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Targeted fluorination of a non-steroidal anti-inflammatory drug to prolong metabolic half-life

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In drug design, one way of improving metabolic stability is to introduce fluorine at a metabolically labile site. At the early stages of drug design identification of such sites is challenging, and a rapid method of assessing the effect of fluorination on a putative drug's metabolic stability would be of clear benefit. One approach to this is to employ microorganisms that are established as models of drug metabolism in parallel with the synthesis of fluorinated drug analogues. In this study we have used the filamentous fungus *Cunninghamella elegans* to identify the metabolically labile site of the non-steroidal anti-inflammatory drug flurbiprofen, to aid in the design of fluorinated derivatives that were subsequently synthesised. The effect of the additional fluorine substitution on cytochrome P450-catalysed oxidation was then determined via incubation with the fungus, and demonstrated that fluorine substitution at the 4' position rendered the drug inactive to oxidative transformation, whereas substitution of fluorine at either 2' or 3' resulted in slower oxidation compared to the original drug.

Fluorine's unique physicochemical properties, such as its size, electronegativity and strength of the carbon-fluorine bond, make it an important element in the design of drugs and other biologically active compounds. Consequently, fluorine is present in approximately 20 % of pharmaceuticals, including the blockbuster drugs Prozac and Lipitor, and 30 % of agrochemicals [1]. Owing to fluorine's size, incorporation into biologically active compounds has only minor steric effects, but its high electronegativity leads to significant electronic consequences. One reason for incorporating fluorine into drug molecules is to slow their metabolic processing though the cytochrome P450 (CYP)-mediated detoxification steps in the liver, thus extending their potency [2]. This requires knowledge of where the metabolically labile sites in the drug compound are, which is available for drugs that have already undergone ADME studies, and also increasingly for compounds that are at the very

early stage of development (hit-to-lead). One approach to fluorinating metabolically labile sites is to activate the site via CYP-catalysed oxidation enabling further functionalization via deoxofluorination [3]. We have recently reported a straightforward chemical-microbial method for identifying metabolically sensitive sites on drug-like compounds, which can be fluorinated and then readily re-assessed for biotransformation[4]. By employing a microorganism, such as the zygomycete fungus *Cunninghamella elegans* or the actinomycete *Streptomyces griseus*, both of which express CYP and are known to metabolise drugs in a similar fashion to mammals[5], it was possible to identify the metabolically labile sites of drug-like biphenyl molecules. Using Suzuki-Miyaura coupling reactions with relevant fluorinated benzene boronic acids, it was possible to generate fluorinated biphenylderivatives that, upon subsequent re-incubation with the microorganism, were stable to metabolic attack (Scheme 1).

In this paper we describe the next stage in the development of our technique, by demonstrating its applicability to a drug currently in use. Flurbiprofen [(RS)-2-(2-fluoro-4-biphenyl)propionic acid] **1** is a non-steroidal anti-inflammatory drug used to manage arthritic pain, is an active ingredient in some throat lozenges and the *R*-enantiomer is in clinical trials as a treatment of prostate cancer [6]. When incubated with *C. elegans* and *S. griseus* flurbiprofen is transformed to the main human metabolite, 4'-hydroxy-flurbiprofen**2**, and to the minor metabolites 3',4'-dihydroxyflurbiprofen **3** and 3'hydroxy, 4'methoxy-flurbiprofen **4** [7](Scheme 2); in *C. elegans* a sulfated conjugated metabolite is also generated. Therefore, in this paper we report the synthesis of various fluorinated derivatives of flurbiprofen **5**-**9**, which were subsequently incubated with *C. elegans*, so that the effect of fluorine substitution on metabolically labile sites could be evaluated.

