Natural production of fluorinated compounds and biotechnological prospects of the fluorinase enzyme

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ABSTRACT

Fluorinated compounds are finding increasing uses in several applications. They are employed in almost all areas of modern society. These compounds are all produced by chemical synthesis and their abundance highly contrasts with fluorinated molecules of natural origin. To date, only some plants and a handful of actinomycetes species are known to produce a small number of fluorinated compounds that include fluoroacetate (FA), some ω-fluorinated fatty acids, nucleocidin, 4-fluorothreonine (4-FT), and the more recently identified (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid. This largely differs from other naturally produced halogenated compounds, which totals more than 5000. The mechanisms underlying biological fluorination have been uncovered after discovering the first actinomycete species, *Streptomyces cattleya*, that is capable of producing FA and 4-FT, and a fluorinase has been identified as the enzyme responsible for the formation of the C–F bond. The discovery of this enzyme has opened new perspectives for the biotechnological production of fluorinated compounds and many advancements have been achieved in its application mainly as a biocatalyst for the synthesis of [^{18}F]-labeled radiotracers for medical imaging. Natural fluorinated compounds may also be derived from abiogenic sources, such as volcanoes and rocks, though their concentrations and production mechanisms are not well known. This review provides an outlook of what is currently known about fluorinated compounds with natural origin. The paucity of these compounds and the biological mechanisms responsible for their production are addressed. Due to its relevance, special emphasis is given to the discovery, characterization and biotechnological potential of the unique fluorinase enzyme.

KEYWORDS Natural fluorinated compounds; fluoroacetate; 4-fluorothreonine; nucleoci-din; fluorinated fatty acids; *Dichapetalum*; fluorinase; *S*-adenosylmethionine; *Streptomyces cattleya*; *Streptomyces calvum*

Introduction

The chemistry of fluorinated compounds is essentially man-made chemistry. The unique properties of the fluorine atom led to the generation of a myriad of fluorinated molecules with a wide range of applications and increasing uses [1–3]. Fluorine is the most electronegative of all elements [4]. When bound to carbon, its extreme electronegativity is responsible for creating a highly polarized bond with the highest energies found in organochemicals and its small atomic size can easily mimic a hydrogen atom or a hydroxyl group [5]. Due to their peculiar characteristics, fluorinated compounds have now a role in almost all aspects of our lives, with their applications thriving in industry, agriculture and medicine [6]. Despite all of this success, the widespread utilization of fluorinated compounds has also a dark face, as the same characteristics that make these compounds so attractive from a commercial point of view are also responsible for their accumulation in the environment, where they are becoming ubiquitous contaminants [1,2]. For many fluoro-organics biodegradation is completely unknown. Monofluorinated compounds like fluorophenols [7–10], fluorobenzenes [11–15], fluorobenzoates [16–18], fluoroanilines [19–21], and fluoroacetate (FA) [22–24] are more likely to be biodegraded, while polyfluorinated compounds are more prone to be recalcitrant, as is the case with perfluorinated compounds.[1,25,26]

In contrast to the countless number of man-made fluorinated molecules, fluorine is rarely found in natural compounds, in spite of constituting the 13th most abundant element on Earth. Natural fluorinated compounds may be produced as a result of both biotic and abiotic mechanisms. For example, it is now known that

volcanoes constitute an emission source of fluorinated compounds. The biological production of fluorinated compounds is extremely rare, which highly contrasts with other halogenated compounds, for which more than 5000 natural chlorinated, brominated, and iodinated products have been described.[27] In fact, the number of natural fluorinated compounds is so low that any new discovery on this subject is of great interest. Aqueous fluorine has a very low bioavailability (ca. 1.3 mg L^{-1} in contrast with e.g. 3000 mg L^{-1} for chloride ion) and is quite inert. Its high redox potential constitutes a strong hindrance to the activity of haloperoxidases, which are commonly involved in the biological synthesis of chlorinated and brominated compounds, since the necessary redox potential for fluoride oxidation ($E^0 = -3.06$ V, as opposed to -1.36 V for chloride and -1.07 V for bromide) cannot be parallelized by that produced by hydrogen peroxide reduction ($E^0 = +1.71$ V).[28,29] In addition, aqueous fluoride is highly hydrated which constitutes a high obstacle to nucleophilic reactions.[29] These properties resulted in the evolution of the majority of life without the inclusion of fluorine in biological mechanisms. Despite this, nature has found way to the development of a few secondary biochemical reactions involving the fluorine atom. Table 1 shows the main developments in this field.

Table 1. Landmarks in the history of biogenic organofluorines.

Date	Landmark	Reference
1943	Isolation of potassium fluoroacetate from the plant *Dichapetalum cymosum*, constituting the first known naturally occurring fluorine containing molecule	[30,31]
1957	First identification of a microbial species (Streptomyces calvus) capable of producing a fluorinated compound (nucleocidin)	[32]
1986	*Streptomyces cattleya* was discovered to produce fluoroacetate and 4-fluorothreonine as secondary metabolites, providing a suitable system to study biological fluorination	[33]
2002	Discovery in *S. cattleya* of the first fluorinase enzyme capable of catalyzing the formation of the C–F bond	[34]
2003	First synthesis of an [^{18}F]-radiolabeled organic molecule using the fluorinase enzyme	[35]
2004	Elucidation of the fluorination mechanism catalyzed by the fluorinase enzyme	[28]
2006	Sequencing of the gene cluster responsible for the synthesis of fluoroacetate and 4-fluorothreonine in *S. cattleya*	[36]
2010	First biosynthesis of a fluorinated natural product analog (fluorosalinosporamide) through genetic engineering	[37]
2014	Discovery of four additional bacterial fluorinases, one of them isolated for the first time from a marine source	[38,39]
2015	Discovery of a new fluorometabolite – (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid – produced by Streptomyces sp. MA 37	[40]

As far as is known, biogenic fluoro-organics exist in a few monofluorinated compounds that are produced by some tropical and subtropical plants and by a small number of bacterial species.[38,41] These products are often toxic, presumably produced as a defense strategy. The relatively recent and small list of known biosynthesised fluoro-organics only consists of about 20 compounds,[42] which include the mammalian toxin FA, some ω-fluorinated fatty acids, the antibiotics nucleocidin and 4-fluorothreonine (4-FT) and the recently identified (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid (Figure 1). In all cases, these compounds only possess in their structure one fluorine atom and most are carboxylic acids, greatly contrasting with the fluorinated compounds of anthropogenic origin that are frequently polyfluorinated and have a diverse range of functional groups.

To date, the mechanism by which plants produce fluorinated compounds is unknown. The discovery of the biological fluorinating mechanism of the fluorinase from the actinomycete *Streptomyces cattleya* has opened new possibilities for the biotechnological production of synthetic fluorinated chemicals as well as fluorinated analogs of relevant natural products. This is particularly significant given the increasingly commercial importance of fluorochemicals in diverse areas.

This review presents a comprehensive and updated overview of the discoveries made in the field of fluorinated compounds with natural occurrence. For further reading related with this subject the reader may refer the references.[43–47]

Due to its peculiarity, special emphasis is given to the identification, characterization, and biotechnological relevance of the unique bacterial enzyme, fluorinase.

