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^{19}F NMR metabolomics for the elucidation of microbial degradation pathways of fluorophenols

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Of all NMR-observable isotopes ^{19}F is the one most convenient for studies on the biodegradation of environmental pollutants and especially for fast initial metabolic screening of newly isolated organisms. In the past decade we have identified the ^{19}F NMR characteristics of many fluorinated intermediates in the microbial degradation of fluoroaromatics including especially fluorophenols. In the present paper we give an overview of results obtained for the initial steps in the aerobic microbial degradation of fluorophenols, i.e. the aromatic hydroxylation to di-, tri- or even tetrahydroxybenzenes ultimately suitable as substrates for the second step, ring cleavage by dioxygenases. In addition we present new results from studies on the identification of metabolites resulting from reaction steps following aromatic ring cleavage, i.e. resulting from the conversion of fluoromuconates by chloromuconate cycloisomerase. Together the presented data illustrate the potential of the ^{19}F NMR technique for (1) fast initial screening of biodegradative pathways, i.e. for studies on metabolomics in newly isolated microorganisms, and (2) identification of relatively unstable pathway intermediates like fluoromuconolactones and fluoromaleylacetates. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 22–34.

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Introduction

Of all NMR-observable isotopes ^{19}F is the one perhaps most convenient for studies on biodegradation of environmental pollutants [18,23]. This originates from several advantages of the ^{19}F NMR isotope compared to other nuclei. First, the intrinsic sensitivity of the ^{19}F nucleus is high and almost comparable to that of the ^{1}H nucleus. Sensitivity is an important issue, because xenobiotics and their metabolites are usually present at relatively low concentrations. Second, for fluorine the sensitivity is further increased due to the absence of background signals, because biological systems do not contain ^{19}F NMR visible fluorinated endogenous compounds. This implies that all resonances observed can be unambiguously ascribed to the xenobiotic compound and its biodegradation products. Third, the ^{19}F nucleus has a broad chemical shift range of about 700 ppm. This is large compared to the chemical shift range of, for example ^{1}H (15 ppm) and that of ^{13}C (250 ppm). The chemical shift of a ^{19}F nucleus is highly sensitive to its molecular surroundings resulting in widespread changes in chemical shifts upon biotransformation of fluorinated organic compounds, thereby reducing the chances of peak overlap. The number of fluorine-containing xenobiotics and environmental contaminants has strongly increased during recent decades [16,33]. A number of fluorine-containing drugs are currently in clinical use, a large number of fluorinated compounds are intermediates or end products in the synthesis of industrial and agrochemicals, and many fluorine-containing biodegradation products result from the chemical and/or microbial degradation of these fluorinated chemicals [1–3,9].

The objective of the present paper is to give an overview of the potential of ^{19}F NMR as a technique to study the aerobic microbial degradation of fluorinated environmental pollutants, and to summarise the ^{19}F NMR signals of metabolites identified over the last decade in the course of our studies on the initial steps in the metabolic pathways for fluorophenol degradation [4,6,10,12,20, 24,25]. In addition the paper presents new data on ^{19}F NMR studies of intermediates formed beyond these first two steps, i.e. formed upon conversion of fluoromuconates by a next enzyme in the pathway, chloromuconate cycloisomerase.

Experimental procedures

Chemicals

Phenol was purchased from Merck (Darmstadt, Germany). 2-Fluoro-, 3-fluoro- and 4-fluorophenol were obtained from Janssen Chimica (Beerse, Belgium). 2,4-Difluoro-, 2,5-difluoro-, 3,4-difluoro-, 2,4,5-trifluorophenol and 2,3,4,5-tetrafluorophenol were purchased from Aldrich (Steinheim, Germany). 2,3-Difluoro-, 2,6-difluoro-, 3,5-difluoro-, 2,3,4-trifluoro-, 2,3,5-trifluoro-, 2,3,6-trifluoro- and 3,4,5-trifluorophenol were obtained from Fluorochem (Derbyshire, UK). Pentafluorophenol and catechol were obtained from Sigma (St. Louis, MO, USA). Fluorocatechols and fluoromuconates were prepared as previously described [6] from the corresponding fluorophenols using purified phenol hydroxylase from *Trichosporon cutaneum* and by incubating the fluorocatechols thus formed with catechol 1,2-dioxygenase from *Pseudomonas arvilla* C-1.

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Purification of enzymes

Chloromuconate cycloisomerase from *Rhodococcus opacus* 1cp was purified essentially as reported before [29]. Phenol hydroxylase was purified from *T. cutaneum*, essentially as described by Sejlitz and Neujahr [27]. Catechol 1,2-dioxygenase was purified from *P. arvilla* C-1 as described by Nakai *et al.* [19].

Isolation and growth of Rhodococcus strains

The various *Rhodococcus* strains were isolated, maintained and grown as described previously [8,14].

Incubations with purified enzymes

Incubations of fluorophenols with purified phenol hydroxylase and catechol 1,2-dioxygenase were performed at 30°C in closed reaction vessels to prevent evaporation of phenolic substrates. Incubations contained (final concentrations) 0.1 M potassium phosphate pH 7.6 unless indicated otherwise, 0.7 mM fluorophenol added as 1% (v/v) of a 70 mM stock solution in dimethyl sulphoxide, 10 μM FAD, 1 mM ascorbic acid and 1 mM NADPH. The incubations were started by the addition of catalytic amounts of the purified enzymes (5.3×10^{-3} U/ml phenol hydroxylase and 7.2×10^{-3} U/ml catechol 1,2-dioxygenase, respectively). The samples thus obtained were analysed by ^{19}F NMR, showing significant formation of the corresponding fluoromuconate(s), and then used for the incubation with purified chloromuconate cycloisomerase. Upon addition of a catalytic amount of chloromuconate cycloisomerase (9.6×10^{-3} U/ml) the conversion of fluoromuconate was followed in time by recording ^{19}F NMR spectra while incubating the sample in the NMR spectrometer at 30°C.

Incubations with whole cells

Incubations with whole cells were performed as previously described [8] with 0.7 mM (final concentration) of the

Table 1 Overview of ^{19}F NMR resonances of phenolic substrates and metabolites formed in the biotransformation of fluorophenols identified so far. Chemical shift values were obtained in 0.1 M potassium phosphate pH 7.6 and presented in parts per million relative to $CFCl_3$

