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Research Paper

# Antioxidant properties in a non-polar environment of difluoromethyl bioisosteres of methyl hydroxycinnamates

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**Keywords**
antioxidant activity; bioisosteres; difluoromethyl group; hydroxycinnamic acids; lipid peroxidation

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## Abstract

**Objectives** Many natural antioxidants have poor pharmacokinetic properties that impair their therapeutic use. For hydroxycinnamic acids (HCAs) and other phenolic antioxidants, their major drawback is their low lipophilicity and a rapid metabolism. The difluoromethyl group may be considered as a 'lipophilic hydroxyl' due to its hydrogen bond donor and acceptor properties; this prompted us to assess it as a bioisosteric replacement of a phenolic hydroxyl for increasing the lipophilicity of HCAs.

**Methods** Six difluoromethyl-substituted methyl cinnamates (**4a-c**, **5a-c**) related to caffeic acid were synthesized and their antioxidant activity evaluated by chemical (FRAP, DPPH scavenging, inhibition of β-carotene bleaching, at 1–200 μM), electrochemical (differential pulse voltammetry, cyclic voltammetry) and cell-based (inhibition of lipid peroxidation in erythrocytes, at 1 and 50 μM) assays.

**Key findings** Analogues **4a-c** and **5a-c** were inactive in FRAP and DPPH assays and only those containing a free phenolic hydroxyl (**4a** and **5a**) exhibited electrochemical activity although with high redox potentials. Compounds **4a**,**b** and **5a**,**b** were active in the inhibition of β-carotene bleaching assay and all analogues inhibited lipid peroxidation in the human erythrocytes assay.

**Conclusions** Lipophilic difluoromethyl-substituted cinnamic esters retain radical scavenging capabilities that prove useful to confer antioxidant properties in a non-polar environment.

## Introduction

Reactive oxygen species (ROS) are produced in living organisms under normal physiological conditions and comprise both free radicals as the superoxide anion and hydroxyl radical and non-radical species as peroxides and singlet oxygen. Several defence mechanisms are present in biological systems to eliminate most of these species, as well as to repair the damage they produce in cells, mostly to their DNA, but also to proteins and lipids. Thus, endogenous antioxidant compounds as glutathione, or enzyme systems as catalase or superoxide dismutase, can convert ROS into harmless compounds. When these defence mechanisms are impaired or an excessive production of ROS exceeds their capacity, the accumulated highly reactive species will damage most cell components and may also disrupt cellular signalling. This condition, termed oxidative stress, is implicated in the pathogenesis of several human diseases including cancer, certain cardiovascular diseases and neurodegenerative diseases and may also contribute to the ageing process.[1–4] Besides the endogenous antioxidants mentioned above, compounds with antioxidant properties from exogenous sources are normally incorporated through the human diet, with polyphenols being the most abundant. Although much controversy exists around the benefits of incorporating this type of compounds as dietary supplements in a regular basis, their ability to counteract the harmful effects of ROS and regulate the

physiological defence systems makes them useful candidates for prevention or treatment of oxidative stress-related diseases.[5,6]

Phenolic acids are natural hydrophilic antioxidants derived from benzoic or cinnamic acids that occur in fruits, vegetables, spices and herbs.[7] Hydroxycinnamic acids (HCAs) and their derivatives have drawn particular attention, as a large number of beneficial health effects have been correlated with their ingestion as part of the human diet. This has led to extensive studies of their antioxidant properties.[8,9] The most common substituted cinnamic acids found in fruits and vegetables are shown in Figure 1. Most of these compounds also present antibacterial, antiviral, antisclerotic and antitumor activities, among others.[5] HCAs can prevent or minimize oxidative damage processes essentially by scavenging free radical species and/or boosting the endogenous antioxidant system capacity, by stimulating the synthesis of endogenous antioxidants. However, the major drawback of HCAs and other phenolic compounds lies in their low lipophilicity and a rapid metabolism that result in limited ADME properties (absorption, distribution, metabolism and excretion).[2,5,10]