Biogenic fluorinated compounds

Natural fluoro-organics produced by plants

Fluoroacetate

FA was the first reported fluorinated compound with biological origin. This compound was isolated from the highly toxic South African plant *Dichapetalum cymosum* ("gifblaar") in 1943.[30] The fatal effect in grazing animals caused by the ingestion of the leaves of this plant was known for a long time, but the explanation for this phenomenon only became available after the study conducted by Marias,[31] which demonstrated that FA was the main component responsible for the plant's toxicity. Since the discovery of FA production by *D. cymosum*, other plants belonging to the *Dichapetalum* genus as well as with other phylogenies, and growing in tropical and semitropical regions, namely in Africa,

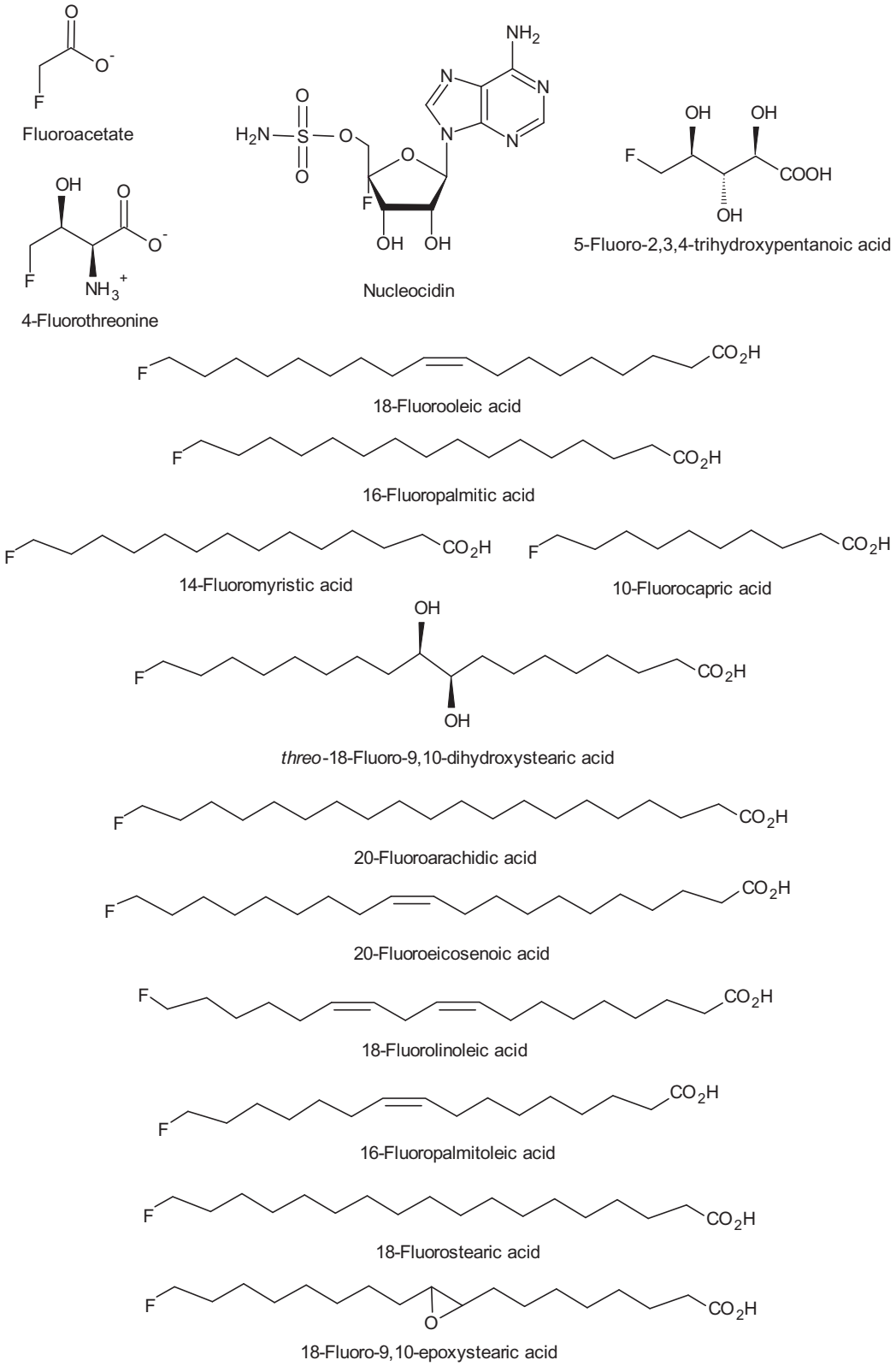

Figure 1. Structure of biogenic fluoro-organics hitherto discovered.

Australia, and South America, were later found to also produce this fatal compound. Examples include *D. toxicarium*,[48] *D. stuhlmanii*,[49] *D. braunni*, *D. guineense*, *D. heudelotti*, *D. macrocarpum*, *D. michelsonii*, *D. ruhlandii*, *D. venenatum*,[50] *D. barteri*,[51] *D. edule*,[52] *Acacia georginae*,[53,54] *Palicourea marcgravii*,[55,56] *P. aeneofusca*,[57] *Arrabidaea bilabiata*,[58] several species of *Amorimia* (formerly belonging to the genus

Mascagnia),[57,59] *Gastrolobium grandiflorum*,[60] *G. parviforum* (formerly *Oxylobium parviforum*),[49,61] *Spondianthus preussii* [62] and *Cyamopsis tetragonolobus*.[63] Altogether, the known FA producing plants account for more than 40 species. Interestingly, these plants were found to grow in soils containing low levels of fluorominerals, and FA concentrations in the tissues of the different plant species was found to vary considerably.[49,61] For example, *D. cymosum* leaves can accumulate up to 250 ppm of FA, while young leaves and seeds of *D. braunni* were found to contain 7200 and 8000 ppm of this compound, respectively, it being the highest concentrations so far reported.[52,64] In addition, FA levels in a given plant species can change significantly with the season and age of the plant, with young plants being generally more toxic than mature ones and levels of FA being significantly higher in spring than during other seasons.[29,64] FA has severe effects in the central nervous system of mammalians causing tetanic convulsions and fulminant heart attacks.[65] Its toxicity is due to the blockage of the tricarboxylic acid cycle caused by the *in vivo* conversion of this compound first to fluoroacetyl-CoA and then to (2*R*,3*R*)-fluorocitrate which is, coincidently, the only toxic form of fluorocitrate, with the remaining three stereoisomers being inoffensive (Figure 2). Fluorocitrate is a strong inhibitor of the enzyme aconitate hydratase through its conversion to 4-hydroxy-*trans*-aconitate, preventing citrate metabolism which accumulates in the living tissues.[66–69] In addition, it seems that fluorocitrate is also implicated in the inhibition of mitochondrial transmembrane citrate transport, bonding covalently to citrate carrier proteins, and studies indicate that this mechanism may be even more relevant to citrate toxicity than the previous one.[70]

It is interesting to mention that some edible plants also have the ability to synthesize small, non-toxic levels of FA (and consequently of fluorocitrate) in the presence of fluoride. Trace amounts of these compounds were detected in the leaves of some tea plants, oatmeal, alfalfa, soya bean, and crested wheat grass,[71–73] suggesting that the capacity of plants to produce FA may be more or less generalized and that, among these, certain plants have evolved mechanisms that allowed them to produce high amounts of this compound.[74]