Compound	Chemical shift	Reference
2-Fluorophenol	−141.9	[24]
3-Fluorophenol	−116.5	[24]
4-Fluorophenol	−129.1	[20]
2,3-Difluorophenol	−167.1 (F2); −143.1 (F3)	[20]
2,4-Difluorophenol	−137.4 (F2); −126.3 (F4)	[20]
2,5-Difluorophenol	−147.4 (F2); −122.5 (F5)	[20]
2,6-Difluorophenol	−139.1	[20]
3,4-Difluorophenol	−140.9 (F3); −154.2 (F4)	[20]
3,5-Difluorophenol	−114.7	[20]
2,3,4-Trifluorophenol	−162.5 (F2); −164.9 (F3); −153.6 (F4)	[20]
2,3,5-Trifluorophenol	−173.0 (F2); −142.0 (F3); −122.2 (F5)	[20]
2,3,6-Trifluorophenol	−164.1 (F2); −144.4 (F3); −148.5 (F6)	[20]
2,4,5-Trifluorophenol	−147.1 (F2); −153.5 (F4); −143.7 (F5)	[6]
3,4,5-Trifluorophenol	−139.7 (F3/5); −177.9 (F4)	[20]
2,3,5,6-Tetrafluorophenol	−170.5 (F2/6); −149.3 (F3/5)	[25]
Pentafluorophenol	−171.7 (F2/6); −173.2 (F3/5); −186.0 (F4)	[6]
2-Chloro-4-fluorophenol	−127.7	present study
3-Chloro-4-fluorophenol	−132.6	[20]
4-Chloro-2-fluorophenol	−138.7	present study
4-Chloro-3-fluorophenol	−118.7	[20]
3-Fluorocatechol	−140.4	[20,24]
4-Fluorocatechol	−126.7	[20]
3,4-Difluorocatechol	−164.0 (F3); −152.1 (F4)	[20]
3,5-Difluorocatechol	−137.9 (F3); −125.8 (F5)	[20]
3,6-Difluorocatechol	−145.3	[20]
4,5-Difluorocatechol	−152.2	[20]
3,4,5-Trifluorocatechol	−162.2 (F3); −176.1 (F4); −154.1 (F5)	[20]
3,4,6-Trifluorocatechol	−171.5 (F3); −153.1 (F4); −146.0 (F6)	[20]
Tetrafluorocatechol	−170.9 (F3/6); −180.8 (F4/5)	[20]
3-Chloro-4-fluorocatechol	−129.4	[20]
4-Chloro-3-fluorocatechol	−141.9	[20]
4-Chloro-5-fluorocatechol	−129.9	[20]
2-Fluoro-*p*-hydroquinone	−138.7	[24]
2,3-Difluoro-*p*-hydroquinone	−163.4	[24]
2,5-Difluoro-*p*-hydroquinone	−144.9	[10]
2,6-Difluoro-*p*-hydroquinone	−137.1	[24]
2,3,5-Trifluoro-*p*-hydroquinone	−169.7 (F2); −162.2 (F3); −144.4 (F5)	[24]
Tetrafluoro-*p*-hydroquinone	−171.2	[25]
4-Fluoro-1,2,3-trihydroxybenzene	−149.2	[12]
5-Fluoro-1,2,3-trihydroxybenzene	−125.9	[12]
3-Fluoro-1,2,4-trihydroxybenzene	−161.7	[4]
5-Fluoro-1,2,4-trihydroxybenzene	−149.8	[4]
6-Fluoro-1,2,4-trihydroxybenzene	−138.4	[4]
6-Fluoro-1,2,3,5-tetrahydroxybenzene	−170.1	[4]

Table 2 Overview of ^{19}F NMR resonances of fluoromuconates formed in the biotransformation of fluorophenols identified so far. Chemical shift values were obtained in 0.1 M potassium phosphate pH 7.6 and are presented in parts per million relative to $CFCl_3$

Compound	Chemical shift	Reference
2-Fluoromuconate	−111.8	[6]
3-Fluoromuconate	−108.2	[6]
2,3-Difluoromuconate	−142.3 (F2); −134.5 (F3)	[6]
2,4-Difluoromuconate	−107.4 (F2); −102.5 (F4)	[6]
2,5-Difluoromuconate	−111.1	[6]
3,4-Difluoromuconate	−121.1	[6]
2,3,4-Trifluoromuconate	−137.0 (F2); −131.4 (F3); −106.2 (F4)	[6]
2,3,5-Trifluoromuconate	−141.9 (F2); −106.9 (F3); −127.5 (F5)	[6]
Tetrafluoromuconate	−134.5 (F2/5); −130.2 (F3/4)	[6]

fluorophenol. Samples were taken at different time intervals. The reaction was stopped by freezing the samples into liquid nitrogen. Samples were stored at −20°C until analyzed. Before ^{19}F NMR analysis, samples were defrosted and centrifuged (5 min 13,000×*g* at 0°C).

^{19}F NMR measurements

^{19}F NMR measurements were performed on a Bruker DPX 400 NMR and some measurements on a Bruker AMX 300 spectrometer as previously described [6,30]. The temperature during the measurements was 7°C. A dedicated 10-mm ^{19}F NMR probehead was used in both NMR instruments. The spectral width used for the ^{19}F NMR measurements was between 20.000 and 50.000 Hz depending on the sample of interest. The number of datapoints used for data-acquisition was 65536. Pulse angles of 30° were used. Between 2000 and 60 000 scans were recorded, depending on the concentrations of the fluorine-containing compounds and the signal to noise ratio required. The sample volume was 1.72 ml, containing 1.4 ml sample, 200 μl 0.8 M potassium phosphate, pH 7.6, 100 μl of $[^2H]_2O$, used as deuterium lock and 20 μl of 8.4 mM 4-fluorobenzoate, added as an internal standard. Concentrations of the various metabolites were calculated by comparison of the integrals of the ^{19}F NMR resonances of the metabolites to the integral of the 4-fluorobenzoate resonance. Chemical shifts are reported relative to $CFCl_3$. The resonance of the internal standard 4-fluorobenzoate was set at −114.2 ppm with respect to $CFCl_3$. The Lorentzian lineshape of the resonances was converted to a Gaussian-type lineshape in order to improve resolution for careful determination of the coupling constants. In the cases where the coupling constants could be determined directly from the datasets, the resolution was at least 0.2 Hz. ^{1}H decoupling was achieved with a Waltz16 decoupling sequence. ^{19}F NMR chemical shift values of the various fluorine-containing compounds were identified on the basis of added authentic reference compounds or as described previously, see reference numbers in Tables 1 and 2.

Results and discussion

Aromatic hydroxylation

The initial step in the aerobic microbial degradation of (halogenated) phenol derivatives is their aromatic hydroxylation to metabolites suitable for subsequent ring fission. Thus, formation of *ortho*- and *para*-dihydroxy- and even the formation of tri- and tetrahydroxybenzenes may occur. In the course of our studies using isolated enzymes many mono-, di- and even tri- and tetrahydroxybenzenes have been identified by ^{19}F NMR. The use of purified enzymes greatly facilitated identification of the various intermediates. Table 1 summarizes the ^{19}F NMR characteristics of these phenolic intermediates identified. Combined ^{1}H coupled ^{19}F NMR measurements proved a valuable tool to identify the exact hydroxyl substituent patterns. Figure 1a presents an example of using ^{1}H coupled ^{19}F NMR to identify the substituent patterns in a new type of monofluorotrihydroxybenzene (m/z=144.0223) identified in the biodegradation of 3-fluorophenol and 4-fluorophenol by cells from *R. opacus* 1cp [12]. Only the fluorine in 5-fluoro-1,2,3-trihydroxybenzene will give rise to a triplet due to splitting by two *ortho* protons ($^3J_{F-H}$=10.1 Hz).

Figure 2 presents another example of a study in which ^{19}F NMR proved to be an efficient tool to identify the first two steps for the catabolism of 4-hydroxybenzoate in the yeast *Candida parapsilosis*. Using the fluorinated substrate analogue, 2-fluoro-4-hydroxybenzoate it could be demonstrated that, in contrast to the well-known bacterial pathways and to what was previously assumed, metabolism of (2-fluoro)-4-hydroxybenzoate in *C. parapsilosis* proceeds through initial oxidative decarboxylation to give a 1,4-dihydroxybenzene followed by conversion to a 1,2,4-trihydroxybenzene type ring fission substrate [4,10]. From these examples it follows that ^{19}F NMR provides an efficient analytical tool for screening and elucidation of the aromatic hydroxylation steps of metabolic biodegradation pathways preceding ring cleavage.