A short series of flurbiprofen analogues **5-9**was synthesised by a 5-step strategy, adapting literature procedures [8] (Scheme 3). Reaction of diethyl 2-methyl malonate ester with 2,4-difluoronitrobenzene **10** mediated by sodium hydride proceeded in high yield to give desired product **11** (97%) arising from nucleophilic substitution of fluorine *para* to the nitro substituent. Small quantities of product arising from competing *ortho*-substitution were detected by ^{19}F NMR but the isomeric mixture was used in the following stages without purification. Hydrogenation of the nitro group of **11** to give corresponding aniline **12** was accomplished by reaction with hydrogen over a palladium-on-carbon catalyst and subsequent diazotisation-iodination gave the key iodoaromatic intermediate **13** in good yield. Palladium-catalysed Suzuki-Miyaura coupling reactions between iodoaromatic **13** and a range of fluoroaryl boronic acids **14a-f** gave corresponding biphenyl diesters **15a-f**. Subsequent decarboxylation and hydrolysis gave a short series of the desired fluorinated flurbiprofen analogues **5-9**. Recrystallisation of the acid analogues removed trace quantities of *ortho* substituted products carried through the synthesis from the first stage and, for example, the structure of **7** was confirmed by X-ray crystallography (Fig. 1).

The fluorinated flurbiprofen analogues **5-9** (0.1 mg/ml) were incubated with *C. elegans* as we described previously [4]. Briefly, the fungus was cultivated for 72 h in 250 ml Erlenmeyer flasks containing 45 ml sabouraud dextrose broth prior to the addition of the drug. After further 72 h incubation, the culture was extracted with ethyl acetate and the solvent removed under reduced pressure. The oxidation products were analysed by gas chromatography-mass spectrometry after silylation with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA). Only the starting compounds **5**, **8** and **9** were detected after incubation. The absence of transformation was expected based on the knowledge that CYP-

catalysed hydroxylation occurs at the 4'-position of flurbiprofen, and is consistent with our previous observations concerning metabolism of model fluorinated biphenyl derivatives,[4] and confirms the effectiveness of this methodology for identifying metabolic labile sites for fluorinated drug design.

2'-Fluoro- and 3'-fluoro-flurbiprofen were transformed in varying degrees by the fungus (Fig 2). 3'-Fluoro-flurbiprofen **6** was almost entirely converted to a single hydroxylated product as observed by GC-MS analysis whereas 2'-fluoro-flurobiprofen **7** was incompletely transformed (approx. 30 % starting material remained) yielding three products all with the expected mass of corresponding hydroxylated derivatives.

To identify whether any products are formed by incubation of **6** and **7** with *C. elegans* and to aid oxidation products identification, ^{19}F NMR analysis of the extracts was conducted and, as expected, one fluorinated product was observed from the extracts recovered from incubation with **6** and three products from **7** (Fig. 3).

^{19}F NMR substituent chemical shift (SCS) analysis can be used to identify the hydroxylated products arising from oxidation of **6** and **7** by *C. elegans*. Peelen et al. [9] conducted extensive ^{19}F NMR experiments when investigating enzymatic hydroxylation of fluorophenol, and demonstrated that hydroxylations *ortho*, *meta* and *para* to the fluorine resulted in substituent shifts from the starting substrate of -23, +1.3 and -11 ppm, respectively, upon substitution of hydrogen atom by a hydroxyl group. Consequently, simple calculations allow a reasonable prediction of ^{19}F NMR chemical shifts for all possible hydroxylated products (Fig. 4).

Using the data in Figs. 3 and 4, it is reasonable to conclude that 3'-fluoro-flurbiprofen **6** is most probably converted to the 4'-hydroxylated product **10a**, consistent with our earlier

findings concerning metabolism of model biphenyl derivatives at the 4'-position [4] although **10b** is a possible product based upon the 19F NMR shift. In contrast, 2'-fluoro-flurbiprofen **7** gives three hydroxylated products by GC-MS and ^{19}F NMR analysis, the main product probably being **11b** arising from hydroxylation of the 4'-position consistent with earlier findings [4] with minor products **11a** and **11c**, in the approximate ratio **11a** : **11b** : **11c** 1 : 8 : 2 by ^{19}F NMR resonance intensities. Again the major product is most probably hydroxylation at the least metabolically stable 4'-position **11b** although significant quantities of **11a** and **11c** are formed reflecting the stabilising effect of fluorine *ortho* and *para* to the sites of oxidation.