ω-Fluorinated fatty acids

Apart from the production of FA, the seeds of the shrub *D. toxicarium* were also found to contain several ω-fluorinated fatty acids, namely 18-fluoro-oleic acid, which is

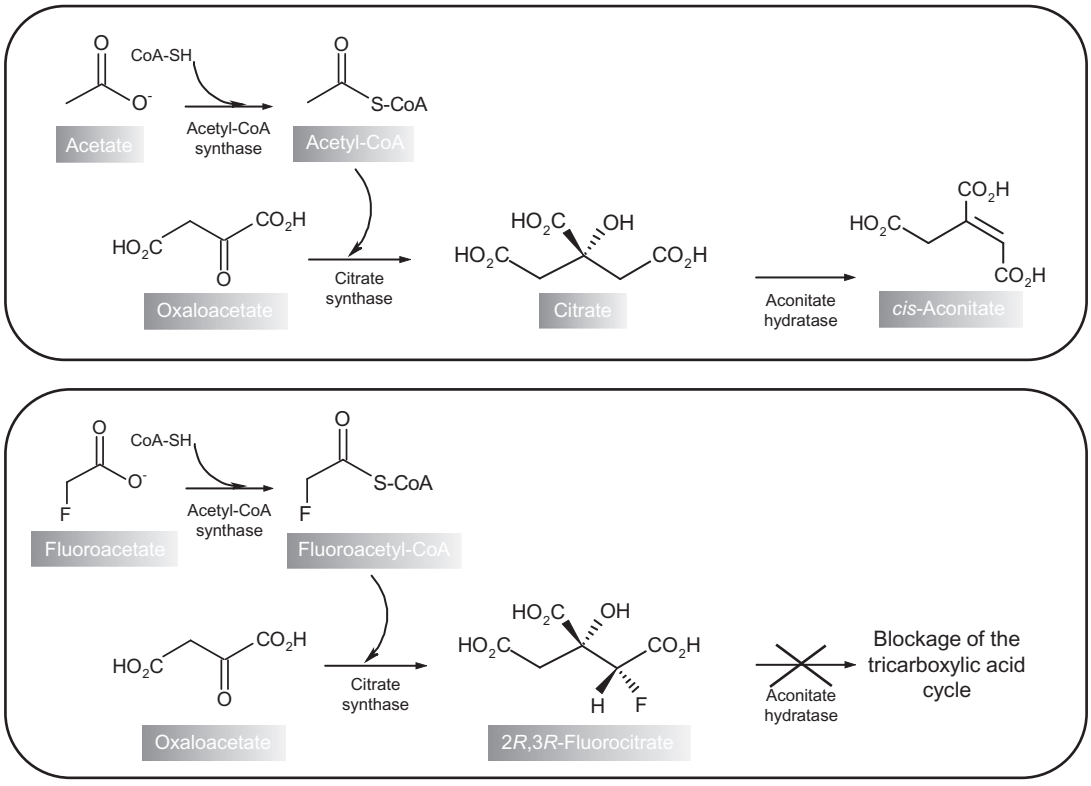

Figure 2. Metabolic pathway leading to the production of fluorocitrate, which is responsible for the blockage of the tricarboxylic acid cycle (TCA). Upper panel: productive TCA cycle generating *cis*-aconitate through the action of the enzyme aconitate hydratase; lower panel: inhibition of aconitate hydratase as a result of fluorocitrate production.

the main fluorinated fatty acid present in the seeds, 16-fluoropalmitic acid, and small quantities of 10-fluorocapric acid, 14-fluoromyristic acid, and *threo*-18-fluoro-9,10-dihydroxystearic acid which is believed to be a metabolite from the 18-fluoro-oleic acid (Figure 1).[75–78] These compounds are also very toxic to mammals since they are metabolized *in vivo* to FA.[65] In addition, six other fluorinated fatty acids were later found in the seed oil of the same plant and identified as 20-fluoroarachidic acid, 20-fluoroeicosenoic acid, 18-fluorolinoleic acid, 16-fluoropalmitoleic acid, 18-fluorostearic acid, and 18-fluoro-9,10-epoxystearic acid.[79] These fluorinated fatty acids are assumed to be biosynthesized by the plant from FA and their production appears to be restricted to the aforementioned *Dichapetalum* species.[52]

Fluoroacetone

Peters and Shorthouse [72] found that homogenates of the native Australian plant *Acacia georginae* contained a fluoro-organic compound identified as fluoroacetone. According to the authors, this volatile compound is thought to be an abnormal product derived from the biosynthesis of the ω-fluorinated fatty acids. Subsequent studies indicated, however, that the identification of fluoroacetone might be doubtful, with new data suggesting fluoroacetaldehyde as a more probable metabolite.[74] Thus, fluoroacetone cannot be reliably considered as a true natural organofluorine compound.

Resistance mechanisms to fluoroacetate

It seems logical that the plants producing fluoro-organic compounds hold mechanisms that allow them to resist their nefarious effects; however, these mechanisms are not completely known. A few studies reported in the literature provide some evidence of possible fluorocitrate protective mechanisms in plants. Some authors suggested that the citrate synthase of *D. cymosum* has a high capacity to discriminate between fluoroacetyl-CoA and acetyl-CoA, having a very low affinity for the first substrate.[68,80,81] Later, Meyer et al. [82] identified in tissue cultures of this same plant a fluoroacetyl-CoA hydrolase that was capable of hydrolyzing fluoroacetyl-CoA but not acetyl-CoA, a mechanism that could prevent the accumulation of fluoroacetyl-CoA and its subsequent conversion into fluorocitrate. Louw et al. [83] and Treble et al. [84] suggested that the aconitases of the fluoro-organic producing plants are much less sensitive to fluorocitrate than the animal aconitases. In addition, it seems that these plants are capable of synthesizing and catabolizing organic fluorine, a strategy that may also constitute a detoxification mechanism.[61] This fluoro-organics recycling capacity was demonstrated by Preuss and Weinstein [85] in an experiment showing that *Acacia georginae* is able to defluorinate FA.

The increased resistance of fluoro-organic producing plants to fluorinated compounds seems to be also shared by some animal species, particularly those that forage in regions abundant in FA-producing plants. Studies revealed that animals which graze in areas where these plant species do not grow are ca. 40–150-fold more sensitive to FA than those which are in contact with this type of vegetation.[86,87] The biological mechanisms responsible for the higher FA resistance in these animals are not yet completely known, but it seems that these organisms have a FA-specific defluorinase (FSD) capable of metabolizing FA.[88–92] Detoxication studies of FA in mammals revealed that FSD occurs mainly in the liver and that this enzyme is glutathione dependent and converts FA into *S*-carboxymethylcysteine and fluoride ion.[88] This enzyme was initially thought to belong to the glutathione *S*-transferases family; however, subsequent studies suggested that FSD has unique properties and is distinguishable from other glutathione *S*-transferases.[90,91] More recent studies allowed the purification of two forms of FSD from a cytosolic fraction of rat liver. One of these forms was identified as Zeta 1 glutathione-*S*-transferase, although it only represented 3% of the total cytosolic FSD activity. However, the major FSD form has not yet been undoubtedly identified but studies suggest that this may not be a glutathione-*S*-transferase isoenzyme, rather constituting a unique dehalogenase or dehydrogenase enzyme.[92,93] Further studies are still necessary to identify this latter FSD.