Comparison of the ^{19}F NMR data obtained for halogenated phenolic derivatives revealed systematic effects of the introduction of hydroxyl groups at respectively the *ortho, meta* or *para* position with respect to a fluorine substituent in the aromatic ring. A hydroxyl group introduced at the position *ortho, meta* or *para* with respect to a fluorine substituent shifts the ^{19}F NMR resonance by −23.1±0.3 ppm, +1.3±0.4 ppm and −11.2±0.7 ppm, respectively [20]. This observation can, in addition to ^{1}H coupled ^{19}F NMR splitting patterns, often be of help in identifying the nature of as yet unidentified hydroxylated metabolites. Thus the ^{19}F NMR resonance of 5-fluoro-1,2,3-trihydroxybenzene (5-fluoropyrogallol), identified in Figure 1a was predicted to be at −125.4 ppm, whereas the ^{19}F NMR resonances of other monofluorotrihydroxybenzene isomers were expected at different positions than the −125.9 ppm of the resonance actually observed and identified also on the basis of its ^{1}H coupled ^{19}F NMR splitting pattern, as that of 5-fluoro-1,2,3-trihydroxybenzene (Figure 1b) [12]. Clearly this preliminary assignment on the basis of expected chemical shift modifications due to hydroxyl incorporation, provides an efficient and additional tool in the identification of unknown hydroxylated aromatic metabolites.

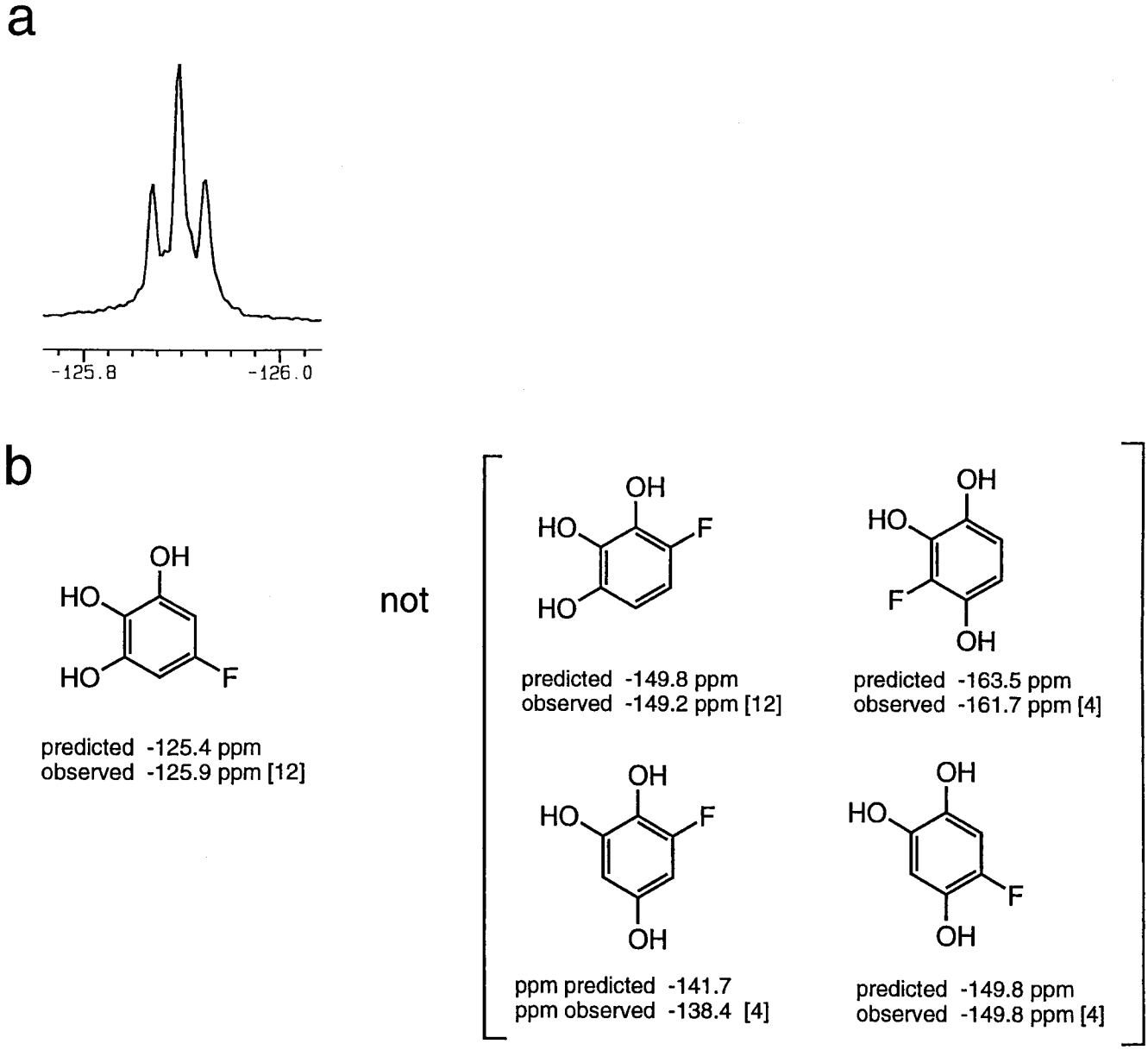

Figure 1 Example of identification of the substituent pattern in a trihydroxyfluorobenzene metabolite ($m/z = 144.0223$) by ^{1}H coupled ^{19}F NMR, (a) ^{1}H–^{19}F NMR splitting pattern and (b) ^{19}F NMR chemical shift values predicted and/or observed for different monofluorotrihydroxybenzenes. For further details see Finkelstein *et al.* [12].

Ring cleavage by intradiol dioxygenases: conversion of fluorocatechols to fluoromuconates

The subsequent metabolic step in the degradation of (halogenated) aromatics consists of ring cleavage by intra- and extradiol dioxygenases. Especially the action of catechol intradiol dioxygenases has been studied by ^{19}F NMR. Table 2 summarizes the various fluorinated muconate metabolites identified so far [6]. Most of these metabolites could be identified using purified enzymes and or specific microorganisms in which the subsequent reaction step converting the halogenated muconates proved to be rate limiting. Figure 3 illustrates that ^{19}F NMR provides an efficient tool to characterise the metabolomics of 2,3-difluorophenol in three species of *Rhodococcus*. The figure also illustrates that the method quickly reveals the nature of the rate-limiting step in the overall biodegradation of the compound. Subsequent studies on the biodegradation of chlorinated analogues were then facilitated and more easily focused on the species and compound of interest [7,13,14].

Reaction steps following aromatic ring cleavage: conversion by chloromuconate cycloisomerase to give fluoromuconolactones

Figure 4 presents an overview of the reaction steps possibly following intradiol cleavage of fluorocatechol metabolites. Depending on the nature of the (chloro)muconate cycloisomerase involved and the position of the halogen substituent, different muconolactones can be expected [5,6,11,26,28,29,31,32]. Iden-

