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Review

^{19}F NMR applications in chemical biology

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ABSTRACT

Although fluorine plays only a minor role in biology, its physicochemical properties have proven incredibly useful in the design of analogues of biologically important molecules. Analysis of organofluorine compounds by ^{19}F NMR was largely confined to synthetic chemists but this technique is finding increasing applications in biological systems. The fluorine atom with its relative small size and 100% natural isotope abundance represents an attractive option for biological NMR studies. In this paper we review the recent literature highlighting the exploitation of ^{19}F NMR in a range of research areas at the interface of chemistry and biology.

Contents

1. Introduction

The physicochemical properties of fluorine distinguish it from the other halogens, and led Pauling to describe it as a 'super-halogen' [1]. Although fluorine is the most abundant halogen in the earth's crust, it plays a very minor role in biology; fluorinated compounds, biosynthesised *de novo*, are rare in nature. This reflects the properties of the fluoride ion, such as its high redox potential and its propensity to become hydrated, but also the predominantly insoluble character of fluorine containing minerals, such as fluorspar (CaF_2), which renders fluorine biologically unavailable compared with the other halogens. Nevertheless, the uniqueness

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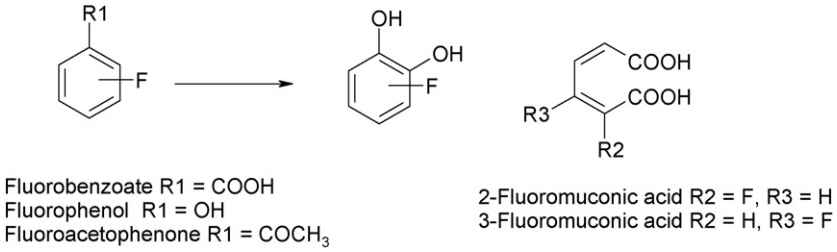


Scheme 1. Microbial transformation of fluoroaromatic compounds.

of fluorine has resulted in this element being increasingly important in the area that is broadly defined as 'chemical biology'. The fluorine atom is isosteric with hydroxyl and its Van der Waal's radius (1.47 Å) is close to that of hydrogen (1.20 Å), thus its substitution in organic compounds results in a sterically unchanged product. However, these substitutions have profound effects on the electronic properties of the compound, since fluorine's electronegativity (4.0 on the Pauling scale) is the greatest of all the elements, and the bond dissociation energy of C–F (110 kcal mol^{-1} in CH_3F) is greater than that of C–H (99 kcal mol^{-1}). Thus fluorinated derivatives of naturally occurring compounds, such as metabolic intermediates, have proved extremely useful in medicine and agriculture, and the numbers of fluorinated compounds used in these areas are ever increasing [2]. In concert with the increased interest in preparing fluorinated compounds that have biological effects, has been the technological advancement in the detection of fluorinated compounds. ^{19}F nuclear magnetic resonance is an invaluable tool for determination of the structure of fluorinated compounds, but the characteristics of ^{19}F NMR spectra make it very useful to investigate the interaction of fluorinated compounds with biological systems: the sensitivity of the fluorine nucleus is only slightly less than hydrogen, thus it is possible to detect μM concentrations; the resonances of organofluorine compounds do not overlap with those of carbon-13 and hydrogen, accordingly analysis is possible without the need for purification; and the relatively large chemical shifts resulting from minor changes in the chemical environment, such as those occurring during enzyme-catalysed reactions or interactions with other macromolecules, means that there is little or no peak overlap.

In this paper, the application of ^{19}F NMR in aspects of chemical biology is reviewed, with particular emphasis on the monitoring of organofluorine metabolism and the probing of macromolecular interactions. For information on aspects of ^{19}F NMR employed in clinical applications, the reader is referred to refs. [3–5].

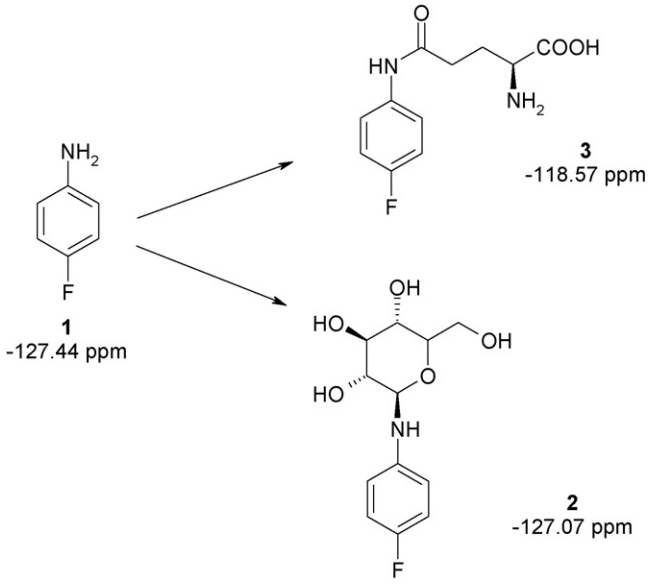
2. Metabolic studies employing ^{19}F NMR

2.1. Biotransformation of fluorinated xenobiotics

The wide range of applications that use organofluorine compounds has resulted in their accumulation in the environment. Therefore, research has focussed on the catabolism of model fluorinated pollutants, in particular fluoroaromatic compounds, by bacteria and fungi, and in this context ^{19}F NMR has proven to be an extremely useful tool for monitoring the degradation of the fluorinated compounds, and determining the catabolic pathways involved by shedding light on the identities of the fluorometabolites that accumulate in the culture medium. ^{19}F NMR has been used to monitor the biodegradation of fluorophenols [6–8], fluorobenzoates [9], fluorobiphenyl [10,11], fluorotoluene [12] and fluoroacetophenone [13]. The oxidative products of aromatic degradation, such as fluorocatechols and fluoromuconic acids (Scheme 1), could be detected in the culture supernatants; however, in some cases it was not possible to fully identify the accumulated fluorometabolites, since no authentic standards were available to compare chemical shift and coupling constant data. For a comprehensive list of chemical shifts and coupling constants of the most common fluorometabolites arising from microbial degradation of fluoroaromatic compounds, the reader is directed to Boersma et al. [14].

^{19}F NMR has also been used to study the metabolism of fluorinated xenobiotics in animals. For example, the metabolism of model pollutants 4-fluoroaniline **1** and 4-fluorobiphenyl was examined in the earthworm *Eisenia veneta* using ^{19}F NMR as a key technique to determine the biotransformation of these compounds [15,16]. 4-Fluorobiphenyl was not catabolised, but 4-fluoroaniline **1** was converted to the N-β-glucoside conjugate **2** when doses of 125 μg cm^{-2} were used and the γ-glutamyl **3** conjugate at lower doses (Scheme 2). More recently the catabolism of 2-fluoro-4-iodoaniline in *E. vaneta* was investigated, and 12 catabolic products were observed using ^{19}F NMR, none of which was fluoride ion [12]. In these studies, it was noted that while valuable data was obtained by ^{19}F NMR analysis regarding the extent of degradation and number of metabolites, it was necessary to use additional analytical techniques, such as HPLC-^{1}H NMR and HPLC-MS, to firmly identify the products of Phase II (conjugative) metabolism. ^{19}F NMR was also used to monitor the biotransformation of 2-fluoroaniline to the excretory products 4-acetamido- and 4-amino-3-fluorophenyl sulphate, and 4-acetamido-and 4-amino-3-fluorophenyl glucuronide in rats [17].