FA can also be metabolized by numerous soil microorganisms through the action of a dehalogenase enzyme, fluoroacetate dehalogenase, that specifically hydrolyzes the highly stable C–F bond, yielding fluoride ion and glycolate.[22,24,94–98] More recently, this compound has also been found to be degraded under anaerobic conditions by a bacterial species belonging to the phylum Synergistetes.[99] The genes encoding fluoroacetate dehalogenase have been explored for the protection of animals from the toxic effects of FA producing plants, by genetically engineering rumen bacteria and inoculating them into grazing animals. In studies developed by Gregg et al. [100] a FA dehalogenase gene isolated from a *Moraxella* species was used to construct a dehalogenase expression plasmid (pBHf) which was introduced into the rumen bacterium *Butyrivibrio fibrisolvens*. Although this genetically modified bacterium showed specific dehalogenation activity ca. 20-fold lower compared with that of *Moraxella*

species, the expression plasmid was found to be retained in the transformed cells for more than 500 generations, even under non-selective growth conditions. In another study, Gregg et al. [101] transformed three *B. fibrisolvens* strains with the plasmid pBHf and a fourth strain with a slightly modified derivative of this plasmid and inoculated a combination of these strains into the rumens of test sheep, showing an increased resistance of the animals to the toxic effects of FA. Transfer of FA degrading bacteria or the rumen fluid containing these bacteria to animals susceptible to FA also resulted in a higher resistance to intoxication by this compound.[102,103] The natural occurrence of FA degrading bacteria in the gastrointestinal system of ruminant animals has also been reported.[104] The protection of livestock animals from FA toxicity is of particular importance, given the significant economic losses caused by the ingestion of FA contaminated plant materials.

Natural fluoro-organics produced by micro-organisms

Nucleocidin

The capacity to biogenically produce fluorinated compounds is not restricted to plants. A few of actinomycetes species are also capable of this capacity. Thomas et al. [32] isolated from an Indian soil sample a *Streptomyces calvus* capable of producing an antibiotic named nucleocidin and later identified 4′-fluoro-5′-*O*-sulphamoyladenosine.[105] This broad-spectrum antibiotic was shown to have a high effect especially against a trypanosomal agent but, unfortunately, it was revealed to be too toxic for clinical use.[106,107] Nevertheless, the chemical structure of this compound generated a special scientific interest due to the position of the fluorine atom at the C-4 of the ribose ring, which seemed to indicate that, unlike the other biogenic fluorinated compounds discovered to date, nucleocidin was not biosynthesised from a fluoroacyl molecule. The possibility of a new fluorinating mechanism in *S. calvus* could never been proven since, for a long time, attempts to reisolate this natural fluoro-organic compound from frozen cultures of different culture collections have always failed.[74,108] On one hand, this scenario may, however, change with the restoration in the genome of this micro-organism with the function of the gene bldA, known to have a relevant role in the production of secondary metabolites by *Streptomyces*, and the identification of nucleocidin biosynthetic genes.[109–111] The fact that *S. calvus* was able to produce nucleocidin when its genome was complemented with a copy of a functional bldA gene opens good perspectives for the biosynthesis of other cryptic secondary metabolites through the expression of this gene, especially in poorly sporulating *Streptomyces* strains. On the other hand, the identification of the gene cluster responsible for the biosynthesis of nucleocidin may allow the creation of genetically engineered bacteria capable of producing new nucleocidin derivatives with relevant bioactivities.

4-Fluorothreonine and fluoroacetate

A major finding in the field of natural fluorinated compounds was made in 1986 during the course of an experiment conducted by Sanada et al. to increase the production efficiency of the antibiotic thienamycin by *S. cattleya*. It was found that in the presence of fluoride, *S. cattleya* was capable of synthesizing, during stationary growth phase, a novel amino acid, identified as 4-FT, and that during this process FA was also co-produced.[33] The synthesis of 4-FT could not be accomplished by all thienamycin producing strains and the production of halogenated analogous of this compound could not be obtained when the corresponding halide ions were added to the culture medium. This threonine analog was found to exhibit mild antimicrobial activity that could be reversed by L-serine or L-threonine and its structure was later confirmed as having the 2S,3S configuration.[112] The production of this natural fluorinated amino acid by *S. cattleya* was particularly relevant since it provided a suitable means to study the biological mechanisms underlying the synthesis of organofluorines. In their study, Sanada et al. [33] found that resting cells of *S. cattleya* were able to produce 4-FT when inorganic fluoride, FA or 4-fluoroglutamate were used as a fluorine source, but fluorocitrate was not used as a precursor of this compound. Conversely, both fluoroglutamate and fluorothreonine (but not fluorocitrate) were metabolized to FA. These observations led the authors to suggest FA as the initial metabolite involved in the biosynthesis of 4-FT. It was also postulated that FA would be further converted to fluoracetaldehyde which, by condensation with glycine, would result in the production of 4-FT. However, further studies on this subject revealed results that were not consistent with this hypothesis. Reid et al. [113] used ^{19}F NMR spectroscopy and isotopically labeled precursors to study the biosynthesis of the fluorinated secondary metabolites in *S. cattleya* and concluded that 4-FT was not synthesized from FA. In that study, it was found only low levels of interconversion between FA and 4-FT, and they defended that this interconversion could be misleading and could be attributed to a small degree of microbial

defluorination of both compounds. This resulted in the liberation of fluoride which would be subsequently incorporated by the fluorinating system into an organic form. This theory was supported by the fact that when resting cells of *S. cattleya* were incubated with different fluorinated intermediates, the amounts of FA and 4-FT produced were similar to those observed in cell suspensions supplemented only with fluoride. Studies with ^{14}C-labeled precursors led to the assumption that glycolate or an activated derivative of this compound was the substrate for the fluorinating enzyme. The experiments conducted by Tamura et al. [114] showed contradictory results with the demonstration that [2–^{13}C] glycerol was more efficiently incorporated into FA than glycolate and that the main pathway responsible for the synthesis of this compound was via β-hydroxypyruvate. These results led to the suggestion that the glycolytic pathway was responsible for the biosynthesis of FA and 4-FT. The studies, carried out by Hamilton et al. [115,116] with ^{13}C- and ^{2}H-labeled precursors, demonstrated a high rate of conversion of glycines and pyruvates into FA and 4-FT, with the carbon atoms of FA and C-3 and C-4 of 4-FT being derived from C-2 of glycine or from C-2 and C-3 of pyruvate, indicating that the substrate for the fluorinating enzyme should be an intermediate between glycerol and pyruvate. It was also shown that the integration of ^{13}C in the C-1 and C-2 of FA always paralleled that in the C-3 and C-4 of 4-FT, indicating the existence of just one fluorinating enzyme in *S. cattleya*. Later, Moss et al. [117] showed that fluoroacetaldehyde is a common precursor of both FA and 4-FT. According to these authors, FA was produced by direct oxidation of fluoroacetaldehyde, mediated by an NADH-dependent aldehyde dehydrogenase, and ^{2}H-labeled fluoroacetaldehyde was found to be integrated into C-3 and C-4 of 4-FT. The enzyme accountable for the biosynthesis of FA, fluoroacetaldehyde dehydrogenase, was isolated, purified, and characterized by Murphy et al.[118] This enzyme showed a high affinity for fluoroacetaldehyde ($K_m = 0.08$ mM), oxidizing this compound much more effectively than acetaldehyde ($K_m = 0.81$ mM) and, regardless of its similarity with other bacterial acetaldehyde dehydrogenases, it appears to be an enzyme specifically evolved for the metabolism of FA. Further studies on the biosynthesis of 4-FT demonstrated that this compound is obtained by condensation of fluoroacetaldehyde with L-threonine, mediated by a pyridoxal 5′-phosphate (PLP) dependent-transaldolase.[119] This enzyme was partially purified and assayed with a range of amino acids, including glycine, and the results obtained were in agreement with those previously achieved by Hamilton et al. [116] indicating that glycine is not a substrate of the enzyme responsible for 4-FT production and not directly involved in the production of this fluorinated amino acid. Accordingly, it has been concluded that the production of 4-FT is mediated by a novel threonine transaldolase, as this enzyme is dependent on PLP and uses L-threonine, instead of glycine, as a substrate.[119]