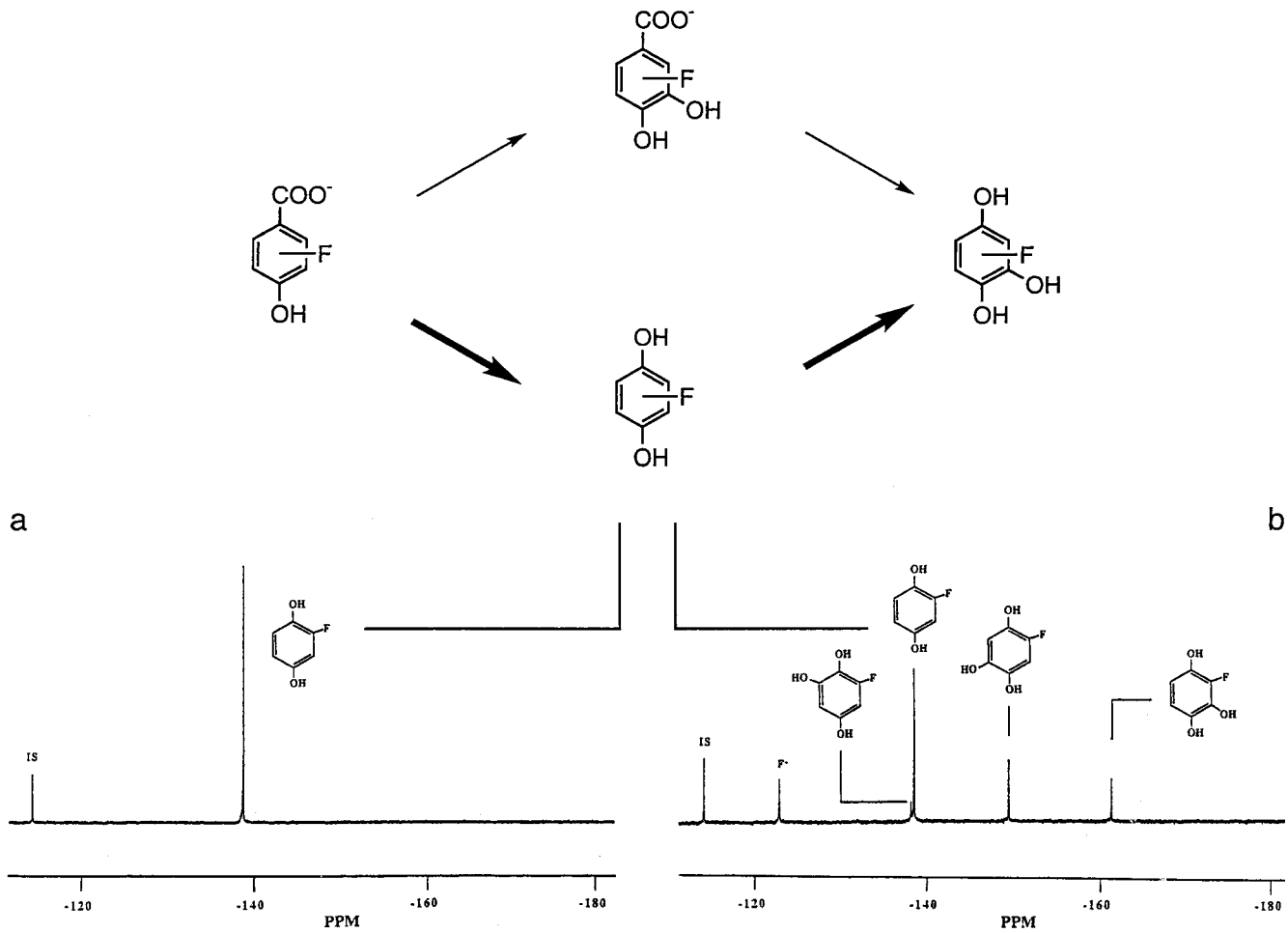

Figure 2 Use of ^{19}F NMR for elucidation of the pathway for formation of a hydroxyquinone metabolite from a 4-hydroxybenzoate by *C. parapsilosis*. Incubation of the fluorinated substrate analogue 2-fluoro-4-hydroxybenzoate with (a) purified 4-hydroxybenzoate hydroxylase from *C. parapsilosis*, and (b) purified 4-hydroxybenzoate hydroxylase plus 1,4-dihydroxybenzene hydroxylase from *C. parapsilosis*, identifying the pathway indicated with bold arrows and eliminating the upper one. For further details see van Berkel *et al.* [4].

tification of these muconolactones has generally been reported to be hampered by their unstable nature, although 4-fluoromuconolactone may be detected at decreased pH values with a half-life in the order of 30–50 h [28]. Thus, in order to extend the application of ^{19}F NMR metabolomics beyond the aromatic ring cleavage step, experiments were initiated on the conversion of fluoromuconates by chloromuconate cycloisomerase. Figure 5a presents the production of 3-fluoromuconate by incubation of 4-fluorophenol with purified phenol hydroxylase and catechol 1,2-dioxygenase in 0.1 M potassium phosphate pH 7.6. Upon addition of purified chloromuconate cycloisomerase from *R. opacus* 1cp to this incubation a swift conversion of all 3-fluoromuconate was observed, accompanied by formation of a fluorinated metabolite with its resonance at -113.1 ppm. The insert in Figure 5b presents the ^{1}H coupled ^{19}F NMR peak of this resonance, indicating J_{F-H} coupling constants of 19.5 and 14.5 Hz. Taking into account that (i) due to the essential axial position of the fluorine substituent with respect to the lactone ring, the vicinal C3–H, C4–F coupling of 4-fluoromuconolactone was not detected [28], and that (ii) the anisochrony in the diastereotypic methylene protons results in unequal $^{3}J_{H-F}$ coupling values [28], the metabolite with its resonance at -113.1 ppm formed from 3-fluoromuconate by chloromuconate cycloisomerase in potassium phosphate at pH 7.6 can be identified as 4-fluoromuconolactone. The difference in the $^{3}J_{H-F}$ values for the methylene protons in the present ^{19}F NMR study (19.5 and 14.5 Hz) and in the previously published ^{1}H NMR measurements (8.1 and 23.1 Hz) [28] may be related to a change in conformation due to the different solvents used, i.e. phosphate buffer versus $CDCl_3/CD_3COCD_3$. Of interest is that when the same experiment was performed in Tris–HCl pH 7.2, accumulation of 4-fluoromuconolactone was no longer observed and the conversion of 3-fluoromuconate was accompanied by formation of fluoride anions only. This suggests that the stabilisation of 4-fluoromuconolactone reported before in different buffers of various pH values [28] was not only caused by decreased pH [21] but also by a change in buffer type from Tris to phosphate. In Tris buffer, known to be optimal for chloromuconate cycloisomerase activity [17,29], stability of the fluoromuconolactone appeared significantly lower than in phosphate buffer. The time-dependent degradation of 4-fluoromuconolactone presented in Figure 6 supports that also in phosphate buffer 4-fluoromuconolactone is ultimately defluorinated resulting in stoichiometric formation of fluoride anions. The fact that the rate of defluorination of 4-fluoromuconolactone was unaffected by the addition of a ten-times-extra amount of