The metabolism of xenobiotic compounds, such as herbicides and pesticides in plants is of considerable interest because of agricultural concerns. One early study illustrated the usefulness of



Scheme 2. Metabolism of 4-fluoroaniline in *Eisenia veneta*.

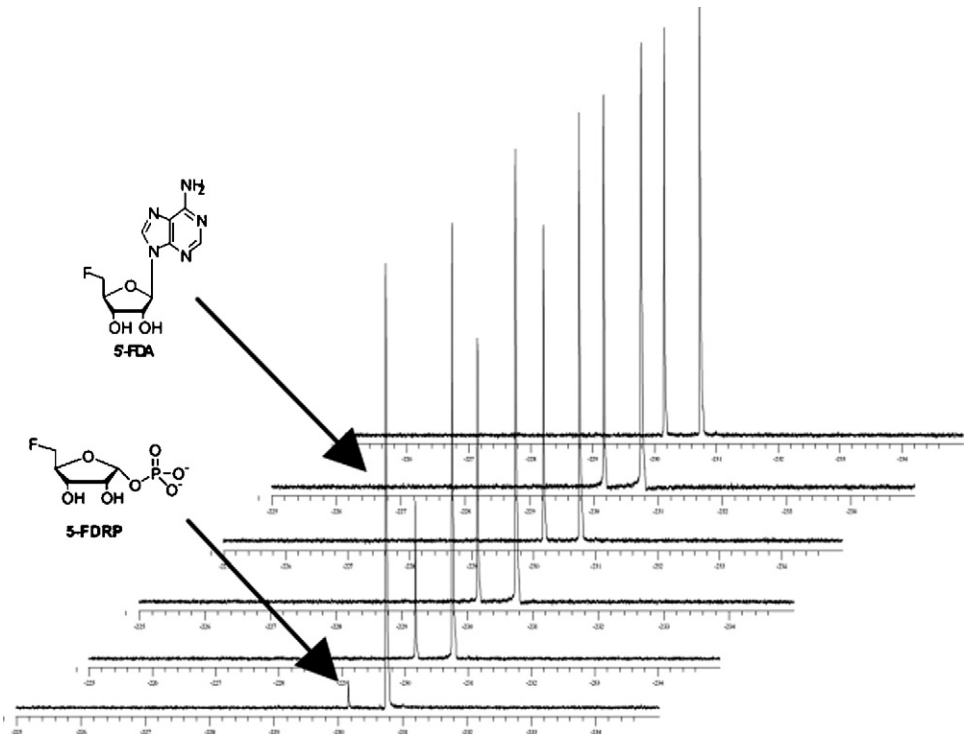


Fig. 1. ^{19}F NMR spectra of compounds formed upon incubation of *S. cattleya* cell extract with *S*-adenosyl methionine (SAM) and F^- ion [30].

^{19}F NMR in this area by examining the *in vivo* accumulation of the mild herbicide trifluoroacetate in the tomato plant *Lycopersicon esculentum*. By surrounding the stem and leaves with the spectroscopy coil [18], it could be demonstrated that the compound accumulates in the leaves and is not further metabolised. The accumulation and transformation products of 4-chloro-2-fluorophenol, an analogue of 2,4-dichlorophenol, in acetonitrile extracts of the aquatic plant *Lemna minor* was investigated by ^{19}F NMR [19]. The compound was most likely conjugated, since new signals appeared downfield (−126 to −126.5 ppm) of that from the parent compound (−130.0 ppm), indicating an increased shielding of the fluorine nucleus, and the splitting pattern of the metabolites indicated that the aromatic nucleus was un-modified.

The degradation of the fluorinated pesticides *N*-ethyl-*N*-methyl-4-(trifluoromethyl)-2-(3,4-dimethoxyphenyl) benzamide (fungicide) and isoxaflutole (herbicide) in *Acer pseudoplantus* by was evaluated using ^{19}F NMR, and some of the fluorometabolites could be identified from their chemical shifts [20,21].

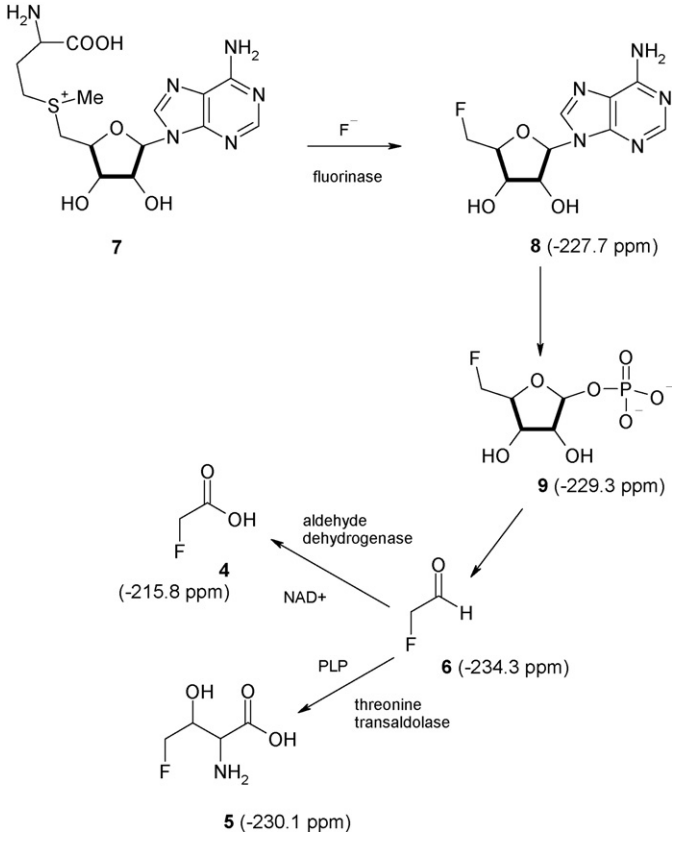
2.2. Detection and biosynthesis of natural organofluorine compounds

Although fluorine is the most abundant halogen in the earth's crust, it is not readily biologically available since most of it exists in insoluble minerals, such as CaF_2. As a consequence of this, and the physicochemical properties of fluorine, such as the high redox potential of fluoride ion and its propensity for hydration in aqueous solution, fluorinated natural products are much less abundant that those containing chlorine or bromine, of which there are over 3000 known [22]. Fluoroacetate **4**, which is produced by plant species native to Australia, South Africa and Brazil and by the bacterium *Streptomyces cattleya*, is the most common, and ^{19}F NMR has made the detection of this compound more convenient [23,24].

The study of fluoroacetate **4** and 4-fluorothreonine **5** biosynthesis in *S. cattleya* relied heavily on ^{19}F NMR. Early experiments using stable isotope-labelled precursors were made possible because the incorporation of deuterium from isotope-enriched glycerol or succinate into the fluoromethyl group of the fluorometabolites led to a shift in the fluorometabolite signals of 0.6 ppm (single label) and 1.2 ppm (double label). Similarly, incorporation of ^{13}C from isotope-enriched precursors resulted in characteristic splitting of the fluorometabolite signals [25]. In cell extracts it was possible to observe the formation of fluorometabolites from the common fluorinated precursor fluoroactealdehyde **6** in enzyme assays for fluoroacetaldehyde dehydrogenase [26] and threonine transaldolase [27]. Crucially, measurement of the activity of the fluorinase enzyme in this strain was only made possible by continuous ^{19}F NMR analysis after incubation with *S*-adenosylmethionine **7** and fluoride ion, and demonstrated that 5′-fluoro-5′-deoxyadenosine (5-FDA, **8**) was the initial product of fluorination [28,29]. Additional signals present in the ^{19}F NMR spectra recorded during these experiments suggested the accumulation of fluorinated intermediates in the biosynthetic pathway. Subsequently, 5-fluoro-5-deoxy-D-ribose-1-phosphate **9** was identified as the next intermediate, which probably resulted from the action of a nucleoside phosphorylase on 5-FDA (Fig. 1) [30]. The fluorometabolite biosynthetic pathway in *S. cattleya* is summarised in Scheme 3.