Biosynthetic route of 4-fluorothreonine and fluoroacetate – the discovery of the fluorinase enzyme

Although the importance of the results achieved on the biosynthesis of fluorinated secondary metabolites by *S. cattleya* cells was undeniable, the process by which these cells could biosynthesise the metabolites from fluoride was still unknown. A large step in this direction was made by O'Hagan et al. [34] who first discovered the fluorinase enzyme responsible for the conversion of the fluoride ion and *S*-adenosylmethionine (SAM) into 5′-fluoro-5′-deoxyadenosine (5′-FDA) and L-methionine (Figure 3(a)). In that study, the authors could also show that crude cell-free extracts of *S. cattleya* were capable of producing FA from fluoride and SAM, indicating that this micro-organism also contained the enzymes that mediate the conversion of 5′-FDA to FA. In another study, Schaffrath et al. [120] confirmed the involvement of the metabolites 5′-FDA and fluoroacetaldehyde in the biosynthesis of FA and 4-FT and demonstrated the production of these secondary metabolites in cell-free extracts of *S. cattleya* from a synthetic sample of 5′-FDA. Later, Cobb et al. [121] found that 5′-FDA was converted to 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) in a reaction catalyzed by a purine nucleoside phosphorylase (Figure 3(a)). They also identified a fluorinated dead-end metabolite, 5′-fluoro-5′-deoxyinosine (5′-FDI) that accumulated only in cell-free extracts of *S. cattleya* and, even though 5′-FDI is not an intermediate of 4-FT and FA biosynthesis, its production is relevant in that it increases the number of enzymatically generated organofluorinated compounds.[122] The discovery of the third fluorinated metabolite involved in the production of 4-FT and FA occurred in 2007, when Onega et al. identified (3R,4S)-5-fluoro-5-deoxy-D-ribulose-1-phosphate (5-FDRulP) in a cell-free extract of *S. cattleya*. It was concluded that 5-FDRulP is produced from 5-FDRP in a reaction mediated by an isomerase, and further metabolized to fluoroacetaldehyde by a Zn^{2+}-dependent aldolase [123] (Figure 3(a)).

The identification of an enzymatic reaction involved in the generation of fluorinated compounds had already been reported ca. 60 years ago by Flavin et al. [124,125] who found that in the presence of fluoride, ATP could be enzymatically converted, in a CO_2-dependent

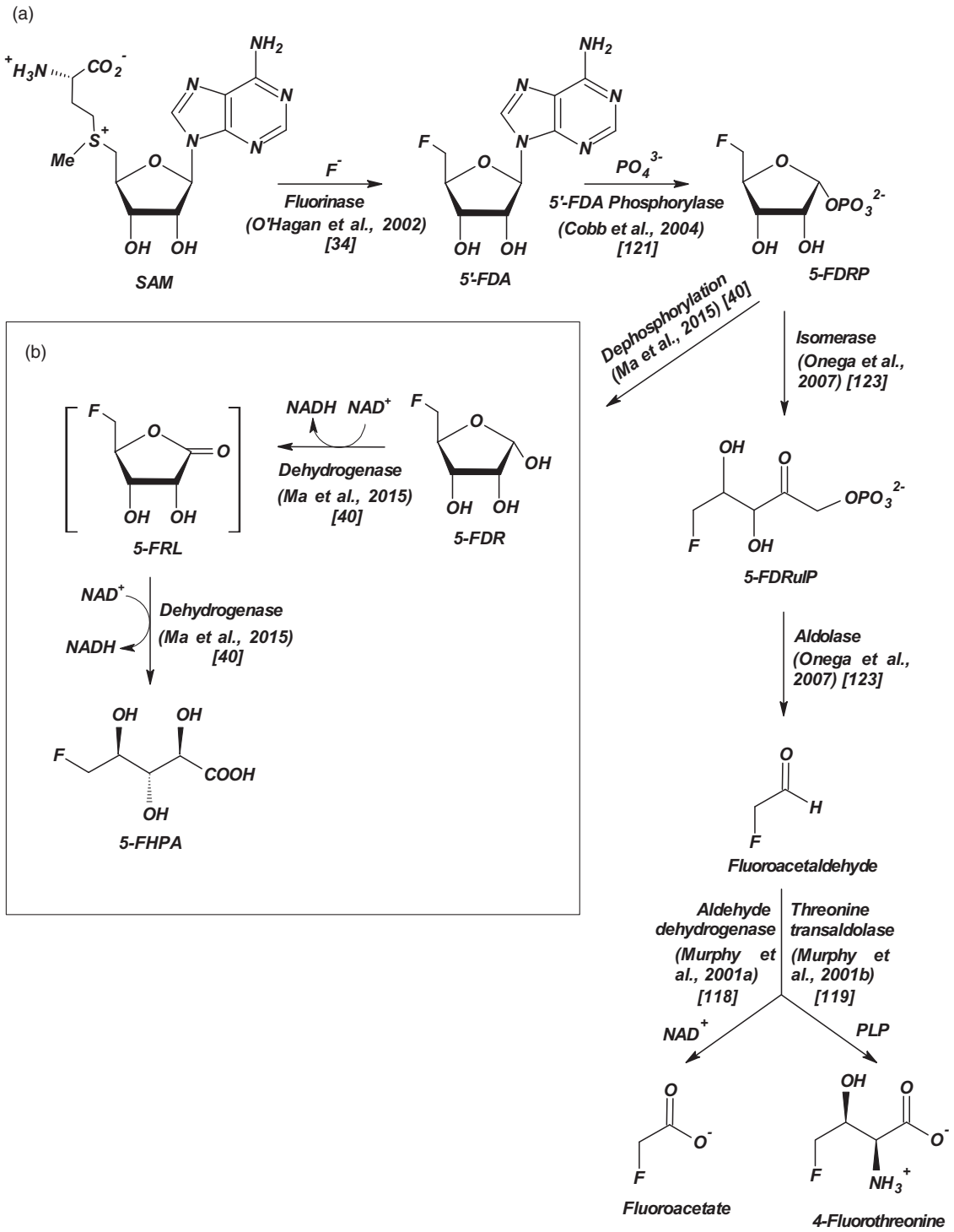

Figure 3. Metabolic pathway and enzymes involved in the production of (a) 4-fluorothreonine and fluoroacetate by *Streptomyces cattleya* and (b) (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid (5-FHPA) by *Streptomyces* sp. MA37.