The structure–activity relationships for a large number of HCAs and synthetic derivatives have been the subject of recent reviews.[8,9,11] The antioxidant activity has been shown to be strongly dependent on certain structural features as the unsaturated side chain and is usually associated with the presence of free phenolic hydroxy groups. However, a clear distinction can be made between the direct antioxidant capacity that may be evaluated in non-biological systems based on redox reactions or radical scavenging processes and the effective antioxidant activity in whole cells or complex matrices. The latter is related not only to the redox or radical scavenging properties but also to lipophilicity and to the presence of enzymes that can expose masked phenolic hydroxyls, as demethylases and esterases.[12,13] Increased lipophilicity that allows the passive crossing of the blood–brain barrier is particularly important for antioxidants that may be potentially useful in the prevention or minimization of oxidative neuronal damage in neurodegenerative diseases.[14] In this respect, most approaches to improve antioxidant activity of HCAs *in vivo* have sought to increase lipophilicity by conversion of the carboxylic acid moiety into different kinds of esters or amides.[14–18] However, it has been proposed that the incorporation of long lipophilic chains into the HCA molecules could lead to undesirable effects as self-aggregation, reduced mobility or internalization into the lipid core that may hamper the antioxidant efficacy in a living organism.[19]

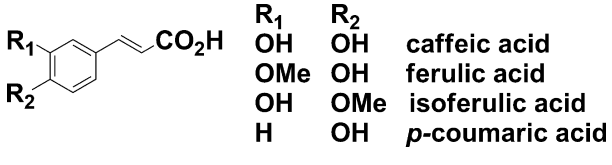



**Figure 1** Common hydroxycinnamic acids found in fruits and vegetables.

The difluoromethyl group can be used as a lipophilic isostere of the hydroxyl group taking advantage of its hydrogen bond donor and acceptor capacities.[20,21] It can also generate radicals by homolytic cleavage of the C-H bond, as the fluorine atoms stabilize the difluoromethyl radicals due to their π-donating ability and participate in the formation of *o*-quinone methides in basic conditions.[22–24] However, there are few reports of its use as a replacement for a phenolic hydroxyl[25–29] and to the best of our knowledge, none pertaining its radical scavenging properties. We envisaged that this modification applied to HCAs would render analogues with enhanced lipophilicity that should retain at least in part the properties of the parent phenolic compound. Specifically, we were interested in evaluating to what extent the difluoromethyl group could mimic the antioxidant properties conferred by a phenolic hydroxyl group, as well as its effect on the antioxidant properties of neighbouring phenolic hydroxyls. In this work, we report for the first time the synthesis of difluoromethyl analogues of simple HCA esters and their antioxidant properties evaluated in different systems with special emphasis on the radical scavenging properties and their performance in non-polar media.

## Materials and Methods

Melting points were taken on a Fisher-Johns apparatus and are uncorrected. $^{1}H$ and $^{13}C$ NMR spectra were measured in a Bruker Avance II 500 NMR spectrometer (Bruker BioSpin, Karlsuhe, Germany) at 500.13 and 125.72 MHz, respectively. Exact mass spectra (HRMS) were measured on a Bruker micrOTOF-Q II mass spectrometer with positive electrospray ionization. Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates (Merck KGaA, Darmstadt, Germany; F254, 0.2 mm thickness); compounds were visualized under 254 nm UV light. Flash column chromatography (FCC) was performed on silica gel Merck 9385 (0.0040–0.0063 mm) eluting with mixtures of ethyl acetate–hexane of increasing polarity. Homogeneity of all compounds was confirmed by TLC and NMR. Compounds **6b**,**c** were synthesized from 2-hydroxy-5-iodobenzaldehyde, and compounds **9b**,**c** were obtained from methyl 4-iodosalicylate (see supporting information for details).

## Chemistry

### *Chemical data of synthesized compounds*

Structures of all compounds were confirmed by NMR and HRMS (see Appendix).

### *Methyl 3-[(3-(difluoromethyl)-4-methoxyphenyl)]-(E)-propenoate (4b)*

Deoxofluor® 50% in toluene (0.061 ml, 0.248 mmol) was added dropwise to a solution of **6b** (0.028 g, 0.124 mmol) in dry dichloromethane (0.5 ml) under argon. The mixture was stirred at room temperature for 1.5 h and then percolated through a silica gel pad eluting with dichloromethane. The percolate was evaporated to dryness and the residue purified by FCC to give **4b** (0.029 g, 96%).

### *Methyl 3-[4-acetyloxy-(3-(difluoromethyl)phenyl)]-(E)-propenoate (4c)*

Compound **4c** was prepared from **6c** (0.072 mg, 0.289 mmol) and Deoxofluor® 50% in toluene (0.142 ml, 0.578 mmol) following the procedure described for **4b** (0.070 g, 90%).

