeed, the two enantiomers may have different bioactivity, toxicity, metabolism and degradation in the environment. Consequently, the enantioselective analysis becomes important in order to define the environmental safety of chiral agrochemicals. Moreover, the enantioseparations of chiral agrochemicals are important for avoiding or reducing the adverse effects of agrochemicals on the environment and for leading to the production of enantiopure products.

Diclofop-methyl (DM) is a widely used herbicide of the aryloxyphenoxy propionate (AOPP) class. Its hydrolysate form (DC) also has herbicidal activity. Moreover, both compounds are suspected to be carcinogens and in particular the (*S*)-enantiomers present a stronger environmental toxicity than the (*R*)-enantiomers. In this context, Zhou reported the simultaneous HPLC chiral enantioseparation of DM and DC by using Chiralpak IC (250 x 4.6 mm). The best resolutions were achieved with *n*-hexane/IPA/TFA 96:4:0.1 (1 ml/min) with $R_s > 2$.[57] The two pairs of enantiomers could be eluted in about 10 min.

Recently, Lin studied the enantiomeric separations of ten chiral arylphenoxypropionate herbicides (Table 11) on Sino-Chiral OJ, Chiralcel OD-H, Chiralpak IB and Chiralpak AD-H (250 × 4.6 mm, 5 μm) by SFC.[58] Supercritical CO_2, modified with MeOH, EtOH or IPA was used as MP (2 ml/min).

Among the selected herbicides, clodinafop-propargyl and haloxyfop-methyl were not enantioseparated. The authors discussed about the recognition mechanism and they recognized to the halogen substituents a double role: a) both the halogen atoms (F, Cl) and C=O groups can interact with the –NH of the CSP by hydrogen bonding; b) the stereoelectronic properties of the halogens (electronwithdrawing inductive effect) can influence close sites.

The nitrogen-heterocyclic fungicides are important agrochemicals that get a wide application due to their excellent antifungal activity and a relatively low resistance risk. Nitrogen-heterocyclic fungicides usually consist of imidazole, hydroxy (keto) group, and substituted benzyl. Most of them have one or two stereogenic centers in molecular structure and consequently are formed by one or two pairs of enantiomers, which can have big different bioactivity and toxicity. In this regard, Qui performed the stereoselective separations of 11 nitrogen-heterocyclic chiral fungicides including the nine halogenated simeconazole, diclobutrazol, nuarimol, carfentrazone-ethyl, cyproconazole, etaconazole, metconazole, bromuconazole, and fenbuconazole by RP-HPLC on Lux Amylose-2 as chiral column (250 × 4.6 mm, 5 μm) (Table 12).[59]

It is worth mentioning that diclobutrazol, cyproconazole, etaconazole, and metconazole were separated as two peaks, although they have four stereoisomers and only bromuconazole got full separation. In this regard, the bromine atom close to the stereogenic centre might have a role in the chiral discrimination of the four stereoisomers. The same series of fungicides was enantioseparated also on Lux Cellulose-1 under RP elution conditions.[60]

The pyrethroids are synthetic pesticides obtained from the modification of natural pyrethrins in order to improve their biological activity and stability. Therefore, the introduction of halogen atoms was also considered as a structural modification (Fig. 10). Several pyrethroids including the halogenated ones have been enantioseparated on polysaccharide-based CSPs by HPLC. This field has been reviewed recently.[61]

POLYCHLORINATED BIPHENYLS AND DERIVATIVES

Polychlorinated biphenyls (PCBs) are a class of highly stable chlorosubstituted biphenyls.[62,63] PCBs have been commercially available since 1929 but they were banned in most countries since 1970 due their high persistence, toxicity and bioaccumulation. Nevertheless, they are legacy pollutants and currently measurable levels of PCBs can be found in the environment. According to the number and the position of the chloro substituents onto the biphenyl scaffold, 209 PCB congeners were identified. Among these, only 19 contain three or four chlorine atoms in the *ortho* positions and present stable atropisomers at room temperature (Fig. 11).