2.3. Fate of fluorinated drugs

Increasing numbers of pharmacologically active compounds are fluorinated [31], and ^{19}F NMR is a useful tool for determining the metabolic fate of these compounds *in vivo*. The classical fluorinated drug is 5-fluorouracil **10**, which is therapeutically applied to solid tumours and its metabolism has been extensively studied. The mechanism of action is the *in vivo* conversion of 5-fluorouracil to fluoronucleotides, which inhibit an enzyme required for DNA replication, thymidylate synthase; rapidly dividing cancerous cells are therefore particularly susceptible. The degradation of 5-fluorouracil to excretory products has been closely monitored, and ^{19}F NMR has identified several fluorometabolites in plasma and urine collected from patients treated with this drug (Fig. 2) including 5,6-dihydroxyuracil **11**, α-fluoro-β-ureidopropionic acid



Scheme 3. Biosynthesis of fluorometabolites in *Streptomyces cattleya*.

12, α-fluoro-β-alanine **13**, fluoride ion, 2-fluoro-3-hydroxypropionic acid **14**, fluoroacetate **4**, *N*-carboxy-α-fluoro-β-alanine **15** and the deoxycholic acid conjugate of **13** [32]. The degradation of 5-fluorouracil to fluoride, fluoroacetate **4** and 2-fluoro-3-hydroxypropionic acid **14** probably occurs via the formation of α-fluoro-β-alanine **13**. *N*-carboxy-α-fluoro-β-alanine can be generated by the non-enzymatic reaction of **13** with bicarbonate, and biliary excretion of **13** probably results in the formation of the deoxycholic acid conjugate. Scheme 4 summarises the likely pathway of degradation. 5-Fluorouracil is cardiotoxic, which might be a consequence of the catabolism of the drug to fluoroacetate.

In drug discovery studies, it is important to have an understanding of the metabolism of the compound before proceeding with clinical studies. Since animal models are becoming less attractive, microbial models of mammalian metabolism are increasingly used. Corcoran et al. [33] examined the microbial transformation products of model fluorinated pharmaceutical compounds by ^{19}F NMR. Of the 48 strains examined, four effectively degraded the fluorinated compounds and the number and nature of the catabolites could be readily assessed using this technique, for example, 5-fluoroindole was transformed completely by *Streptomyces* sp. 3992E to yield five fluorometabolites, and no defluorination occurred (Fig. 3). Such a method might be useful in rapidly assessing the metabolic profiles of potential drugs prior to further development. In addition, ^{19}F NMR can be used in screening studies for novel drugs from natural sources. For example, Tarrago et al. [34] used ^{19}F NMR to screen for inhibitors of prolyl oligopeptidase (POP), which is an enzyme implicated in neuropsychiatric disorders, in extracts of plants traditionally used in Chinese medicine. These researchers designed a fluorinated substrate analogue *Z*-Gly-Pro-Phe-4-(CF_3)-NH_2, the hydrolysis of which can be followed by ^{19}F NMR, since the product formed (Phe-4-CF_3) has a different chemical shift to the substrate. This assay system avoids the pitfalls of colorimetric and fluorimetric assays, which can yield false positive and negatives. The investigators screened the extracts from 10 traditional Chinese medicinal plants using this method and determined that seven contained potential POP inhibitors. In a subsequent study using this assay method [35], the alkaloid berberine was identified as the POP inhibitor from extracts of *Rhizoma coptidis*. The same screening principle has also been applied to the discovery of compounds that inhibit HIV-1 protease, which is an enzyme required for the production of infectious virus particles and is a potential therapeutic target. A substrate based on the conserved cleavage site of the protease was synthesised based on the sequence SQNFP, and included a fluoromethyl group on the aromatic ring of the phenylalanine residue since cleavage occurs between phenylalanine and proline. The cleavage of the substrate by HIV-1 protease could be followed using ^{19}F NMR, which recorded distinguishable signals of substrate and product, the intensities of which varied depending if an inhibitor was present or not [36].

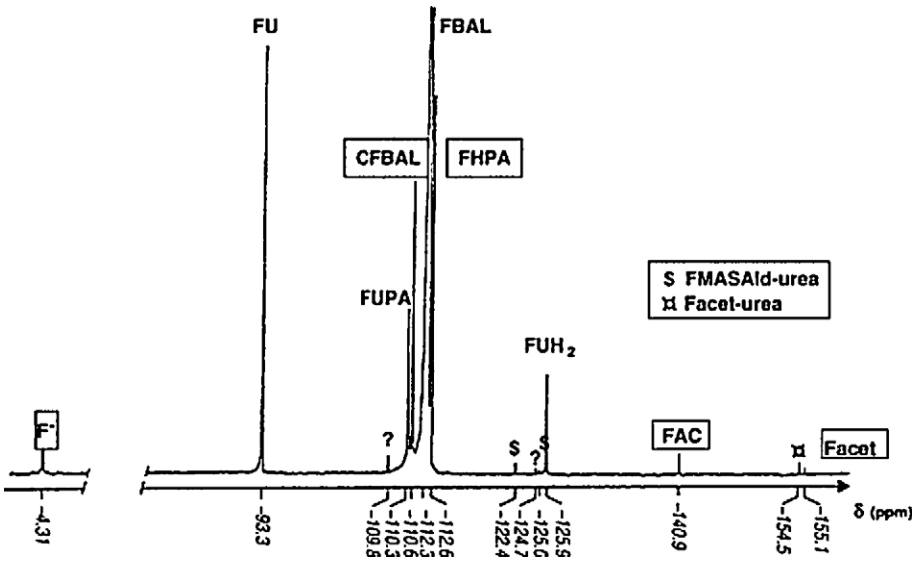
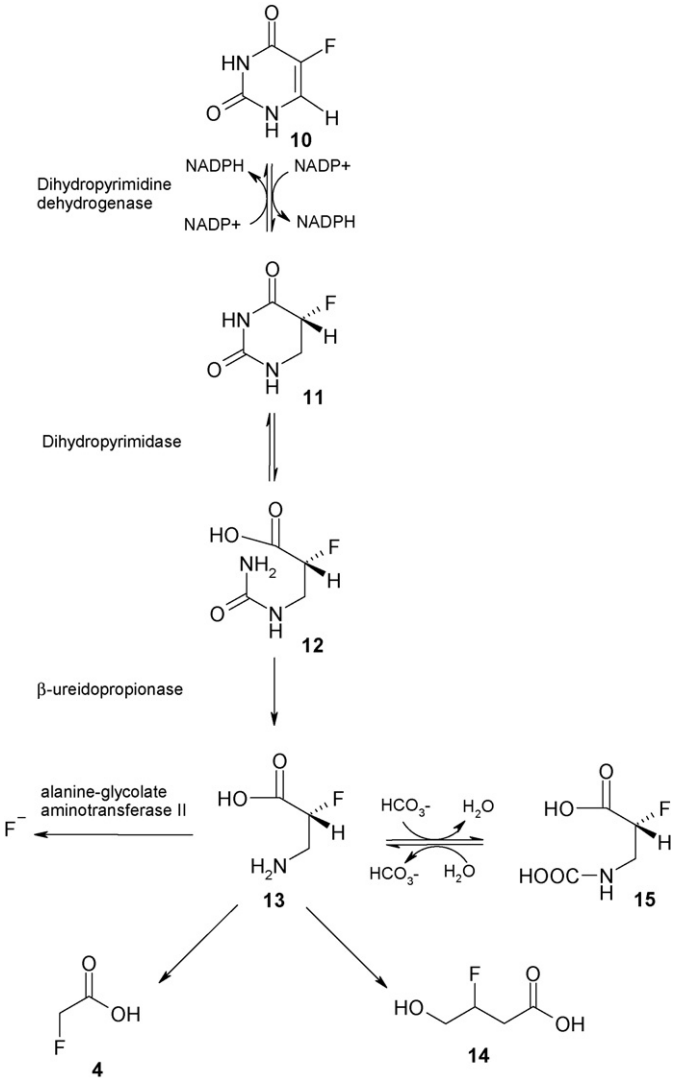