### *Methyl 3-[(3-(difluoromethyl)-4-hydroxyphenyl)]-(E)-propenoate (4a)*

To a solution of **4c** (0.045 g, 0.167 mmol) in methanol (3.0 ml), conc. $H_2SO_4$ (50 µl) was added and the mixture stirred 2 h at 60°C. The reaction mixture was diluted with water (15 ml), concentrated under reduced pressure and extracted with ethyl acetate (2 × 30 ml). The organic layer was dried with anhydrous sodium sulphate and the solvent evaporated. The residue was purified by FCC to give **4a** (0.036 g, 94%).

### *Methyl 3-[(4-(difluoromethyl)-3-methoxyphenyl)]-(E)-propenoate (5b)*

Compound **5b** was prepared from **9b** (0.028 g, 0.127 mmol) and Deoxofluor® 50% in toluene (0.094 ml, 0.381 mmol) following the procedure described for **4b** (0.027 g, 88%).

### *Methyl 3-[3-acetyloxy-4-(difluoromethyl)phenyl)]-(E)-propenoate (5c)*

Compound **5c** was prepared from **9c** (0.055 g, 0.22 mmol) and a solution of Deoxofluor® 50% in toluene (0.164 ml, 0.667 mmol) following the procedure described for **4b** (0.044 g, 73%).

### *Methyl 3-[(4-(difluoromethyl)-3-hydroxyphenyl)]-(E)-propenoate (5a)*

Compound **5a** was prepared from **5c** (0.023 g, 0.016 mmol) following the procedure described for **4a**. Recrystallization from n-hexane/chloroform gave **5a** (0.016 g, 82%).

## Electrochemical measurements

Electrochemical measurements were carried out on 0.1 mM solutions of **1-3**, **4a-c** and **5a-c** in phosphate buffer (0.067 M, pH 7.3) using a homemade two-compartment Pyrex cell. The working electrode was a GC disk (Bioanalytical System, Inc., West Lafayette, Indiana, USA, 3 mm diameter). The counter electrode was a large-area platinum mesh; reference electrode was Ag/AgCl (3 M KCl). Scan rate in cyclic voltammograms was 0.050 V/s. For differential pulse voltammograms, a modulation amplitude of 0.025 V and a step potential of 0.005 V were used. All experiments were recorded at 25°C on an AutoLab PGSTAT 30 potentiostat, controlled by GPES 4.9 electrochemical software (EcoChemie, Utrecht, NL).

## Antioxidant activity

### *Free radical scavenger assay*

Free radical scavenger activity of extracts was assessed by the fading of a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl radical, following the procedure of Tapia *et al.*[30] The tested compounds were assayed at concentrations of 1, 50, 100 and 200 µM. Activities were evaluated in 96-well microplates in triplicate at 517 nm, using a Labsystems microplate reader (Helsinki, Finland). Quercetin was used as reference compound. The percentage of decoloration at each concentration was calculated as follows:

$$\text{decoloration}\,(\%) = \left(1 - \frac{(A_{\text{sample}} - A_{\text{control}})}{A_{\text{DPPH}}}\right) \times 100,$$

where $A_{\text{sample}}$: sample absorbance; $A_{\text{control}}$: control absorbance; $A_{\text{DPPH}}$: DPPH absorbance; values are reported in terms of $IC_{50}$ (concentration of the compound that produces 50% decoloration) as the mean ± standard deviation of three independent assays.

### *Ferric reducing antioxidant power (FRAP) assay*

Ferric reducing antioxidant power was determined by the direct reduction of $[Fe(CN)_6]^{-3}$ to $[Fe(CN)_6]^{-4}$ measuring the absorbance of Perl's Prussian blue complex after addition of excess $Fe^{3+}$.[31,32] The iron reducing power was tested according to Oyaizu.[33] Briefly, 100 µl of

compounds solution or gallic acid solution (1, 50, 100, 200 μM) was added to 250 μl of phosphate buffer (0.1 M, pH 6.6) and 250 μl of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, and 250 μl of 10% trichloroacetic acid was added and vortexed for 20 s. The resulting solution (50 μl) was diluted with distilled water (50 μl) in a microplate and 10 μl of 0.1% $FeCl_3$ added. After 30-min incubation, absorbance was read at 700 nm using a Labsystems microplate reader (Helsinki, Finland). The reducing power was determined by linear regression from a calibration plot and expressed as gallic acid equivalent antioxidant capacity (millimolar concentration of a gallic acid solution with antioxidant capacity equivalent to a 1.0 mM solution of compound).