reaction, to monofluorophosphate and ADP. The reaction was found to be halogen specific, i.e. it did not occur with chloride, bromide or iodide, and the involved enzyme was referred as a "fluorokinase", it being predominantly detected in rat and pig heart tissue extracts and in rat and rabbit skeletal muscle extracts. Later, the "fluorokinase" reaction was additionally found to be catalyzed by a yeast pyruvate kinase.[126] In 2001, Nashiry et al. [127] and Zechel et al. [128] also demonstrated the formation of a C–F bond catalyzed by mutant glycosidase enzymes from *Agrobacterium* sp. and *Cellulomonas fimi*, which were able to transiently produce α-fluoroglycosides.

Characterization of the fluorinase enzyme The fluorinase enzyme (5′-fluoro-5′-deoxyadenosine synthase)

was purified and characterized by Schaffrath et al. [129] revealing to have a relative molecular mass (M_r) of 32 200 and the following kinetic parameters: K_m of 0.42 mM for SAM and 8.56 mM for fluoride and V_{max} of 1.28 U mg^{-1} for SAM and 1.59 U mg^{-1} for fluoride ion.[129] Further studies showed that this enzyme was inhibited by SAM analogs, namely *S*-adenosylhomocysteine, which was shown to be a potent competitive inhibitor, and the antibiotic sinefungin was found to be a weaker inhibitor. The gene responsible for the expression of the fluorinase enzyme (*flA*) was cloned and sequenced by Dong and his colleagues. It has a size of 897 base pairs that code for a protein with 299 amino acids.[28] In the same study, it was determined the crystal structure of the enzyme and the mechanism of the enzymatic fluorination, showing a protein monomer with a unique quaternary structure that acts on the substrate through a rare nucleophilic S_N2 substitution reaction in which fluoride attacks the C-5′ of SAM, displacing methionine. The study also confirmed the mechanism of biological fluorination previously proposed by O'Hagan et al.[130] Curiously, the fluorinase enzyme was able to catalyze a reversible reaction, thus being capable of converting 5′-FDA back into SAM, and also to use chloride ion.[131] More detailed information on the reaction mechanism of this enzyme may be consulted in the papers authored by Cadicamo et al. [132] Cobb et al. [133] Deng and O'Hagan,[134] Senn et al. [135] and Zhu et al.[136] The reaction mechanism of fluorinase was later found to be analogous to a chlorinase (SalL) responsible for the synthesis of salinosporamide in the marine bacterium *Salinispora tropica*, although the latter enzyme is not capable of using fluoride ion as substrate.[137] This micro-organism was, however, shown to produce fluorosalinosporamide when the chlorinase gene was replaced with the gene encoding for the fluorinase.[37]

The genes behind the production of 4-fluorothreonine and fluoroacetate The first gene cluster (*fl*) involved in the synthesis of 4-FT and FA, containing the fluorinase (*flA*) and the 5′-FDA phosphorylase (*flB*) genes, was cloned and sequenced by Huang et al.[36] In that study, a gene encoding a fluoroacetyl-CoA thioesterase (*flK*) was identified capable of catalyzing the conversion of fluoroacetyl-CoA into FA and CoA. This reaction is very interesting not only because of its selectivity (the enzyme is 10^6 more selective for fluoroacetyl-CoA than for acetyl-CoA), but also because it may explain how *S. cattleya* resists the biochemical effects of FA. The catalytic mechanism and kinetics of this enzyme were studied in detail by Dias et al. [138] and Weeks et al.[139–141]

The *in vitro* production of 4-FT through the overexpression and recombination of all the enzymes involved in the biosynthesis of this secondary fluorometabolite was achieved by Deng et al. [142] in a work that not only proved the metabolic steps leading to the bioproduction of this amino acid but also demonstrated the importance that the fluorinase may have in the organofluorine production industry.

The genome of *S. cattleya* (strain NRRL 8057) was completely sequenced by Barbe and colleagues, consisting in one linear chromosome composed by 6 283 062 bp (with a GC content of 72.94%) and one linear megaplasmid composed by 1 809 491 bp (with a GC content of 73.21%).[143] The genes responsible for the synthesis of 4-FT and FA were found to be predominantly located in the bacterial chromosome, with the exception of the aldolase enzyme that mediates the conversion of 5-FDRulP to fluoroacetaldehyde, which could not be identified, and of the transaldolase that catalyzes the production of 4-FT, which was identified in the plasmid of *S. cattleya*. Inactivation of the genes responsible for the production of FA and 4-FT confirmed that they are essential for the biosynthesis of these fluorinated metabolites.[144]

Regulation of fluoro-organics biosynthesis by Streptomyces cattleya Due to the toxicity of FA, one intriguing question is how *S. cattleya* cells are able to avoid the nefarious effects of this compound while producing it. A fluoroacetyl-CoA thioesterase has been implicated in the resistance mechanism of *S. cattleya* to FA due to its high specificity for this metabolite which prevents its entry into the tricarboxylic acid cycle (TCA).[36,139] However, Walker et al. [145] showed that disrupting the gene responsible for the synthesis of this enzyme in *S. cattleya* results in a strain with a normal capacity to produce organofluorine metabolites, indicating the existence of other resistance mechanisms not related to the thioesterase discussed above. In that study, it was found that the production of the enzymes involved in the TCA cycle is dissociated in a temporal scale from the production of the organofluorine metabolites, with *S. cattleya* cells only initiating the synthesis of these compounds after stopping their growth and, therefore, when the TCA cycle is inactive. Also, organofluorines production by *S. cattleya* seems to be regulated by fluoride, as expression of the genes responsible for the production of these compounds was found to be correlated with the presence of fluoride in the culture medium.[145]

The discovery of other fluorinase enzymes The fluorinase enzyme from *S. cattleya* persisted as a singular

finding throughout a period of more than a decade, but very recently four new fluorinases were found by Deng et al. [38] and Huang et al. [39] all being identified from actinomycetes species. By using a genome mining approach, Deng et al. [38] identified three novel fluorinase isolates in the terrestrial actinomycete species, *Streptomyces* sp. MA37 and *Actinoplanes* sp. N902-109, and in the pathogenic actinomycete *Norcardia brasiliensis*. These fluorinases showed to have a high homology (80–87%) to the fluorinase of *S. cattleya* and to share a signature loop of 21 residues, which appear to be a unique feature of the fluorinase enzymes. The activity of all three fluorinases was confirmed by *in vitro* assays and kinetic studies revealed that they are slow enzymes. Culture growth assays showed that *Streptomyces* sp. MA37 produces a series of unknown fluorometabolites along with the synthesis of FA and 4-FT. No fluorometabolites could be detected in cultures of *N. brasiliensis*, while the production of these metabolites could not be tested in *Actinoplanes* sp. N902-109 due to the fact that this micro-organism is not publicly available.[38] In the same timeframe, Wang et al. [146] also reported studies identifying a fluorinase (*Nob A*) in the genome of *N. brasiliensis*. This fluorinase was found to share 79% similarity with the fluorinase from *S. cattleya* and biochemical characterization revealed that this enzyme exhibits similar catalytic properties to FIA, although its efficiency is slightly lower (2.3-fold).[146]