To demonstrate that the fungus is an appropriate model for metabolic stability experiments, rat-liver microsomes [10] were incubated with the flurbiprofen derivatives **5**-**8**, plus NADPH. The same transformation products were detected by GC-MS from **6** and **7**; in contrast, no transformation of **5** and **8** was observed confirming that blocking the 4' position with fluorine prevented metabolism.

In summary, following our previously reported strategy for identifying metabolically labile sites on drug-like compounds for subsequent fluorination, we have demonstrated that this strategy is effective for generating fluorinated derivatives of known drugs that are metabolically stabilised. Flurbiprofen **1** is known to be initially oxidised at the 4' position by both mammals and microorganisms. Analogues of flurbiprofen bearing fluorine atoms at the 2'-, 3'- and 4'-positions **5**-**7** were synthesised. Upon incubation with *C. elegans* to determine if these modifications had any effect on biotransformation, the flurbiprofen analogue that had an additional fluorine in the 4' position **7** was completely stable to metabolism. Analogues of the parent drug with fluorine located at the 2' and 3' positions resulted in

hydroxylation to varying degrees upon incubation; 3'fluoroflurbiprofen **6** was almost completely transformed to a hydroxylated product **10a** whereas 2'fluoroflurbiprofen **7** was transformed to a lesser extent, and to three mono-hydroxylated products. Significantly, the same transformation products were detected after rat liver microsomes were incubated with **5**-**8**, demonstrating the relevance of the microbial model used here. It is often attractive to improve drug efficacy by slowing their metabolism, thus 2'- and 3'-fluoroflurbiprofen might be more useful derivatives as their biotransformation is altered, but not completely prevented, as with the 4' fluorinated analogue.

Experimental Section

Details of the synthetic procedures, characterisation of compounds and microbiological methods can be found in the Supplemental Information.

Acknowledgements

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Keywords: Cross-coupling, cytochromes, drug design, fluorine, metabolism

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Scheme and Figure Legends

Scheme 1. A chemical-microbial method for developing metabolically stable fluorinated drug-like molecules [3].

Scheme 2. Oxidative metabolites of flurbiprofen via incubation with *C. elegans* and *S. griseus*.

Scheme 3**.** Synthesis of fluorinated Flurbiprofen analogues **5-9**

Figure 1. Molecular structure of **7**

Figure 2. Total ion chromatograms from incubation of **6** and **7** with *C. elegans*: A, **6** without fungus; B, biotransformation of **6** to hydroxylated product; C, **7** without fungus; biotransformation of **7** to three hydroxylated products. All of the extracts were derivatised with MSTFA prior to GC-MS analysis.

Figure 3. F-19 NMR spectra of extracts from *C. elegans* cultures incubated with 3'-fluoroflurbiprofen **6** (top) and 2'fluoroflurbiprofen **7** (bottom).

Figure 4. Calculated and observed ^{19}F NMR shifts of hydroxylated flurbiprofen derivatives **10** and **11**

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By applying a chemical-microbiological approach for the design of drugs with enhanced metabolic stability, a series of fluorinated derivatives of the NSIAD flurbiprofen was synthesised that were more resistant to P450-catalysed transformation than the original drug.