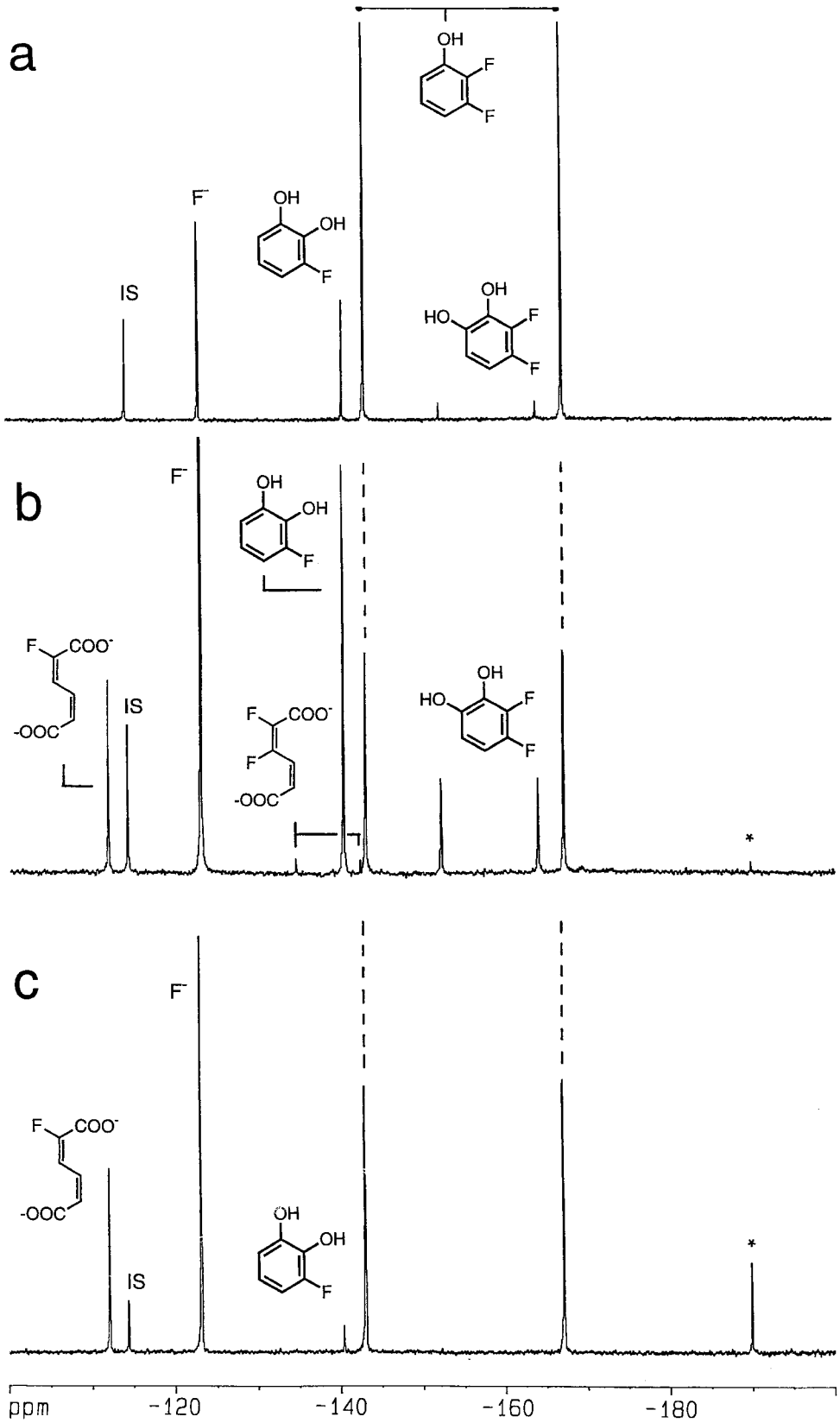

Figure 3 ^{19}F NMR spectra showing the differential metabolic pathways and intermediate accumulation upon conversion of 2,3-difluorophenol by (a) *R. opacus* 1G, (b) *R. corallinus* 135 and (c) *R. opacus* 1cp. This illustrates the use of ^{19}F NMR for the fast initial screening of metabolic pathways in various organisms defining the species and enzymes of highest interest for further studies. The resonance marked IS is from the internal standard 4-fluorobenzoic acid. For further identification of the resonance marked with an asterisk see figure 7.

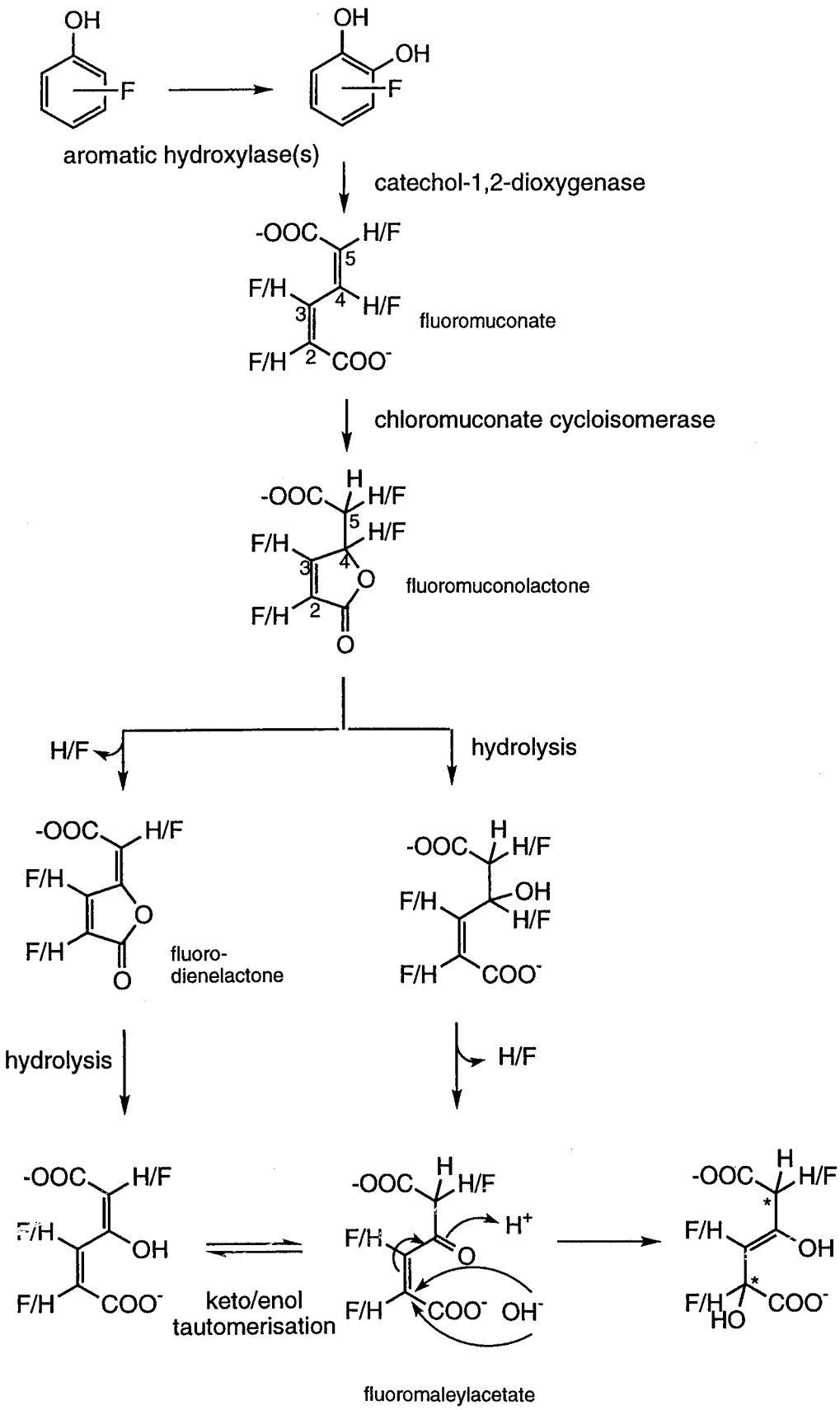

Figure 4 Schematic presentation of the degradation pathway of fluorophenols, including the chemical conversion of unstable fluoromuconolactones and fluoromaleylacetates formed upon the conversion of fluoromuconates by chloromuconate cycloisomerase. Note the formation of the set of diastereoisomers upon hydroxyl anion addition to the fluoromaleylacetate. For further details see text.