A fluorinase derived from a marine source was reported for the first time by Huang et al. [39] being isolated from *Streptomyces xinghaiensis* NRRL B-24674. The fluorinase of this bacterium has a high similarity (84%) to the other known fluorinases and also presents the 21 residues loop characteristic of these enzymes. When cultured, this micro-organism was able to produce FA and this capacity was found to be sea-salt dependent. Deeper characterization of the fluorinase from *S. xinghaiensis* revealed that this enzyme is the most efficient among the known fluorinases.[147] Similar to the fluorinase of *S. cattleya*, in the presence of *L*-amino acid oxidase, the fluorinase of *S. xinghaiensis* is capable of using chloride ion as a substrate to produce 5′-chloro-5′-deoxyadenosine. The enzyme was found to be highly suitable for industrial biocatalysis.[147]

Biotechnological applications of the fluorinase enzyme The potential of the fluorinase enzyme as a natural mechanism to incorporate fluoride ion into organic molecules reveals it to be enormous in the production of useful fluorinated compounds. Current investigations are focused on the application of this enzyme as a catalyst for the generation of [^{18}F]-labeled compounds to be used in positron emission tomography (PET), a state-of-the-art technology widely used for medical and biological imaging (Figure 4(A)). There are numerous studies in the literature illustrating the application of fluorinase in this field.[35,148–156]

The fluorinase enzyme can also be employed as a valuable tool to enlarge the diversity of fluorometabolite structures through genetic engineering synthesis. The study conducted by Eustáquio et al. [37] in which the production of fluorosalinosporamide (Figure 4(B), compound **5**) was achieved by replacing the chlorinase gene with the fluorinase gene in *S. tropica*, constitutes a good example of this. Also, fluorinated metabolites synthesized by the action of fluorinase enzyme can be used as building blocks for the biosynthesis of other fluorinated molecules. For example, Hong et al. [157] demonstrated that FA could be incorporated into a polyketide, after its *in vivo* conversion to fluoroacetyl-CoA, by a minimal polyketide synthase responsible for the production of the antibiotic actinorhodin, generating a full-length fluorinated polyketide named F-SEK4b (Figure 4(B), compound **6**). Walker et al. [158] demonstrated the biosynthesis of a fluorotriketide molecule (Figure 4(B), compound **7**) from FA or fluoromalonate and also showed that fluorine may be regioselectively incorporated into the backbone of polyketides using fluoromalonate as a building block (Figure 4(B), compounds **8** and **9**). These results are particularly important in the light of the relevant role that fluorinated molecules and polyketides play in human medicine synthesis such as antibiotics, anticancer agents, and anti-inflammatory agents.

(2R3S4S)-5-Fluoro-2,3,4-trihydroxypentanoic acid

The production of a number of unknown fluorinated metabolites by the actinomycete *Streptomyces* sp. MA37, recently reported by Deng et al. [38] to contain a functional fluorinase, instigated Ma et al. to study the identity of these compounds. To this end, the authors identified a new fluorometabolite – (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid (5-FHPA) – resulting from a new biosynthetic pathway derived from the fluorinase pathway leading to the production of FA and 4-FT, with 5-FDRP being the common metabolite between the two pathways.[40] Phosphorolysis of this compound produces 5-fluoro-5-deoxyribose (5-FDR), which by oxidation generates 5-fluoro-5-deoxy-D-ribono-ϒ-lactone (5-FRL) that is subsequently hydrolyzed to 5-FHPA (Figure 3(b)). Also a gene cluster (*fdr*) has been identified involving in the biosynthesis of the novel fluorinated metabolite, 5-FHPA, and a gene (*fdrC*) encoding a NAD^+ dependent dehydrogenase responsible for the biosynthesis of this metabolite from 5-FDR has been