### *β-Carotene bleaching assay*

The β-carotene bleaching assay was conducted according to the method described by Mogana with modifications and recommendations by Prieto.[34,35] Briefly, a solution of β-carotene (1 mg) in chloroform (5 ml) was mixed with 20 μl of linoleic acid and 250 μl of Tween-20. Chloroform was removed under vacuum at 35°C and deionized water (50 ml) added with vigorous stirring to give the β-carotene–linoleic acid emulsion that was used immediately. Twenty microlitre of methanolic solutions of the compounds (1, 5, 10, 20, 50, 100 μM) was plated out in duplicate in a 96-well microtiter plate and 180 μl of β-carotene–linoleic acid emulsion added to each of the test samples. Absorbance was measured at 470 nm against a blank (linoleic acid emulsion without β-carotene) and a control (emulsion and 20 μl of methanol) every 5 min for 4 h at 45°C using a PHERAstar F5 multiplate spectrophotometer reader (BMG Labtech, Cary, North Carolina, USA) with stirring. The antioxidant activity was evaluated in terms of bleaching of β-carotene as:

$$\%\beta\text{-carotene bleached} = \frac{(A_0 - A_t)}{A_0} \times 100,$$

where $A_0$: initial absorbance and $A_t$: absorbance at the different incubation times for the test samples and control.

### *Lipid peroxidation in human erythrocytes*

Studies on erythrocytes lipid peroxidation were conducted as described by Tapia *et al.*[30] Cells were washed three times in cold phosphate-buffered saline (PBS) by centrifugation at 1200g, suspended in PBS and the density adjusted to 1 mM haemoglobin. The cell suspension was incubated with different concentrations (1 and 50 μM) of the test compounds dissolved in DMSO and PBS for 10 min at 37°C. The final concentration of DMSO in samples and controls was 1%. After incubation, cells were exposed to *tert*-butyl hydroperoxide (1 mM) for 15 min at 37°C under vigorous shaking. Lipid peroxidation was determined indirectly by TBARS formation at 540 nm using a Labsystems microplate reader (Helsinki, Finland). Quercetin (1 and 50 μM) was used as reference antioxidant. Percentages of inhibition relative to controls were expressed as means ± standard error of three independent experiments. Statistical analyses were performed with STATISTICA 6.0 (StatSoft Inc., Tulsa, Oklahoma, USA) and consisted in one-way ANOVA followed by Tukey's multiple comparisons tests. Differences were regarded as significant at $P < 0.05$ (bars with different superscript letters are significantly different from each other). Before statistical analysis, Q-Q plot and Shapiro–Wilk test were performed for normality. Homoscedasticity was assessed with Levene's test.

## Results

### Chemistry

Three representative HCA methyl esters, methyl caffeate (**1**), methyl ferulate (**2**) and methyl *iso*ferulate (**3**), were chosen as templates for the bioisosteric replacement of hydroxyl by the difluoromethyl group, leading to the fluorinated analogues **4a,b** and **5a,b** (Figure 2). The acetylated derivatives **4c** and **5c** were also selected, considering that they could act as precursors of the free phenols in biological systems.

The 3'-difluoromethyl analogues **4a-c** were obtained from salicylaldehyde as depicted in Scheme 1. Iodination with iodine monochloride in glacial acetic acid gave 5-iodo-2-hydroxybenzaldehyde[36] that was coupled with