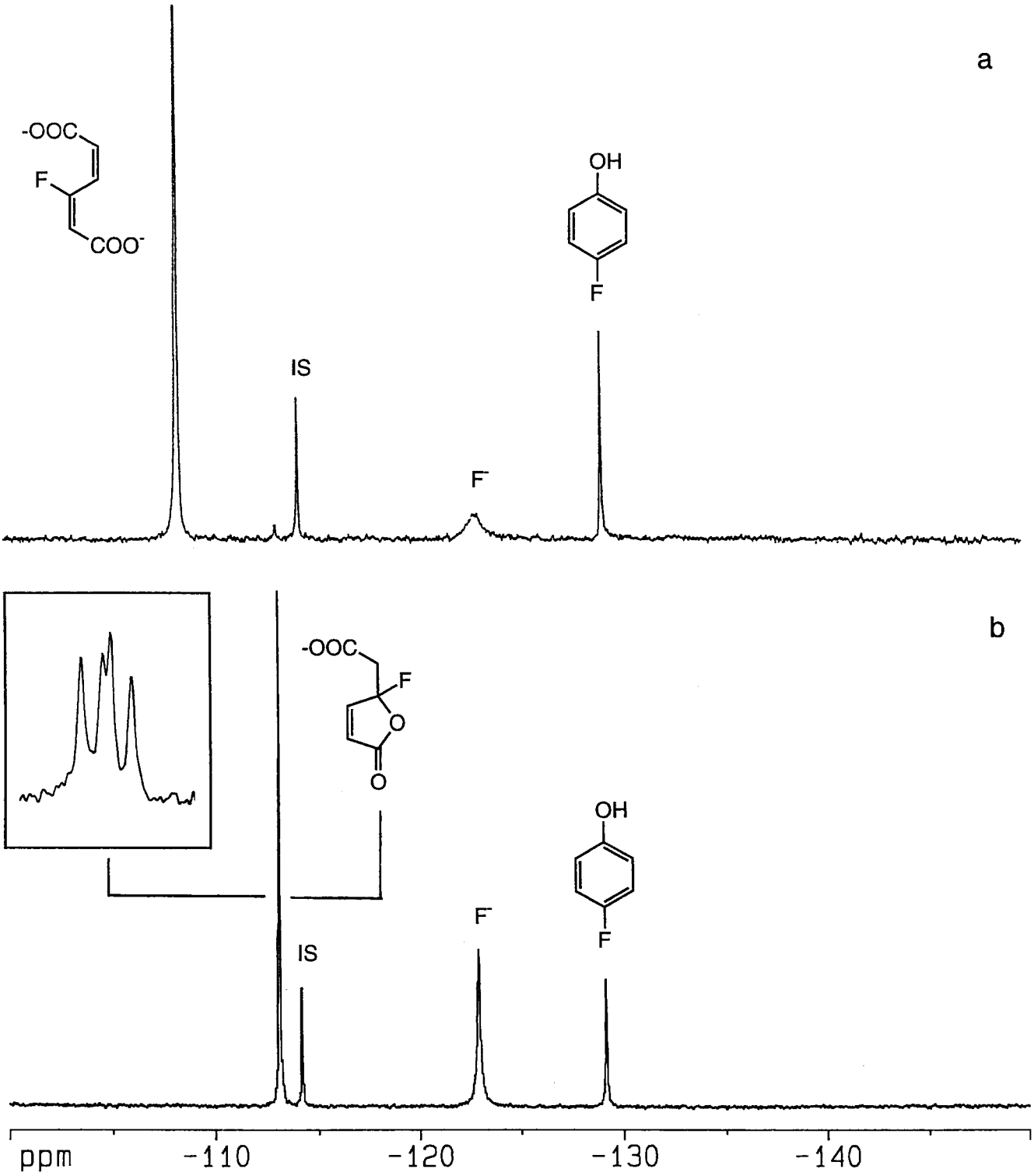

Figure 5 ^{19}F NMR spectra of incubations for (a) the formation of 3-fluoromuconate by incubation of 4-fluorophenol with purified phenolhydroxylase and catechol 1,2-dioxygenase and (b) the subsequent conversion of 3-fluoromuconate with chloromuconate cycloisomerase from *R. opacus* 1cp, all in phosphate buffer pH 7.6. The resonance marked IS is from the internal standard 4-fluorobenzoic acid. The insert in Figure b shows the ^{1}H coupled ^{19}F NMR splitting pattern of the resonance at − 113.1 ppm.

chloromuconate cycloisomerase indicates that the dehalogenation proceeds by a chemical not an enzymatic reaction. The fluoride anion elimination from 4-fluoromuconolactone and its further chemical conversion may proceed by the reaction routes depicted in Figure 4 analogous to the pathways suggested before [6,22,28].

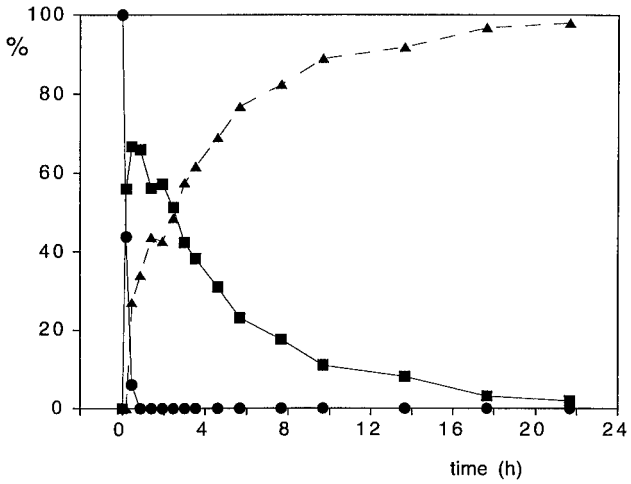

Figure 6 Time-dependent conversion of 3-fluoromuconate (– ● –) by chloromuconate cycloisomerase from *R. opacus* 1cp, accompanied by formation of 4-fluoromuconolactone (– ■ –) and stoichiometric formation of fluoride anions (– ▲ –) measured in potassium phosphate pH 7.6, at 30°C in the NMR spectrometer.

Reaction steps following aromatic ring cleavage: conversion by (chloro)muconate cycloisomerase to give fluoromaleylacetates and other degradation intermediates as products from fluoromuconolactones

Similar incubations performed with other fluoromuconates, gave different results, mainly due to the fact that the different fluoromuconolactones formed appeared to be even more labile than 4-fluoromuconolactone, and were observed only transiently and/or not at all. Introducing additional fluorine substituents into the molecules, and/or fluorine substituents at other positions enabled the detection of fluorinated intermediates beyond the level of the fluoromuconolactones and resulting from the further chemical degradation/conversion of the unstable fluoromuconolactones.

Thus, Figure 7 presents the time-dependent ^{19}F NMR results obtained for an incubation of 2,3-difluoromuconate prepared by incubation of 2,3-difluorophenol with phenol hydroxylase and catechol 1,2-dioxygenase, with chloromuconate cycloisomerase. The intermediate ultimately formed with resonance at -189.9 ppm and a $^{2}J_{H-F}$ coupling of 50.4 Hz, has recently been tentatively identified as the keto form of 5-fluoromaleylacetate [8]. Formation of this intermediate reflects the chemical hydrolytic dehalogenation of 4,5-difluoromuconolactone, a closed-ring metabolite formed from 2,3-difluoromuconate by chloromuconate cycloisomerase. Figure 7b reflects the transient formation of an unstable difluorinated intermediate (at -127.2 and -195.3 ppm) which may, in analogy to the results obtained for the conversion of 4-fluorophenol to 4-fluoromuconolactone (Figure 5) be tentatively ascribed to the unstable 4,5-difluoromuconolactone. However, closer inspection of the ^{19}F NMR resonance at -189.9 ppm revealed the presence of a second ^{19}F NMR signal only 0.07 ppm shifted with respect to the -189.9 ppm peak and also showing a ^{19}F–^{1}H coupling of 50 Hz (insert Figure 7c). The nature of this second resonance can only be explained upon analysing the ^{19}F NMR characteristics of the conversion of other fluoromuconates by chloromuconate cycloisomerase (see below).