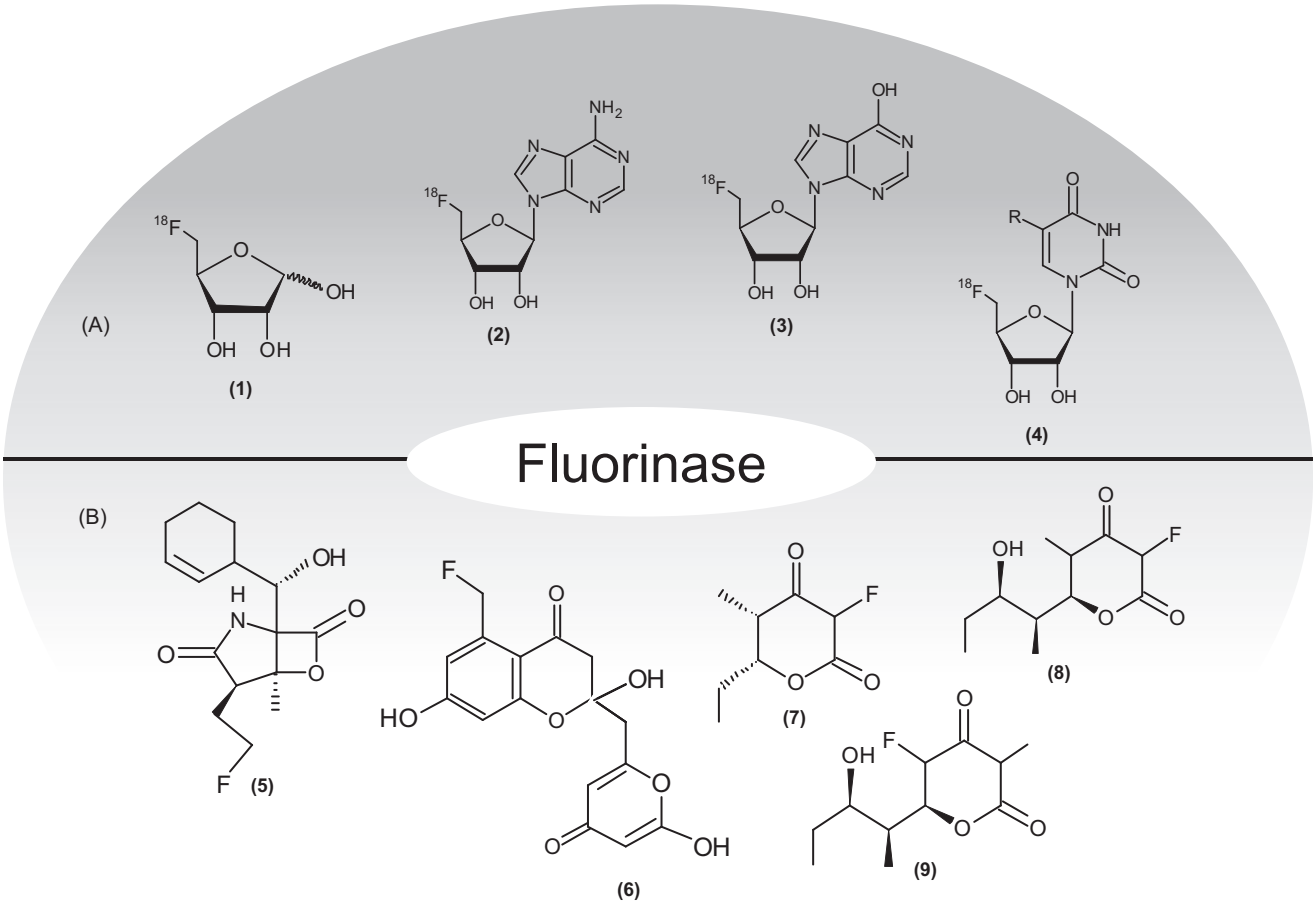

Figure 4. Biotechnological applications of fluorinase for the production of commercially important organofluorines. The fluorinase can be used as a catalyst for the synthesis of [^{18}F]-labeled compounds (A) and to enlarge the diversity of fluorinated structures through genetic engineering or the synthesis of fluorinated building blocks (B). The compounds illustrated are 5-[^{18}F]fluoro-5-deoxyribose (**1**); 5-[^{18}F]fluoro-5-deoxyadenosine (**2**); 5-[^{18}F]fluoro-5-deoxyinosine (**3**); 5-[^{18}F]fluoro-5-deoxyuridines (**4**); fluorosalinosporamide (**5**); F-SEK4b (**6**); 2-fluoro-2-desmethyltriketide lactone (**7**); 2-fluoro-4-methyl-tetraketide lactone (**8**) and 2-methyl-4-fluoro-tetraketide lactone (**9**).

identified.[40] The results obtained in this work constitute an important contribution to the small list of hitherto known biogenic fluoro-organics.

3,5-Di-tert-butyl-4-fluorophenylpropionic acid

Recently, while searching for new naturally produced bioactive compounds, Jaivel et al. [159] reported the isolation, from a *Streptomyces* sp. strain TC1, of a novel fluoroaromatic compound identified as 3,5-di-*tert*-butyl-4-fluorophenylpropionic acid. The discovery was particularly significant given that, by the time the work was published, no enzyme capable of adding fluorine to an aromatic ring was known. However, shortly after the paper by Jaivel et al. [159] Ayoup et al. [160] claimed that the discovered molecule does not contain fluorine in its structure and that the erroneous identification was due to a misinterpretation of the analytical data. This conclusion was also corroborated by Aldemir et al.[161] Thus, in the light of this later evidence, and unless otherwise proven, 3,5-di-*tert*-butyl-4-fluorophenylpropionic acid cannot be considered a true natural fluorinated compound.

Natural fluoro-organics produced by animals

Apart from plants and micro-organisms, a marine animal was also reported to be capable of producing natural fluoro-organics. Xu et al. [162] investigated the marine sponge *Phakellia fusca* Schmidt, obtained from the South China Sea and found that this organism was able to produce five 5-fluorouracil alkaloids, including the well-known anticancer agent, 5-fluorouracil, and 1-(2-hydroxylethyl)-5-fluorouracil, also exhibiting antitumor properties (Figure 1). However, according to Deng et al. [44] the compounds identified in the sponge may

not be derived from biological synthesis and the authors believe that they may rather be a result of industrial contamination. In spite of this uncertainty, this study was the first to report the production of fluorinated compounds by marine animals. As far as it is known, the production of fluorinated compounds by other animals or insects has never been reported.

Abiogenic fluorinated compounds

There are also natural fluorinated compounds which are not produced by biological means, for example, volcanoes produce great quantities of hydrogen fluoride (ca. 11 million tons per year). They are the main contributor for the presence of this compound in the atmosphere.[45] This gas has been postulated to react with organic compounds from sediments or fossil soils, producing CFCs and other organofluorine compounds such as tetrafluoroethylene and hexafluoropropene.[163] Other fluorinated compounds were also identified in the gaseous emissions from fumarole and lava of four volcanoes located in Italy and Japan and included fluorotrichloromethane, trifluoropropene, fluorobenzene, tetrafluorobenzene, and fluorochlorobenzene.[164] Studies indicate that ca. 75% of the most active volcanoes in the world are capable of producing CFCs and other fluoro-organic compounds, although in much less quantity compared with the anthropogenic production.[165] Fluoroalkanes, namely, trifluoromethane, trichlorofluoromethane, dichlorodifluoromethane, and polyfluorinated propane were found, among other halogenated compounds, in rocks derived from mining activities.[166,167] It is thought that the mining industry itself is responsible for atmospheric emission of significant amounts of natural halocarbons, in which organofluorine compounds are included.[45] Natural fluorites have been found to have in their composition: sulfur hexafluoride, nitrogen trifluoride, tetrafluoromethane, dichlorodifluoromethane, trichlorofluoromethane, and tetrafluoroethylene. It is thought that the contribution of natural tetrafluoromethane to the atmospheric pollution is significant.[168–170] In addition to natural fluorites, it seems that these fluorinated compounds also occur in igneous and metamorphic rocks at concentrations similar to the ones released into the atmosphere from human activity.[169] Hydrogen fluoride has been found in the interstellar medium and there is a strong belief that it may also contain fluoro-organic compounds.[171]

Concluding remarks

The number of fluorinated compounds produced by chemical synthesis is increasing, despite their natural genesis being rare. The discovery, isolation, and characterization of the fluorinase enzyme constitute important landmarks in fluorine research and offers new biotechnological perspectives for the biosynthesis of fluorinated products through genetic engineering. The use of fluorinase as a catalyst for the generation of [^{18}F]-labeled compounds for PET analysis, and the cloning of the fluorinase gene into the salinosporamide A-producing bacteria, *Salinispora tropica*, to produce the fluorinated version of this anticancer drug, are two elegant examples of biotechnological applications of this enzyme. The hitherto known small number of microorganisms holding a fluorinase gene suggests that these genes are scantly distributed in the microbial world. However, due to the fact that a huge number of micro-organisms are undiscovered, more fluorinases are expected to be revealed.

The recent identification of the new fluorometabolite – (2R3S4S)-5-fluoro-2,3,4-trihydroxypentanoic acid, is an important contribution for the small list of natural fluorinated compounds and expands the number of enzymatic fluorination reactions and fluorinated scaffolds available for the chemical synthesis of organofluorines. The biological nature of the compounds 5-fluorouracil and 3,5-di-*tert*-butyl-4-fluorophenylpropionic acid has yet to be proven, though evidence points to an anthropogenic origin, in the case of the former compound, and to data misinterpretation of the latter.

Unveiling the mechanisms by which *Streptomyces calvus* synthesizes nucleocidin will certainly be a relevant contribution to the field of natural fluorinated compounds, since there is a high potential to discover a new fluorination reaction. Another important achievement will be to discover how plants growing in Africa, Australia, and South America produce FA and to solve if micro-organisms associated with them are also implicated in the biosynthesis of this compound. Also, exploring the exact mechanisms that protect these plants from the negative effects of fluorinated molecules may allow developing more efficient strategies to deal with episodes of acute intoxications caused by the ingestion of plant material.

Abiogenic sources, such as volcanoes and rocks, also contribute to the natural release of fluorinated compounds into the environment, although its extent and how these compounds are produced still remain unknown.

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