Biological macromolecules can interact enantioselectively with PCBs and chiral PCBs in environmental biota have been found enantiomerically enriched, indicating the existence of stereoselective biodegradation/accumulation. Consequently, the enantiomeric analysis of chiral PCBs is mandatory in order to get information about the degradation or accumulation pattern of each atropisomers. Thus, due to the need to determine the enantiomeric fraction of chiral PCBs in environmental samples, in the last decades the analytical enantioseparation of PCB atropisomers have been extensively investigated by means of different chromatographic techniques. In this field, GC has been generally privileged because it is an easier and more efficient technique for analysis of low-polar analytes. Nevertheless, recently, interesting LC enantioseparations on polysaccharide-based CSPs has been reported by using the SFC technique. In this regard, chiral column containing cellulose tris(4-methylbenzoate) as chiral selectors furnished the best results. Lin reported the baseline enantioseparation of 17 out of 18 chiral PCBs (Table 13) using Sino-Chiral OJ, Chiralcel OD and Chiralpak IB (250 × 4.6 mm) as chiral columns by SCF.[64] In this case, the preliminary screening was performed with a mobile phase of 100% CO_2 at a flow rate of 2.0 ml/min at column temperature of 36 °C. As reported in Table 14, some separations were optimized by adding alcoholic modifiers to the mobile phase.

Recently, the absolute configuration of the atropisomers of PCB 183, 171 and 132 were determined after HPLC enantioseparation on Chiralcel OJ-H (150 × 4.6 mm) by using *n*-hexane as MP (0.5 ml/min).[65]

Methylsulfonyl polychlorinated biphenyls ($MeSO_2$-CBs) (Fig. 11) are terminal metabolites of PCBs. As the PBCs, they are persistent, hydrophobic and lipophilic and tend to be accumulated in tissues. Wong and co-workers reported the enantioseparation of nine congeners on Chiralpak AD-H under NP elution conditions.[66]

In general, the introduction of a carbon-halogen bond in a molecule can have important effects on its chemical and biological properties such as increased thermal and oxidative stability, biological membrane permeability, binding efficacy and selectivity. Unfortunately, the disadvantage is that organohalogens tend to be degraded with great difficulty and, consequently, they are persistent in the environment. Obviously, this problem concerns not only polychlorinated biphenyls but also other agrochemicals or pharmaceuticals such as compounds previously discussed.

NON-NATURAL SYNTHETIC INTERMEDIATES AND PRODUCTS

More than other chromatographic techniques, HPLC on CSPs has been shown to be a useful tool to separate and characterize new chiral compounds. Indeed, enantioselective analysis can assume a key role in the development of new asymmetric reactions in order to explain stereochemical phenomena or mechanisms.

In the last years, chiral molecules characterized by axial-, planar-, or helical chirality, have been widely used in asymmetric synthesis and catalysis due to their enantioselection ability.[67-69] In particular, due to the versatility of the carbon-halogen bond, atropisomeric compounds decorated with halogen atoms as substituents have attracted considerable attention in organic chemistry.

Recently, compounds **17** and **18** were prepared by the molybdenum-catalyzed ring closing metathesis (RCM) of acyclic precursors **16a**,**b** characterized by two 3-butenyl substituents at both sp^3 nitrogen atoms of the bisimidazole scaffold. The RCM reactions furnished **17** and **18** where the *E*-alkenylene

388 moiety bridging the two heteroaromatic rings inhibits the rotation about the carbon-carbon bond.[70] Thus,
389 as reported in Fig. 12, the enantiomeric purity of the products could be measured by HPLC on Chiralpak
390 IC (250 ×4.6 mm). Moreover, the olefinic moiety was transformed into the corresponding epoxide and
391 in this case the enantiomeric purity of the product was measured by HPLC on Chiralpak IF.

392 Atropisomeric 4,4'-bipyridines can function as chiral multifunctional linkers in the preparation of
393 homochiral metal-organic frameworks (MOFs).[71] In this regard, two series of new atropisomeric
394 polyhalogenated 4,4'-bipyridines (Fig. 13) have been synthesized as racemates.[72,73] In both cases, the
395 chemistry of carbon-halogen bond represented a pivotal tool to access to the designed structures through
396 a sequence of a) dimerization via lithiation of halogen-activated pyridine ring,[74] b) halogen-selective
397 cross-coupling reaction.[72,73] The chemistry of atropisomeric 4,4'-bipyridines is in its infancy and
398 therefore, no asymmetric synthesis to produce enantiopure 4,4'-bipyridines is available. When
399 asymmetric procedures are not available due to the novelty of a research field, enantiopure compounds
400 can be obtained only by resolving racemic mixtures of synthesized compounds. On this basis, the
401 combined use of Chiralpak AD-H and Lux Cellulose-2 allowed to achieve baseline (R_s > 1.5)
402 enantioseparations of all 4,4'-bipyridines **20**-**25** under NP and PO elution mode.[26] Chiralpak IA proved
403 to be more versatile for all analytes **20**-**26** compared to Chiralpak IC.[75] Both column showed
404 complementary enantioseparation ability toward 4,4'-bipyridines **27**-**32** under multimodal elution
405 conditions.[76] Optimized enantioseparation conditions for 4,4'-bipyridines **20**-**32** are summarized in
406 Table 15.