Fig. 2. ^{19}F NMR spectrum of fluids from a patient treated with 5-fluorouracil; Facet, fluoroacetaldehyde. Reprinted from [32] with permission from Elsevier.



Scheme 4. *In vivo* catabolism of 5-fluorouracil.

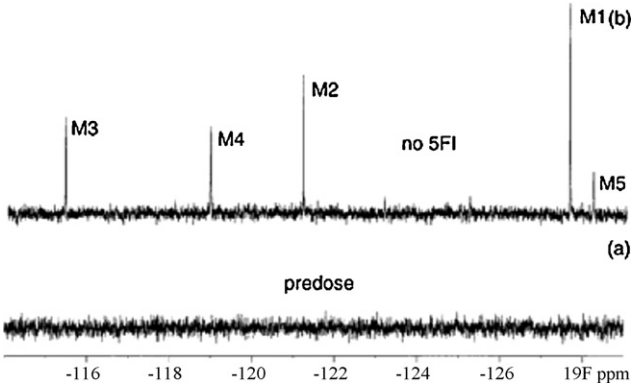


Fig. 3. ^{19}F NMR of transformation products of 5-fluoroindole in cultures of *Streptomyces* sp. 3992E. The fluorinated metabolites are labelled M1–M5 according to their relative concentrations [29]. Reproduced by permission of the Royal Society of Chemistry.

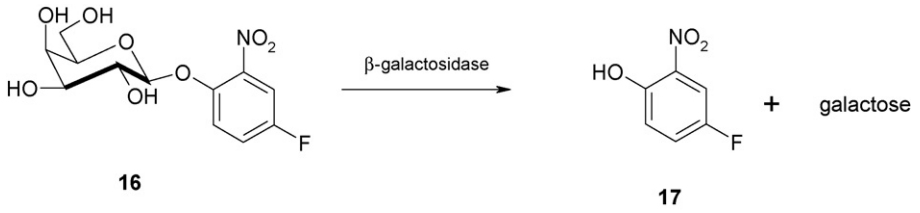
2.4. Assaying gene expression

Reporter genes, such as *lacZ*, which codes for the enzyme β-galactosidase, are inserted into vectors because they are easy to detect and demonstrate that transformation of the host cell has been successful. There are colorimetric methods available to detect the expression of this gene, but these are not appropriate for determining gene expression *in vivo*, since histological processing is required to visualise the colour. The molecule 4-fluoro-2-nitrophenyl-β-D-pyranose **16**, which is the fluorinated analogue of the colorimetric substrate for β-galactosidase, was successfully demonstrated as a possible reporter molecule for detection of β-galactosidase activity, using ^{19}F NMR to detect the product [37]. Cleavage of the glycosidic bond results in the formation of *p*-fluoro-*o*-nitrophenol **17**, which has a chemical shift difference of >3.6 ppm compared with the substrate (Scheme 5). This assay system has been applied to the detection of LacZ in transfected human PC3 prostate tumour cells [38]. Signal-to-noise was improved with the incorporation of a trifluoromethyl group instead of a single fluorine atom [39], but the relative chemical shift response to glycosidic bond cleavage was smaller (<1.2 ppm). One drawback to this assay system is that the product is toxic and causes some cells to lyse. However, other less toxic fluorinated reporter molecules, based on 5-fluoropyridoxol as the aglycone, have been designed, and were found to yield ^{19}F NMR-observable products after hydrolysis by β-galactosidase [40].

Biologically-based cancer therapies have been developed that employ non-toxic 5-fluorocytosine, which is delivered to the site of the tumour together with a recombinant bacterium such as *Salmonella typhimurium* that expresses cytosine deaminase. This results in the intratumoural conversion of 5-fluorocytosine to the toxic 5-fluorouracil. ^{19}F NMR provides a means to monitor the successful conversion of the 5-fluorocytosine by recombinant organism, without the need for biopsy [41].

2.5. Physiological measurement

The application of ^{19}F NMR to the *in vivo* measurement of physiological parameters such as pH, ion concentrations and tissue oxygenation is of major clinical relevance. These measurements rely on changes in the chemical shifts of fluorinated reporter molecules in response to, for example, binding a metal ion, or variation in relaxation rates, for example between anoxic and oxygen rich environments. These aspects of ^{19}F NMR have been recently comprehensively reviewed [42] and will not be elaborated upon further here.



Scheme 5. Hydrolysis of 4-fluoro-2-nitrophenyl-β-D-pyranose by β-galactosidase.

3. Binding studies

3.1. Introduction

Developing a detailed understanding as to the ways in which biological molecules interact with one another is crucial to both elucidating their function and the mechanisms by which act. ^{19}F NMR has become a valuable tool for probing interactions between molecules in a variety of biological systems. The following section provides an overview of how ^{19}F NMR has been used in this area.

3.2. Binding interactions involving proteins

^{19}F NMR represents an attractive option for studying the interactions that can occur between proteins and a variety of biological molecules such short peptides and nucleic acids. The general advantages of ^{19}F NMR have already been discussed but in relation to the study of proteins it has emerged as a useful technique to study large proteins that cannot easily be probed by conventional NMR. An essential element to establishing this particular technique has been the development of procedures that give access to ^{19}F labelled proteins. In general two approaches are routinely used to achieve ^{19}F protein labelling. The first of these involves the chemical modification of existing amino acids residues such as cysteine within the desired protein. An example of this chemical labelling approach was reported by Thomas and Boxer who prepared trifluoroacetyl-labelled cysteine mutants of myoglobin that were then used to probe nitric oxide binding [43]. The reagent 2,2,2-trifluoroethanethiol has also been used in a similar manner to chemically incorporate a fluorine label into the protein rhodopsin via disulfide bond formation with cysteine residues [44]. In both of these examples some biosynthetic manipulation of the native proteins was performed prior to the chemical introduction of the fluorine label. This was required to either remove unwanted cysteines to allow site-specific incorporation of the fluorine label or to introduce a cysteine residue at the desired site of investigation.