Fig 1

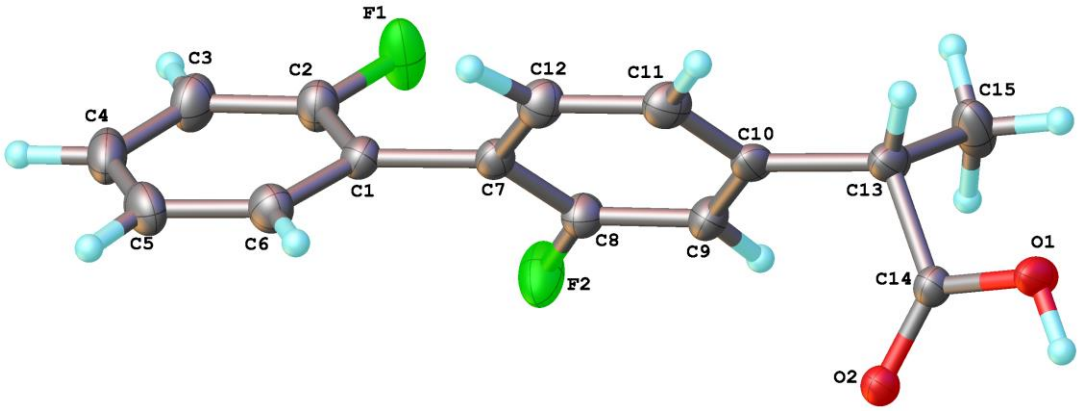
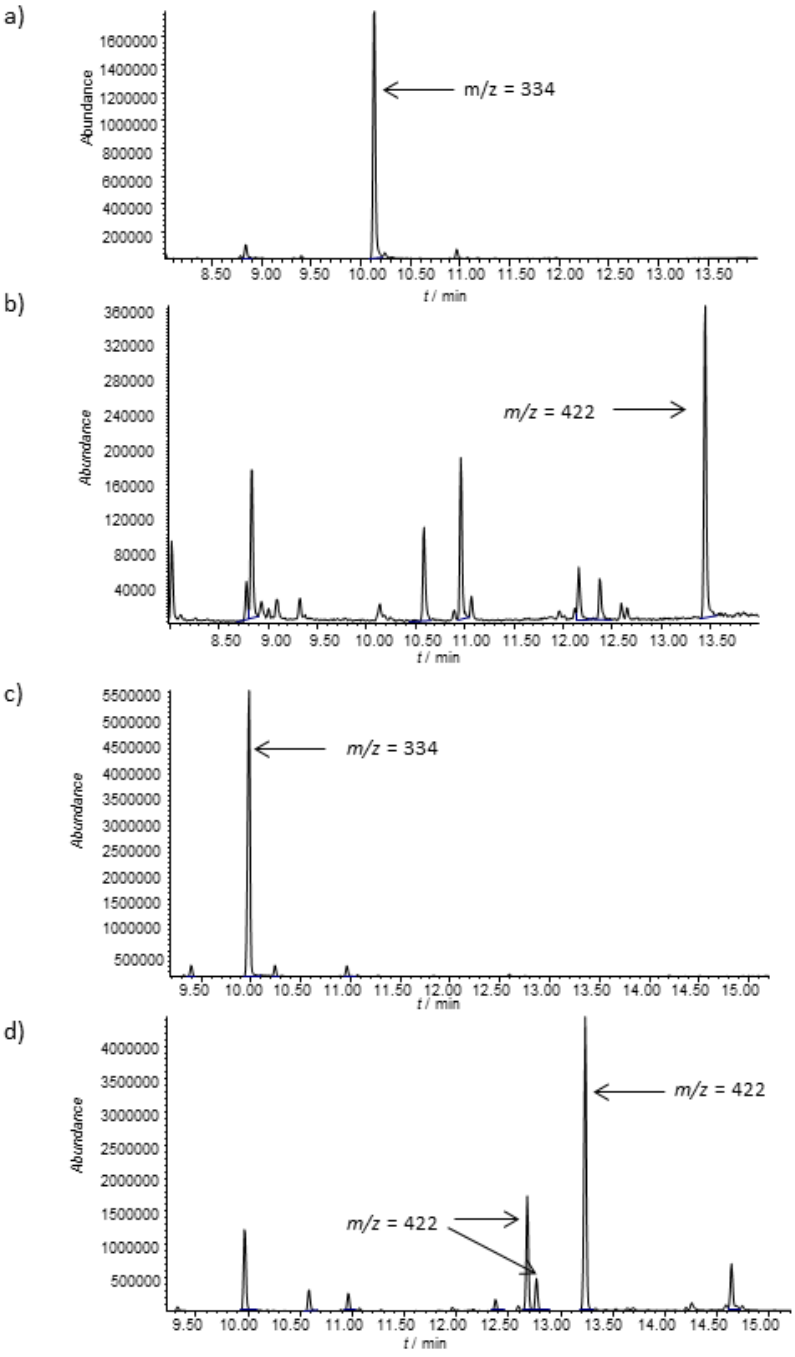