Using the chloromuconate cycloisomerase of the present study, 2-fluoromuconate was not converted which is in agreement with previous observations on its lack of conversion of 2-chloromuconate [29,31]. In contrast, conversion of 2,4-difluoromuconate and 2,3,5-trifluoromuconate by chloromuconate cycloisomerase appeared again feasible. Figure 8a shows the enzymatic production of 2,4-difluoromuconate and 2,3,5-trifluoromuconate upon conversion of 2,3,5-trifluorophenol by phenol hydroxylase and catechol 1,2-dioxygenase. Addition of chloromuconate cycloisomerase to this incubation ultimately results in complete conversion of 2,4-difluoromuconate as well as of 2,3,5-trifluoromuconate (Figure 8b and c). The decrease in 2,4-difluoromuconate is accompanied by formation of a monofluorinated new intermediate with its ^{19}F NMR resonance at -145.4 ppm. Surprisingly, ^{1}H coupled ^{19}F NMR measurements did reveal the absence of any significant ^{1}H coupling for this fluorine resonance (insert Figure 8b). The conversion of 2,3,5-trifluoromuconate proceeds at a lower rate and is accompanied by formation of two difluorinated intermediates giving rise to four ^{19}F NMR resonances at, respectively, -142.0, -142.4, -189.9 and -190.5 ppm (Figure 8c). In analogy to the observations with 4-fluoromuconate and 2,3-difluoromuconate, conversion of 2,4- and 2,3,5-trifluoromuconate by chloromuconate cycloisomerase is accompanied by formation of a significant amount of fluoride anions reflecting efficient defluorination. Similar to the ^{19}F NMR resonance of the intermediate formed from 2,3-difluoromuconate, the resonances at -189.9 and -190.5 ppm both reveal a large 50/Hz $^{2}J_{F-H}$ coupling in proton coupled measurements (insert Figure 8c), indicating that these compounds both contain a fluorine substituent at a sp^3 carbon containing one hydrogen substituent [34]. And, in analogy to the resonance of the intermediate formed from 2,4-difluoromuconate, the two resonances formed from 2,3,5-trifluoromuconate at -142.0 and -142.4 ppm, do not reveal a significant proton coupling in ^{1}H coupled ^{19}F NMR measurements (insert Figure 8c). Because literature data and also ^{1}H–^{19}F NMR coupling constants generally reported for $^{3}J_{H-F}$ values over a double bond, such as the $^{3}J_{H-F}$ values in various related fluoromuconates [6,34] generally vary between 10–20 Hz, this absence of the ^{1}H–^{19}F coupling in the resonances at -142.0, -142.2 (Figure 8c) and -145.4 ppm (Figure 8b) exclude the identification of the metabolites formed as fluoromaleylacetates. This also requires reconsideration of the identification of the intermediate formed from 2,3-difluoromuconate at -189.9 ppm upon incubation with chloromuconate cycloisomerase for which, due to the lack of a fluorine in the C2–C3 part of the molecule (Figure 4), the information now obtained for the intermediates formed from 2,4-difluoro- and 2,3,5-trifluoromuconate was not revealed.

This formation of intermediates with almost identical splitting NMR characteristics can best be ascribed to the formation of so-called diastereoisomers, containing two asymmetric carbon atoms in their structure [15]. Figure 4 presents the reaction of (fluoro)maleylacetate with water/hydroxyl anions resulting in such diastereoisomers when at least C2/5 in the muconate contains a fluorine substituent. In the case of 2,3-difluoro- and 2,3,5-trifluoromuconate, formation of 5-fluoromaleylacetate and 2,5-difluoromaleylacetate followed by hydroxyl anion addition to C2 would result in a set of two diastereoisomers that can explain the ^{19}F NMR characteristics observed. The two products resulting from hydroxyl anion addition to 2,5-difluoromaleylacetate also give rise to a set of diastereoisomeric ^{19}F NMR signals around -142.0 and -142.4 ppm which do not show $^{3}J_{H-F}$ coupling because of the dihedral angle between the C3–H and the C2–F,

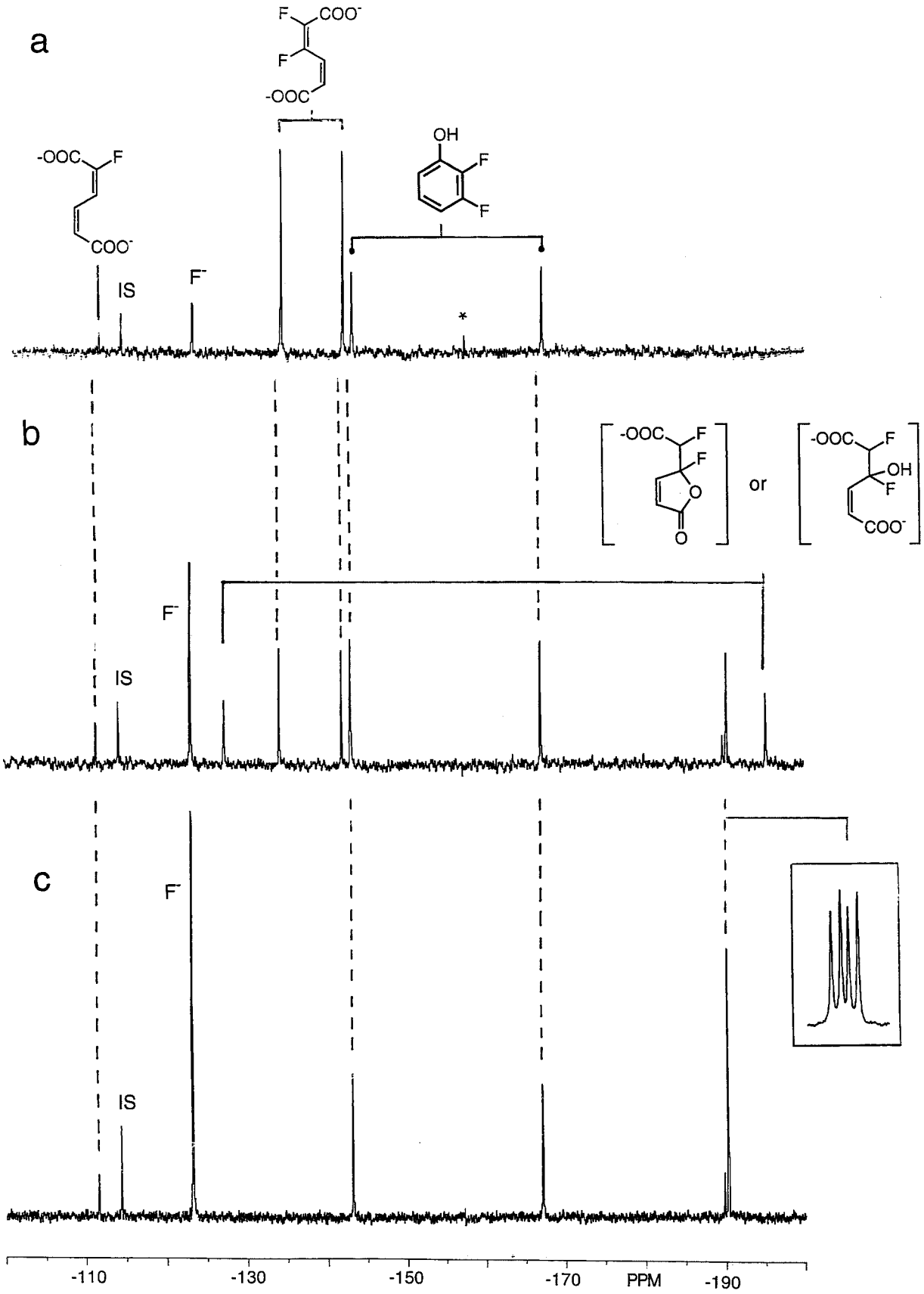

Figure 7 ^{19}F NMR spectra showing the time dependent conversion of 2,3-difluoromuconate by chloromuconate cycloisomerase from *R. opacus* 1cp at (a) $t = 0$, (b) $t = 1.5$ h and (c) $t = 6$ h. The resonance marked IS is from the internal standard 4-fluorobenzoic acid.