The second method involves the biosynthetic incorporation of synthetic ^{19}F-labelled amino acids [45]. The biological methods pioneered by Shultz and co-workers to incorporate unnatural amino acids into proteins are well documented in the literature and will therefore not be discussed in this review [46]. One disadvantage of with this technique is that it has proven difficult to achieve site-specific incorporation of the ^{19}F labelled amino acids. Several groups are currently trying to develop methodology to address this problem and recently the first reported site-specific incorporation of a fluorinated amino acid (trifluoromethyl-L-phenylalanine **20**) was reported by Mehl and co-workers [47]. A variety of ^{19}F-labelled amino acids have been incorporated using protein expression but the majority of the studies that have been carried out have utilised aromatic amino acids (Fig. 4) [48]. This is most likely because they are readily available either commercially or they are easily accessible via synthetic protocols.

The steric demands imparted by a hydrogen to fluorine substitution are minimal but a significant change in the electronic properties of a molecule can be observed. Consequently, the effects that ^{19}F-labelling has on the activity of on the native protein have been investigated and discussed in several papers [49]. In general however incorporation of a fluorine atom does not appear to alter the native protein structure but there are rare exceptions [50].

Although fluorinated aromatic amino acids have been typically favoured as ^{19}F NMR probes in both protein binding and conformational studies there are also examples of fluorinated aliphatic amino acids being utilised in this area. One of the first examples of a fluorinated aliphatic amino acid being used as a ^{19}F NMR probe was reported by Feeney et al. in 1996 [51]. In this study

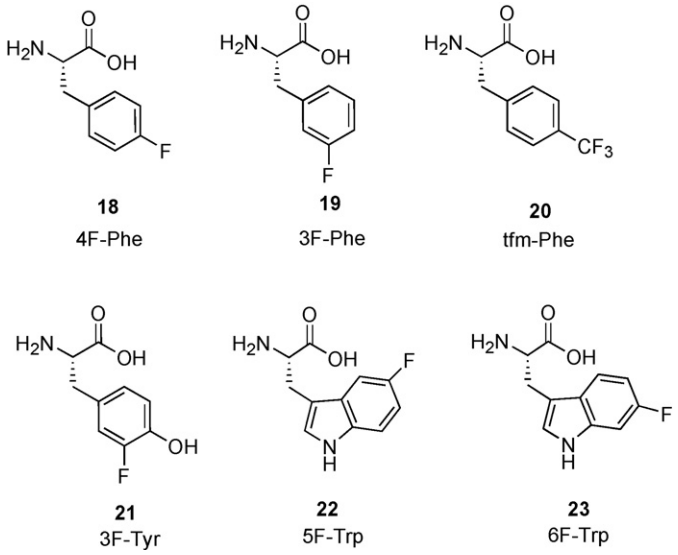


Fig. 4. Chemical structures of ^{19}F-labelled aromatic amino acids commonly used in ^{19}F NMR studies of proteins.

synthetic (2*S*, 4*S*)-5-fluoroleucine **24** (5F-Leu **24**) (Fig. 5) was incorporated into the enzyme dihydrofolate reductase from *Lactobacillus casei*.

In addition to leucine two fluorinated methionine analogues trifluoromethionine **25** [52] and difluoromethionine **26** [53], and 4-fluorohistidine **27** [54] have all been utilised as ^{19}F NMR probes. Recently Kirk and co-workers developed a biosynthetic procedure that could be used to incorporate both 4-fluorohistidine **27** and its isomer 2-fluorohistidine **28** into *E. coli* proteins [55]. The presence of a fluorine atom lowers the pK_a of the side chain of both 4F-His **27** and 2F-His **28** from approximately 6.0–6.5 to 1 and 3 respectively [56]. Thus **27** and **28** offer a novel way in which to directly explore general acid/base catalysis within enzymatic systems. This is not a new concept but its development as a general technique has been hampered by the fact fluorinated histidine had to be incorporated into the protein using synthetic methodology [54,57]. However, it is likely that the development of a biosynthetic approach to incorporate both **27** and **28** into proteins may generate a renewed interest in their application as ^{19}F NMR probes. In recent years developments in the field of synthetic fluorine chemistry have also emerged that have provide routes to a wide range of fluorinated

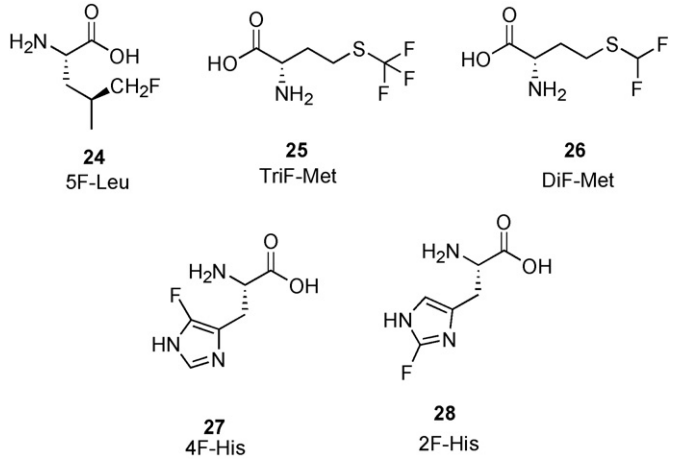


Fig. 5. Chemical structures of ^{19}F-labelled aliphatic amino acids that have been utilised as ^{19}F NMR probes.

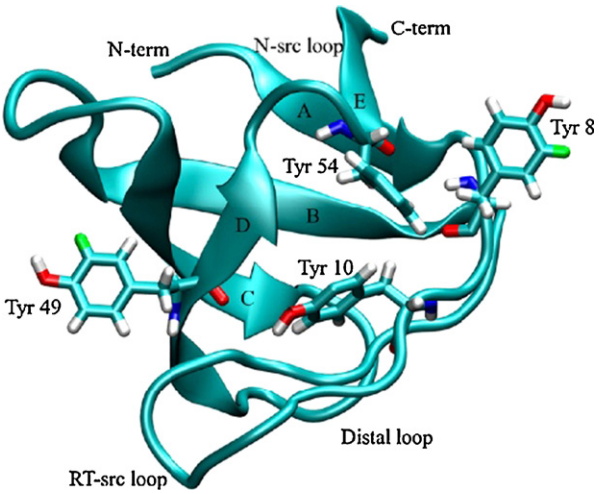


Fig. 6. Ribbon diagram of the Fyn SH3 domain indicating the locations of the four 3F-tyrosine residues. Reprinted from [59]. © 2007, with permission from Elsevier.

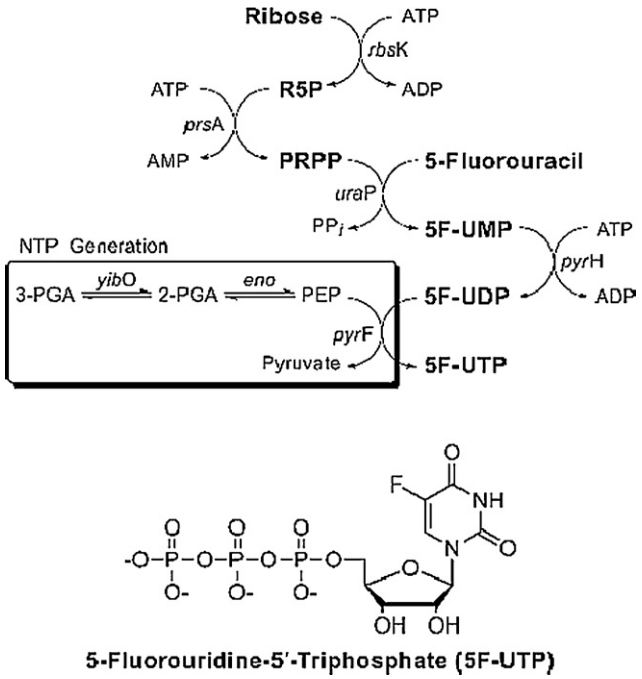
amino acids [58] providing and ever expanding tool-box of ^{19}F NMR probes for protein studies.