Fig 2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Abundance
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t / min
b)
Abundance
m/z = 422
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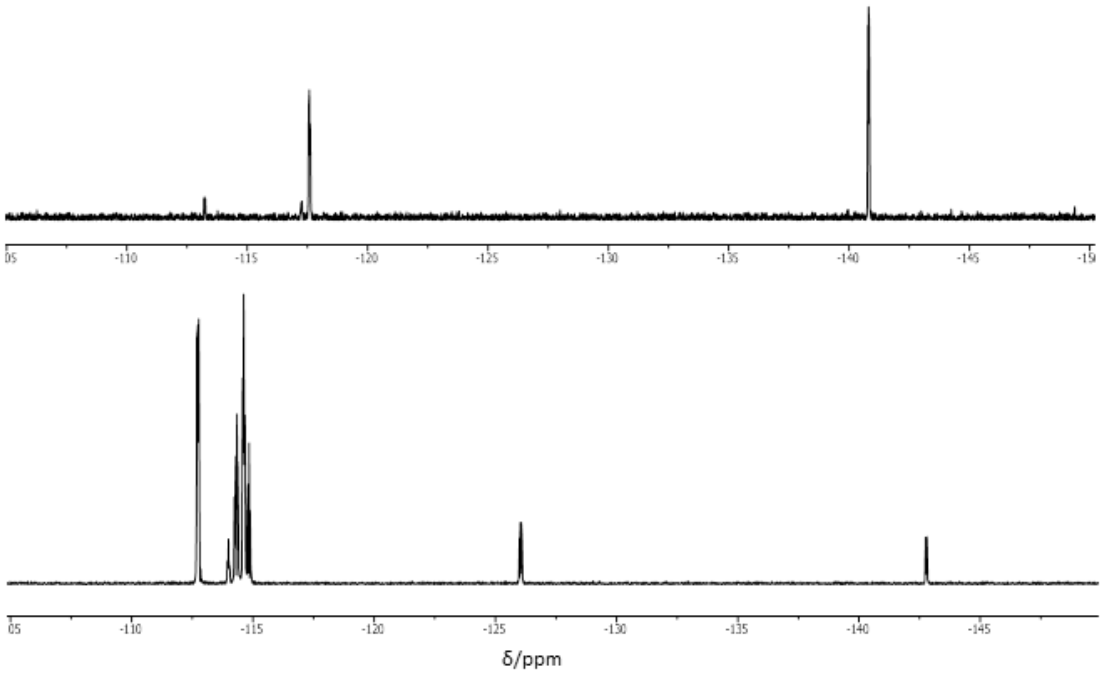
d)
Abundance
m/z = 422
m/z = 422
t / min

Fig 3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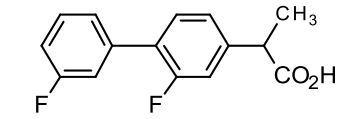
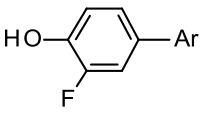
-140
-145
δ/ppm