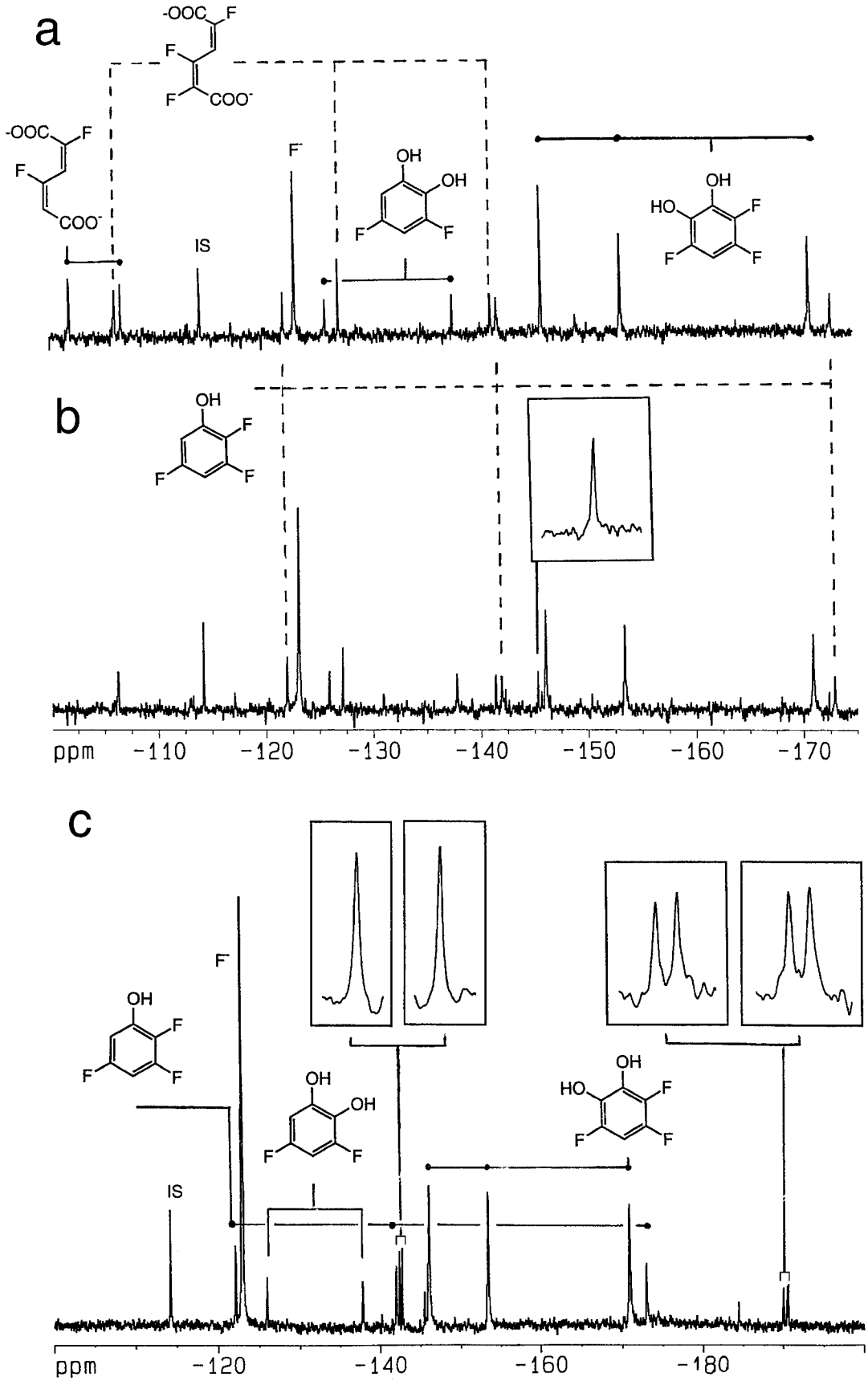

Figure 8 ^{19}F NMR spectra showing the time-dependent conversion of 2,4-difluoro- and 2,3,5-trifluoromuconate by chloromuconate cycloisomerase from *R. opacus* 1cp at (a) t=0, (b) t=3 h and (c) t=16 h. The resonance marked IS is from the internal standard 4-fluorobenzoic acid. Note the extended x-axis in figure c.

similar to what is observed for the vicinal C3–H, C4–F coupling in 4-fluoromuconolactone [28]. The water addition to the fluoromaleylacetates depicted in Figure 4 is also in line with the observation that for the product formed from 2,4-difluoromuconate no set of diastereoisomeric peaks is observed but only a single fluorine resonance at −145.4 ppm. Due to the absence of a fluorine at C5 of the corresponding 2-fluoromaleylacetate the C5 carbon is no longer asymmetric leaving the product mixture resulting from the hydroxyl attack as a set of pure stereoisomers, showing one ^{19}F NMR resonance. Clearly these ^{19}F NMR data indicate that not only the fluoromuconolactones but also the maleylacetates show high chemical instability and reactivity. The electrophilic reaction of the maleylacetates with hydroxyl anions depicted in Figure 4 is in line with the generally known high electrophilicity and reactivity of $\alpha-\beta$ unsaturated ketones including compounds such as ethacrynic acid [15]. The presence of the fluorine substituents in maleylacetate may further increase this electrophilicity of the maleylacetate enhancing its reactivity as depicted in Figure 4. It is important to stress that this chemical reaction of the fluoromaleylacetates now observed in incubations with purified enzymes need not necessarily occur to the same extent in microbial degradation pathways, where enzymes for subsequent conversion of the maleylacetates like maleylacetate reductase are present.

Together these studies reveal some of the ^{19}F NMR characteristics of intermediates formed beyond the mono-, di- and trifluorocatechols, and muconates. The data presented from our studies with the conversion of fluoromuconates by chloromuconate cycloisomerase show that identification of metabolites beyond the level of ring cleavage is possible but hampered by several factors. These include (i) absence of commercially available fluoromuconates and other substrates and reference compounds, and (ii) low chemical stability of several intermediates formed. Nevertheless, the results presented here now contribute to further development of the use of ^{19}F NMR as a direct and noninvasive technique to trap at least some of these intermediates, not only in our incubations with purified enzymes but also in incubations with whole cells. The results obtained so far illustrate the potential of ^{19}F NMR for a fast screening of the metabolomics of fluorinated aromatics by newly isolated strains. This facilitates selection of strains, enzymes and substrates for further studies, setting priorities for further work on enzymes and intermediates involved in the bioconversion of fluoro- and also chlorophenols.

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