^{19}F NMR has been used to study the binding interaction between a variety of biological molecules and ^{19}F-labelled proteins. A recent example of this was reported by Prosser and co-workers who utilised ^{19}F NMR to probe the binding interactions between a short peptide and the enzyme Fyn tyrosine kinase [59]. This enzyme has been found to play a critical role in signal transduction processes and regions of the protein the so called SH2 and SH3 domains, have been identified as being responsible for modulating the signal transduction via various binding interaction [60]. Prosser et al. were able to biosynthetically incorporate four 3-fluoro-tyrosines into the SH3 domain of the protein (Fig. 6) [59]. Each 3-fluoro-tyrosine had a distinct ^{19}F signal and assignment of each signal was achieved by sequential mutation of each tyrosine residue to a non-fluorinated phenylalanine [59]. ^{19}F NMR was then used to study the binding interaction between the ^{19}F-labelled protein and a short proline-rich peptide known to interact with the SH3 domain.

3.3. Protein–DNA binding interactions

To elucidate the mechanisms by which enzymes process DNA substrates, developing a clear understanding about the interactions and conformational dynamics that occur between these two macromolecules is fundamental [61]. However, one of the major challenges in this field is developing analytical techniques that can be effectively employed to monitor dynamic interactions in these large complexes. Again the unique properties of the fluorine atom allow ^{19}F NMR spectroscopy to circumvent the major problems associated with studying molecules of this size. Methods to access fluorinated-proteins have already been discussed but in studying binding interactions in enzyme–DNA complex access to fluorinated DNA are also desirable. A wealth of synthetic chemistry permits access to a range of fluorinated nucleosides that can act as ^{19}F NMR probes [62,63]. Fluorinated nucleoside analogues such as the 2,4-difluorotoluene analogue of thymine have also been used as probes to study various properties of DNA [64].

A DNA strand containing 5-fluoro-cytosine was used as a ^{19}F NMR probe to study the mechanism of DNA methylation [65]. From the ^{19}F NMR data collected it was possible to gain detailed structural information about the conformation that the DNA adopts when in contact with the enzyme's active site. In this particular case it was not possible to obtain similar information for X-ray studies. Stivers and co-workers have also utilised ^{19}F NMR



Scheme 6. Enzymatic synthesis of 5F-UTP Reprinted with permission from [72]. © 2007, American Chemical Society.

spectroscopy to study the conformational dynamics of DNA bound to vaccinia type IB topoisomerase [66]. Topoisomerases are family of enzymes that are essential for relaxing the superhelical strain in genomic DNA [67]. Stivers and co-workers were able to use ^{19}F NMR to show that the ^{19}F-DNA that interacts with the topoisomerase enzyme exists in two conformational states that are in slow exchange. It is worth noting that 5-fluoro-2′-deoxyuridine that was used as the ^{19}F NMR probe in this case did not affect the binding affinity of the topoisomerase enzyme for DNA. It is also possible to utilise ^{19}F NMR to study DNA/enzyme binding interactions by incorporating the fluorinated label onto the protein rather than the DNA. This strategy was employed by Summers and co-workers who investigated the conformational effects that a fluorine labelled mutant of the metalloregulator protein MerR experienced upon DNA binding [68].

Studies that investigate the binding interactions between RNA and proteins have also been reported [69]. The fluorinated nucleosides required as building blocks for either the chemical or biosynthetic preparation of ^{19}F-labelled RNA samples can be accessed by a variety of synthetic procedures [70]. Efficient enzymatic syntheses of 2-fluoroadenine-5′-triphosphate [71] 5-flourouridine-5′-triphosphate (Scheme 6) and 5-fluorocytodine-5′-triphosphate have also been reported [72].

3.4. Small molecule–protein binding interactions

As it has already been shown ^{19}F NMR can be an extremely powerful and versatile tool for investigating binding interactions between biological macromolecules. ^{19}F-labelled proteins can also be used to probe binding interactions between a known/potential substrates or small molecule and an enzyme's active site [73]. Alternatively such binding interactions can also be investigated by incorporating the fluorine-label required for analysis onto the small molecule. This can be achieved with relatively easily as a wide range of both a fluorinating agents and fluorinated building blocks are commercially available [74]. Ease of incorporation coupled with the fact that the fluorine atom displays a large chemical shift dispersion has led to ^{19}F NMR being developed as an

analytical tool to screen libraries of potential ligands for biological targets. This is an expanding area of research and an excellent review of the applications of ^{19}F NMR in the field of drug discovery was recently published by Dalvit [75].

3.5. Small molecule–DNA/RNA binding interactions

DNA has long been a target for the development of drug molecules [76] and consequently considerable effort has been directed towards developing analytical techniques to investigate the binding of small molecules to specific DNA targets. At present molecules that can stabilise DNA G-quadruplexes (quartet of four guanines) and thus act inhibitors of the telomerase enzyme are attracting considerable attention due to their potential anti-cancer properties [77]. Searle and co-workers have shown that ^{19}F NMR can successfully be used as analytical technique in this flourishing field of research [78].

In recent years RNA has also is become an attractive target for the development of drug molecules [79] and analytical techniques that can be easily employed to identify RNA binders are of great interest in this field. A variety of techniques have been utilised to date to try and identify potential RNA binders and these include fluorescence [80] mass-spectroscopy-assisted screening [81] and recently ^{19}F NMR [78]. Micura and co-workers outlined a general approach by which ^{19}F NMR could be used to identify potential RNA binders [82]. The technique involved incorporating 2′-deoxy-2′-fluoro nucleosides into a target RNA sequence. If a small molecule has the ability to bind to the RNA sequence then a shift in the ^{19}F signal is detected.

It was also shown that it was possible to successfully carry out binding studies using this ^{19}F NMR approach even with the larger RNA sequences (53-nt) (Fig. 7), something that is difficult to do with other analytical methods such as ^{1}H NMR. Although this work used known RNA binders such as tobramycin [83] it provided a clear proof of concept showing that the ^{19}F NMR could be applicable to the screening of combinatorial libraries to search of novel RNA binders.

4. Structural analysis of macromolecules using ^{19}F NMR

4.1. Background

Studying the conformational dynamics that macromolecules such as proteins and DNA display in solution is fundamental to developing a greater understanding of the biological processes in which with take participate in their natural environment. ^{19}F NMR again offers several advantages in this area. One of the major advantages that ^{19}F NMR has in this field is that it can be used to study biological macromolecules that would be too large to investigate easily by other analytical techniques such as ^{1}H NMR.

4.2. Protein folding

Since its initial applications in the late 1960s and early 1970s ^{19}F NMR has established itself as powerful and versatile analytical tool for studying protein structure and conformational changes [84]. For further background information regarding the development of ^{19}F NMR in the field of protein studies readers are directed towards the earlier reviews written by Gerig [85] and Danielson and Falke [86].