407 Bipyridyl is a poor π-electron system and scarcely accessible due to the *ortho–ortho*' steric hindrance
408 around the chiral axis. The two nitrogens are poor HB acceptor due to the electronwithdrawing effect
409 exerted by the halogen substituents. Consequently, retention times of 4,4'-bipyridine **21** by eluting with
410 *n*-hexane/IPA 90:10 on Chiralcel OD-H are shorter than those of bromosubstituted biphenyl **34**[77] under
411 similar elution conditions. Moreover, the introduction of a halogen in the *meta* position, by respect to
412 the chiral axis, decreases retention in both systems (**33** vs **21** and **35** vs **34**) (Fig. 14).

Atropisomerism and axial chirality can be evidenced also for unexpected cases such as 1,3-butadienes. The atropisomerism of 1,3-butadienes are due to the hindered rotation around the central C^2-C^3 bond. Three chiral halogenodienes iodo- (**36**), bromo- (**37**) and chlorosubstituted (**38**) were enantioseparated by Roussel on three polysaccharide-based columns, namely Chiralpak IA, IB, and IC (250 × 4.6 mm).[78] The introduction of bromine or iodine onto the 1,3-butadiene core have a beneficial effect on enantioseparation compared to chlorine (Table 16).

In some studies, samples of enantiopure compounds are required to perform *in vitro* or *in vivo* biological assays. In this case, HPLC enantioseparation on CSP allows to access to the needed amounts quickly, whereas asymmetric synthesis can require time-consuming and too expensive procedures.

In this regard, a novel series of mitiglinide derivatives (Fig. 15) were synthesized and their binding affinity for the sulfonylurea receptor 1 of pancreatic islets were evaluated by inhibition studies.[79] Indeed, measuring changes in β-cell mass *in vivo* during progression of diabetes mellitus is important for understanding the pathogenesis, facilitating early diagnosis and developing novel therapeutics for this disease. (+)-(*S*)-*o*-FMIT showed high affinity and it was obtained by radiofluorination of the racemic precursor **36**, hydrolysis and enantioseparation by chiral HPLC using Chiralpak AY-H as chiral column with ACN/MeOH/TFA 95:5:0.1 as MP (2 ml/min, t_1 = 22.9 min; t_2 = 46.4).

As the previous sections illustrated, halogens play an important role in the development of compounds of interest in applicative fields for several decades and the presence of halogen atoms can enhance the potentialities of a compounds in terms of chemical stability, binding activity and selectivity.

Nevertheless, in spite of recently recognized potentialities of halogen bonds to contribute to different types of ligand-receptor binding, the number of compounds containing heavy halides, particularly iodine, in applicative fields is very low.[80] In this regard, the availability of bromo or iodo-containing molecules as test probes could make possible to study important XB-based interactions such as highly directed iodine-driven interactions.

Recently, a family of atropisomeric iodinated 4,4'-bipyridines (Fig. 16) has been synthesized. In this case, iodine could be incorporated into the 4,4'-bipyridine scaffold by a) dimerization of preformed iodopyridine (**37** and **38**),[74] b) deprotolithiation and electrophilic trapping (**39**), c) deprotolithiation, lithium-halogen exchange reaction and electrophilic trapping (**40**).[73] The chromatographic behaviour of this class of 4,4'-bipyridines on polysaccharide-based CSPs was investigated and the enantioseparation of **40** on Lux Cellulose-1 with *n*-hexane/IPA 90:10 as MP could be recognized as the first reported halogen bonding-driven enantioseparation.[27]

Thus, currently, the 4,4'-bipyridine scaffold presents a great potential as starting material for designing and synthesizing a new generation of iodinated test probes to study iodine-driven XB interactions on polysaccharide-based CSPs, in HPLC environment. In particular, 4,4'-bipyridyl is a semirigid poor π-electron system, thus it has suitable stereoelectronic properties to incorporate halosubstituents and tune their strength as XB donor. Indeed, a halogen's σ-hole tends to become more positive with increasing size of the halogen (Cl < Br < I) and, in the meantime, changing neighbouring atoms or groups can dramatically affect the strengths of the XBs.[7] Moreover, the depth of the σ-hole on the halogen atom and thus the strength of the halogen bonds increases as the electronwithdrawing nature of the atom, or moiety, bound to a given halogen increases following the order $Csp{-}X > Csp^2{-}X > Csp^3{-}X$.[81] It is worth noting that halogen-O,N interactions were observed in crystal structures of polyhalogenated 4,4'-bipyridines and pyridones.[73,74]