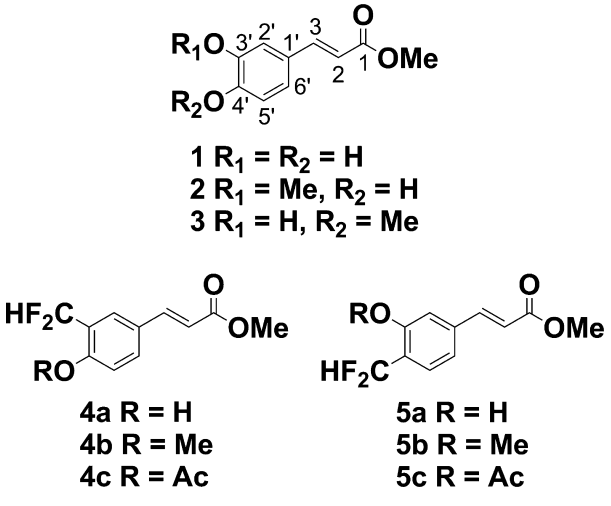
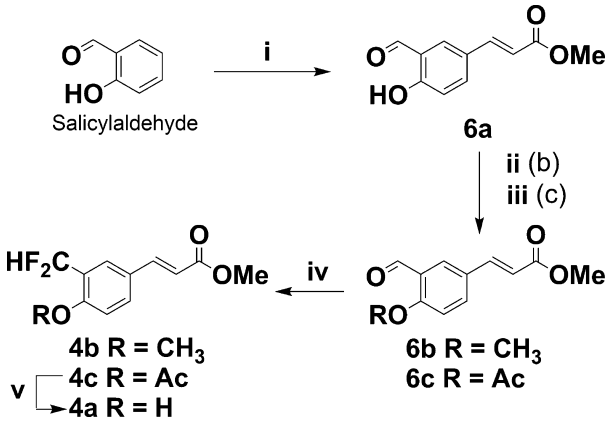



**Figure 2** Structures of methyl hydroxycinnamates **1-3** and difluoromethyl analogues from bioisosteric replacement of the 3′ or 4′ phenolic hydroxyl (**4a-c** or **5a-c,** respectively).




**Scheme 1** *Reagents and conditions:* (i) 1. ICl, AcOH; 2. $Pd(AcO)_2$, (*o*-Tol)$_3$P, methyl acrylate, TEA, MeCN; (ii) MeI, $K_2CO_3$, $Me_2CO$; (iii) $Ac_2O$, $K_2CO_3$, $CH_2Cl_2$; (iv) Deoxofluor®, $PhCH_3$, $CH_2Cl_2$; (v) $H_2SO_4$, MeOH.

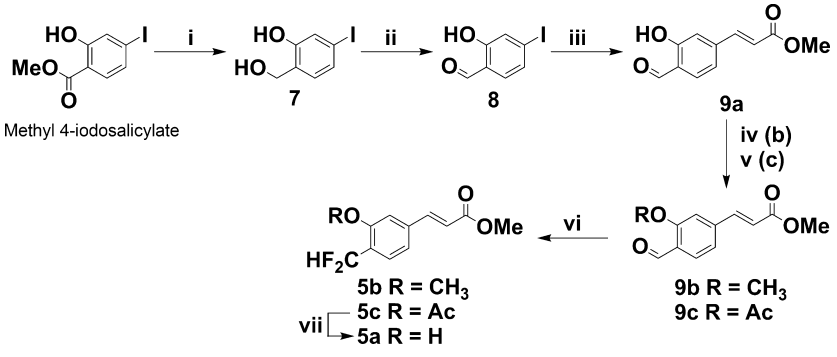
methyl acrylate under Heck reaction conditions to give the methyl cinnamate derivative **6a** in 65% yield.[37] Attempts to carry out the deoxofluorination reaction on the latter compound failed; thus, the phenolic hydroxyl was acetylated and the aldehyde group converted to the difluoromethyl group with Deoxofluor® to give the isoferulate analogue **4c** in 84% yield from **6a**.[38] The $^1H$ and $^{13}C$ NMR spectra of **4c** showed triplets at $\delta_H$ 6.76 ppm ($J_{HF}$ = 55.2 Hz) and at $\delta_C$ 111.5 ppm ($J_{CF}$ = 239 Hz) for the $CF_2H$ moiety. Mild acid hydrolysis of the phenolic acetate gave the difluoromethyl phenol **4a** in 79% yield from **6a**. Methylation of **6a** with methyl iodide followed by reaction with Deoxofluor® gave the isoferulate analogue **4b** (91% from **6a**).

The 4'-difluoromethyl analogues **5a-c** were obtained by a similar sequence but starting from commercially available methyl 4-iodosalicylate (Scheme 2). Reduction to the benzyl alcohol **7** with lithium aluminium hydride followed by oxidation with manganese dioxide gave 4-iodo-2-hydroxybenzaldehyde **8**. Coupling with methyl acrylate under Heck conditions as described above gave cinnamate **9a** in 60% yield. Conversion of the latter compound to the fluorinated analogues **5a-c** was achieved by the same reaction sequence as used for **4a-c**. The $^1H$ and $^{13}C$ NMR spectra of compounds **5a-c** showed the corresponding triplets of the difluoromethyl group as observed in **4a-c**.