Protein folding is a spontaneous process by which linear polypepetides adopt a 3-dimensional structure [87] For many proteins the 3-dimensional structure is essential for proper protein function. Problems with protein folding (i.e. protein misfolding) can lead to a number of human diseases including cystic fibrosis and Alzheimer's, and therefore it is currently an important area of research [88]. Optical techniques such as fluorescence and circular dichroism have widely been used to study protein-folding but the information provided by such approaches relates more to the global properties of the protein during folding or unfolding [89]. In order to obtain more detailed information about localised sites, side chains or specific residues then complementary spectroscopy techniques must be also employed and the most commonly used is NMR [90].

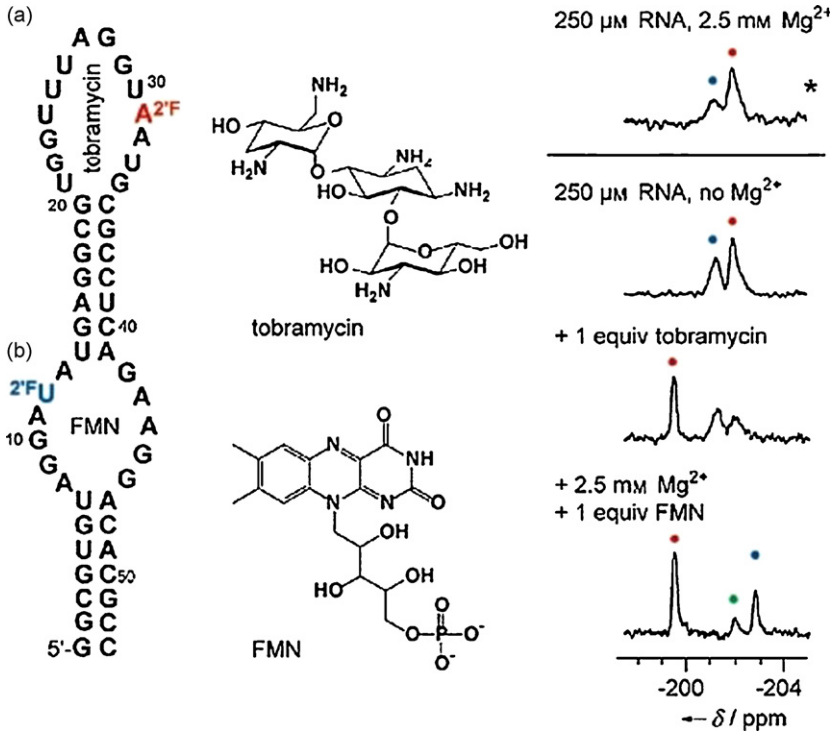


Fig. 7. ^{19}F NMR spectra showing the RNA binding selectivity of two ligands: (a) tobramycin and (b) FMN [82]. © Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.

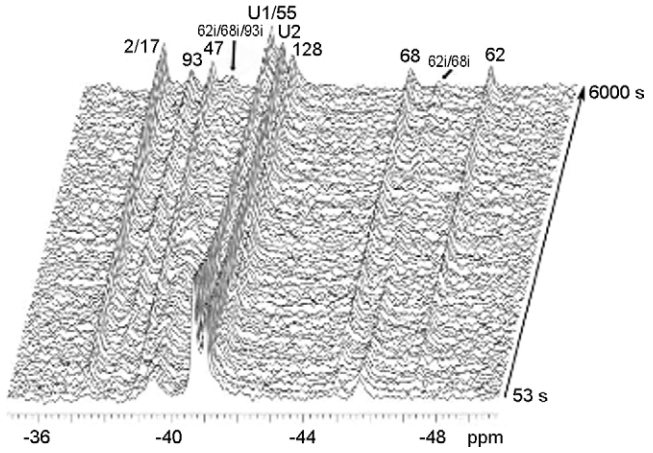
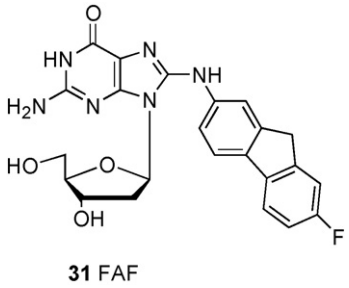


Fig. 8. Real time ^{19}F NMR spectra showing the folding kinetics of a G121 V mutant of intestinal fatty acid binding protein. Reproduced from [95]. © 2007, The National Academy of Sciences.

The techniques that have been developed to produce and study fluorine labelled proteins for binding studies (see Section 3) have also found applications in the field of protein conformational studies [91]. In this field the application of ^{19}F as a probe offers several advantages over the more commonly utilised nuclei (^{1}H, ^{13}C and ^{15}N). Firstly, ^{19}F NMR can be used to provide information about local conformational changes within specific regions of a protein during folding [92]. A recent example of this can be seen in the studies carried out by Hore and co-workers into the native and denatured states of green fluorescent protein [93]. A uniformly 3-fluorotyrosine-labelled GFP was produced biosynthetically that contained ten different 3F-Tyr labels. As fluorine has a large chemical shift dispersion it was possible to differentiate and assign the signals belong to all of the incorporated 3F-Tyr residues within the protein. This allowed information about specific localised regions as well as a global view of the protein structures during folding to be established.

One of the most challenging aspects about studying protein folding relates to trying to identify intermediate structures. The difficulties of detecting these intermediates are largely related to their transient nature and low concentration. However, ^{19}F NMR can successfully provide information about intermediates on the protein-folding pathway that are invisible to other spectroscopic techniques. Here the strength of ^{19}F NMR is due to the fact that 1D-^{19}F NMR signals can be resolved on a short time scale (seconds) allowing the direct detection of intermediates in real time [94]. Furthermore if multiple fluorinated amino acids are incorporated into the proteins in question then multiple localised sites can be monitored at the same time. This then allows a complete picture of the folding within the whole molecule to also be established. An excellent example highlighting the advantages of this technique was recently reported by Li and Frieden [95]. By utilising 4-fluoro-



31 FAF

Fig. 10. Chemical structure of FAF **31**.

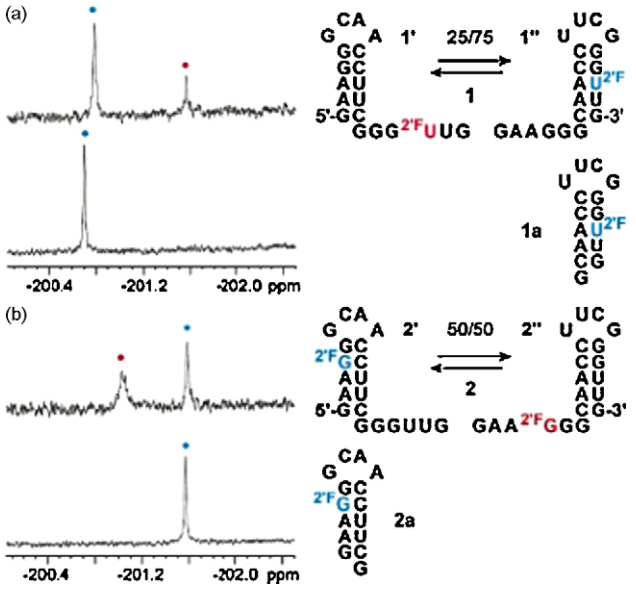
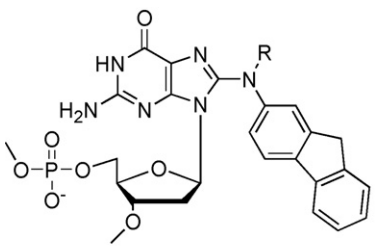


Fig. 11. ^{19}F NMR spectra (^{1}H decoupled) showing that two conformations exisit for (a) 2′-F uridine labelled RNA and (b) 2′-F guanosine RNA. Reprinted with permission from [109]. © 2007, American Chemical Society.