CONCLUDING REMARKS

Halogens are extremely versatile atoms able to drive and control several chemical and biological properties of molecules. For a long time, compounds bearing heavy halogens such as bromine and iodine as substituents have been used mainly as synthetic intermediates due to their stereoelectronic properties (size, polarizability), whereas they were not successful in the market. A few applications of brominated and iodinated compounds emerged in several applicative fields such as medicinal or

agrochemical chemistry compared to the widespread exploitation of fluorinated and chlorinated compounds. Nevertheless, nowadays theoretical studies have fully explained the electrophilic nature of halogen in terms of anisotropic distribution of the electron density around the atoms and along with hydrogen bond, π-π, and dipole-dipole interactions, halogen bonds became an important tool for understanding molecular recognition processes. Recently, a study on the enantioseparation of iodinated atropisomeric compounds proved that halogen bonds can be active also in HPLC environment. In this regard, cellulose tris(3,5-dimethylphenylcarbamate) as CSP appear suitable for the purpose because heavier halogens can form linear interactions with oxygen atoms (C=O) and aromatic π systems as XB acceptors. Moreover, despite the involvement of fluorine as XB donor is a rather rare event, this type of interaction has been recently claimed to explain the retention behaviour of a fluorinated compound analyzed in SFC.[47]

Halogen bond may be considered as a tool for recognition also on different type of selectors. Indeed, a re-examination of some seminal studies of Pirkle[82,83] on the well-known chiral column Whelk-O1 highlighted an unexpected halogen effect on the enantioseparation of halogenated amide derivatives of 1-phenylethylamine[82] (Fig. 17) and 5-methyl-5-phenylhydantoins[83] (Table 17).

At that time, it was described that halogen substituents on benzenoid rings used as π-basic sites by π-acidic chiral stationary phases have been reported to reduce retention and separation factors.[84] Indeed, *it seemed plausible that an electron withdrawing substituent might reduce the π-basicity of an aromatic ring and reduce retention and enantioselectivity when that ring is involved in a π-π interaction essential to the chiral recognition process*.[82] Consequently, in his paper entitled "*The enhancement of enantioselectivity by halogen substituent*",[82] the author said about the strangeness of the observed halogen effect (Fig. 17): *Unexpectedly, para and meta halogen substituents increase both retention and enantioselectivity when nonaqueous organic mobile phases are used. The more polarizable the halogen, the greater the effect*. Next, he argued for a similar behavior observed in the enantioseparation of

hydantoins derivatives (Table 17): *Note that contrary to ones intuition, the electronegative halogen substituents increase enantioselectivity, the effects being greater for the more polarizable halogens […] an understanding of the principle by which halogen substituents give rise to enhanced levels of enantioselectivity can profitably be used in the design of chiral selectors and chiral catalysts*.[83]

It could be acceptable to read in those words a description of that we define today as halogen bond.

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FIGURE CAPTIONS

Fig. 1. Halogen-containing chiral compounds.

Fig. 2. General description of halogen bond (XB).

Fig. 3. Modular nature of derivatized polysaccharide-type selectors.

Fig. 4. Proton pump inhibitors (PPI) drugs.

Fig. 5. Novel antagonists to the human neurokinin-3 receptor: enantioseparation conditions.

Fig. 6. Chlorinated benzothiadiazine-1,1-dioxide.

Fig. 7. Representative marketed halogenated pharmaceuticals.

Fig. 8. Natural polyhalogenated 2,2'-bipyrroles.

Fig. 9. Molecular structures of chiral arylphenoxypropionate herbicides.

Fig. 10. Pyrethrins and pyretroids.

Fig. 11. Absolute configurations of PCB-183 atropisomers and some $MeSO_2$-CB congeners.

Fig. 12. Macrocyclic heterobiaryl derivatives.

Fig. 13. Chiral polyhalogenated 4,4'-bipyridines.

Fig. 14. Enantioseparation conditions of bromosubstituted atropisomeric compounds.

Fig. 15. Structure of ^{18}F-labeled mitiglinide derivative FMIT.

Fig. 16. Enantioseparation parameters of iodinated atropisomeric 4,4'-bipyridines on cellulose tris(3,5-dimethylphenylcarbamate) under NP elution mode.

Fig. 17. Enantioseparation of halogenated amide derivatives of 1-phenylethylamine on Whelk-O 1.