Fig 4



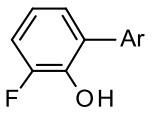
-114.8 -119.1

6



Calc: -137.8
Obs: -140.8

10a



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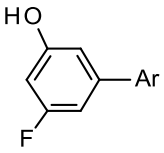
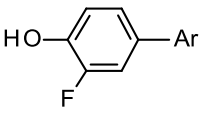
10b



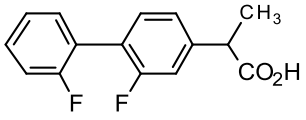
-113.5

10c



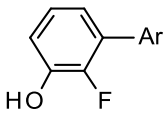
-125.8

10d



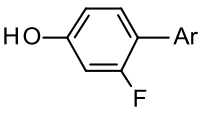
-116.8 -116.9

7



Calc: -139.9
Obs: -142.8

11a



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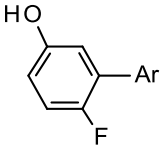
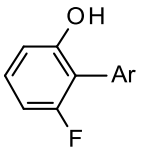
Obs: -112.9

11b



Calc: -127.9
Obs: -126.1

11c



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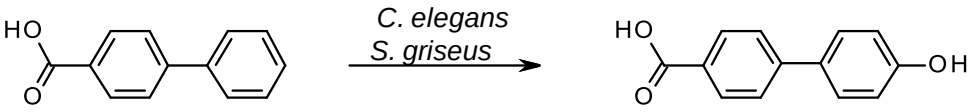
11d

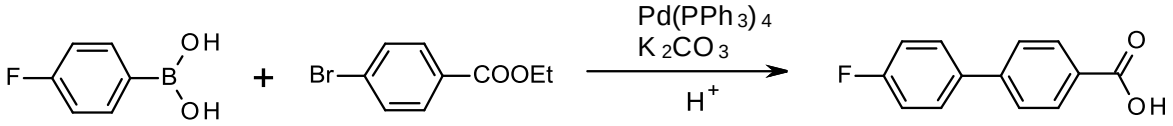
Ar = 

Scheme 1

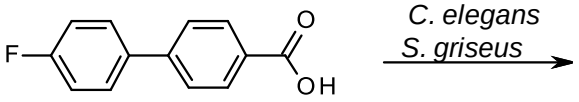
Step 1: Identify metabolically labile site