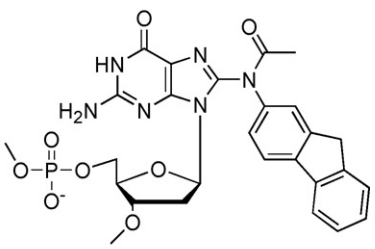
phenylalanine **18** as a ^{19}F NMR probe they were able to monitor the sequential folding of intestinal fatty acid biding protein (Fig. 8) [95].

The position at which the fluorine atom sits on the aromatic ring can also varied to help provide additional information during ^{19}F NMR protein conformational studies. For example aromatic ring-flipping which can provide valuable structural information about protein folding [96] and this can be monitored by using 2-fluoro-phenylalanine as a probe [97]. In contrast if 4-fluoro-phenylalanine is used as a probe then subtle movements of the benzyl ring that cannot be attributed to ring flipping can also be detected [97].

One of the most intense areas of research with relation to protein structure and conformation undoubtedly relates to the



29 AF



30 AFF

Fig. 9. Chemical structures of DNA adducts dG-C8-AF **29** and dG-C8-AAF **30**.

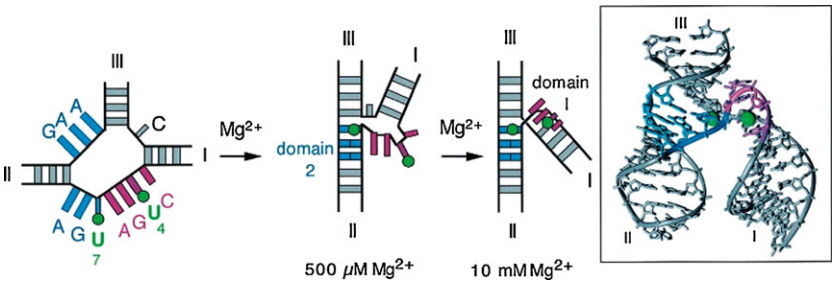


Fig. 12. Folding mechanism of the hammerhead ribozyme. Incorporated 5-fluorouracil probes are shown in green. Reproduced from [110]. © 2001, The National Academy of Sciences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

field of membrane protein [98]. In this field the majority of the work carried out has utilised X-ray crystallography [99] but other spectroscopic techniques such as solution phase NMR [100] have also been investigated. ^{19}F NMR has also been shown to be a method well suited to provide information about immersion depth and topology of membrane proteins. For further information about the applications of ^{19}F NMR in this area readers are directed towards the recent review by Prosser et al. [101]. As an analytical tool in the study of membrane proteins ^{19}F NMR spectroscopy can still be viewed as an emerging technique but it has already shown considerable promise [102] and it will undoubtedly find further and increasing application in this field. It is also worth highlighting that several research groups are actively involved in developing solid-state ^{19}F NMR methodology to obtain structural information about membrane proteins [103].

4.3. DNA and RNA secondary structure

Adduct formation in DNA is a well defined characteristic of mutation and it is also recognised as being an important step in the inhibition of both mutagenesis and carcinogenesis [104]. Arylamines such as 2-aminofluorenes and its analogues are a carcinogenic class of compounds that have been found to target DNA and form C8-substituted dG adducts [105]. Two major dG adducts are formed *in vivo*: AF [N-(2′-dexoyguanosin-8-yl)-2-aminofluorene] **29** and AFF [N-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene] **30** (Fig. 9). Each of these DNA adducts adopt several different conformations that co-exist in a dynamic equilibrium. Developing a detailed understanding of the conformational-heterogeneity of these mixtures is critical as it has believed to be one of the factors that is ultimately responsible for determining the final mutagenic outcome of the DNA adduct.

Although analytical techniques such as ^{1}H NMR [106] and crystallography [107] have provided value structural information about this type of DNA adduct they have proven to be unsuitable for investigating the complex dynamic equilibrium that exists been the various conformations of the adducts. In order to overcome this problem Cho and co-workers investigated the use of a set of fluorine containing arylamines such as FAF **31** (Fig. 10) that could be used as probes in dynamic ^{19}F NMR spectroscopy studies of this type of DNA adduct [108]. Incorporation of fluorine atom was found not to effect the carcinogenic properties of the parent arylamines [108a] and importantly the dG-C8-FAF adduct was shown to adopt a similar conformation to its non-fluorinated analogue [108a]. After incorporation into the target DNA sequence the fluorinated probes were found to be very effective and they can be used to provide valuable information about the conformational heterogeneity of the various DNA adducts.

Understanding the secondary structure dynamics that exist in RNA can also provide valuable information about the function of these molecules. Structural studies on 15-kDa RNAs can routinely carried out using a combination of isotopic labelling (^{13}C and ^{15}N) and multidimensional heteronuclear NMR. However, with RNAs above 15-kDa it is often difficult to interpret the NMR data recorded. To simplify data analysis ^{19}F NMR has been developed by researchers as an alternative spectroscopic technique. As previously discussed a variety of fluorinated nucleoside are readily available and can be incorporated into an RNA sequence to produce a suitably ^{19}F-labelled RNA sample for ^{19}F NMR analysis. The work of Micura and co-workers provides a good example of how this ^{19}F-RNA can then be utilised in conjunction with ^{19}F NMR to probe the secondary structure equilibria of RNA molecules [109]. Micura et al. showed that ^{19}F NMR could easily distinguish between the double helix and single strand regions of various ^{19}F-RNA samples in solution (Fig. 11).

^{19}F NMR has also been used to monitor conformational changes during RNA-mediated catalysis [110]. In order to carry out its catalytic function RNA must be adopt a precise 3-dimemsional structure and understanding the nature of this structure and how it arises is obviously of interest as it underpins the catalytic mechanism. Lilley and co-workers incorporated 5-fluoro-uridine as a ^{19}F NMR sensitive probe into two critical positions within the RNA sequence of interest so that an internal view rather than a global overview of the folding process could be obtained (Fig. 12). By using ^{19}F NMR to monitor the movement of the incorporated fluoro-nucleotides detailed information about localised structural changes that were occurring during the folding of the RNA were investigated. In addition when the ^{19}F NMR data was viewed collectively it was also possible to establish an overview about the sequential steps involved in the RNA folding process. This work demonstrates that ^{19}F NMR provides a relatively straightforward and non-perturbing method to access detailed structural information about RNA folding.

5. Concluding remarks

In this review we have demonstrated that organofluorine compounds, and consequently ^{19}F NMR, have very important roles in research at the chemical–biological interface. In addition to direct applications of organofluorine compounds that are used as pharmaceuticals and agrochemicals, they are employed as probes to explore enzyme mechanism, metabolic pathways and biomolecular interactions. The usefulness of the technique as applied to biological systems is somewhat limited by its sensitivity and the naturally low concentration of biological molecules (e.g. metabolites). Nevertheless, technological advances, such as cryoprobes, allow analysis of metabolites at physiological concentrations. Of minor concern are the differences in reporting chemical shifts by individual researchers; not all chemical shifts are recorded using $CFCl_3$ as a reference, which can lead to confusion for others attempting to confirm observations, or comparison of chemical shifts between separate studies. Future collaborations between fluorine chemists and biologists will be considerably facilitated by an appreciation of, and access to, ^{19}F NMR technology.

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