Step 2: Synthesise flourinated analogue

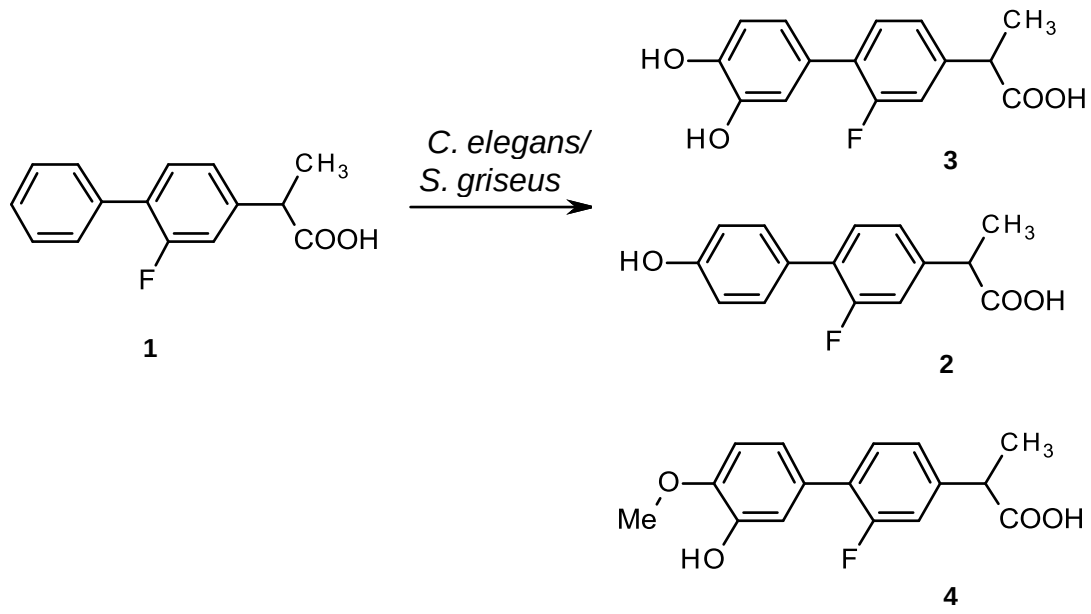


Step 3: Re-test with microorganism



No transformation

Scheme 2



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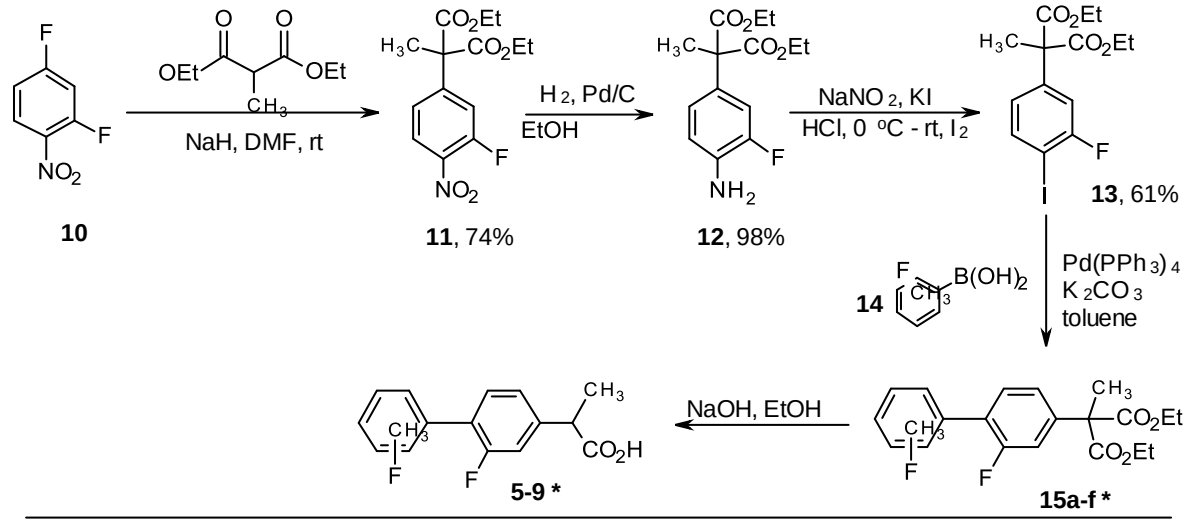
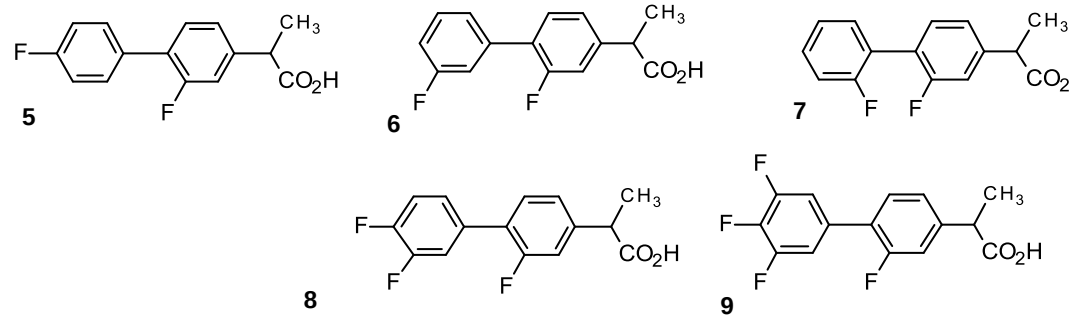


Flurbiprofen analogues synthesised



* For yields of **15a-f** and **5-9,** see supporting information