## Electrochemical measurements

Voltammetric methods have been extensively applied to characterize a variety of natural phenolic antioxidants and synthetic antioxidants, mainly to get an insight of the underlying mechanism.[39,40] The use of differential pulse voltammetry (DPV) and cyclic voltammetry (CV) to investigate the electrochemical behaviour of different kinds of HCA-related antioxidants was recently reviewed.[41] The redox potentials of the synthesized compounds **4a-c** and **5a-c** were measured using differential pulse and cyclic voltammetries at physiological pH (7.3) and their electrochemical behaviour was compared with that of the hydroxylated analogues **1**, **2** and **3** measured under the same conditions (Figures 3 and 4). The difluoromethyl analogues **4b**,**c** and **5b**,**c** did not show any electrochemical activity in the potential range studied, due to the absence of a free phenolic hydroxyl (data not shown).

Consecutive cyclic voltammograms of compounds **1**-**3**, **4a** and **5a** registered in the potential range between −0.2 and 1.2 V showed a marked decrease in the current signals in consecutive sweeps. After the fifth successive sweep, voltammetric signals almost disappeared (data not shown). It is well known that the oxidation of phenols occurs at potentials close to 1 V depending on the reaction medium. This may be represented in principle by an E1C1E2C2 mechanism generating, in some cases, polymeric products that poison or passivate the working electrode surface.[42] Thus, in this work, we only analysed the electrochemical behaviour of the first voltammogram. Compounds **4a** and **5a** had higher redox potentials compared to **1**-**3**.




**Scheme 2** *Reagents and conditions:* (i) $LiAlH_4$, THF; (ii) $MnO_2$, $CH_2Cl_2$; (iii) $Pd(AcO)_2$, (*o*-Tol)$_3$P, methyl acrylate, TEA, MeCN; (iv) MeI, $K_2CO_3$, $Me_2CO$; (v) $Ac_2O$, $K_2CO_3$, $CH_2Cl_2$; (vi) Deoxofluor®, $PhCH_3$-$CH_2Cl_2$; (vii) $H_2SO_4$, MeOH.

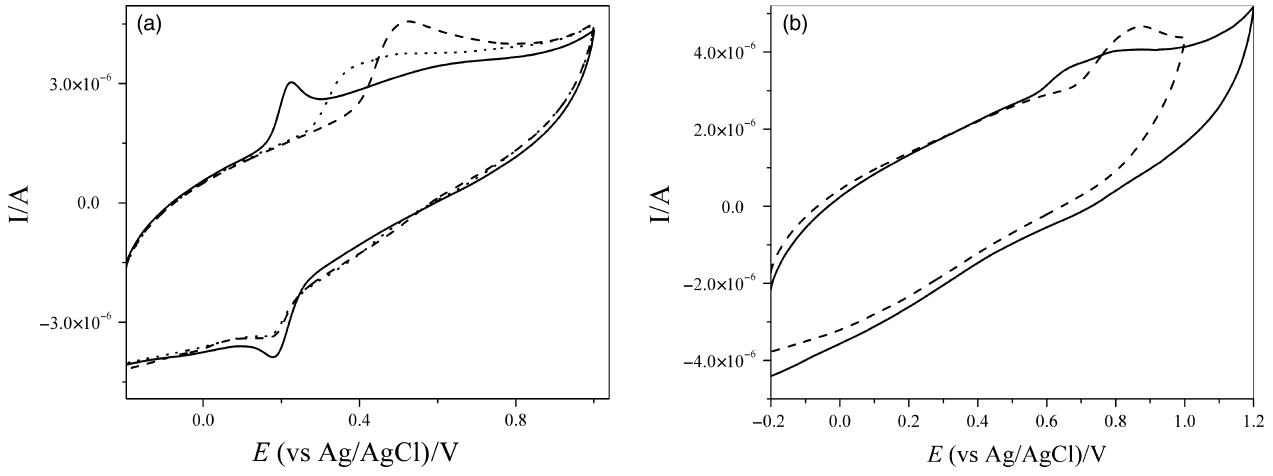



**Figure 3** Cyclic voltammograms for 0.1 mM solutions of the tested compounds (scan rate: 50 mV/s). (a) **1** (—), **2** (. . . .), **3** (---); (b) **4a** (—), **5a** (---). Phosphate buffer (pH 7.3) was used as the supporting electrolyte.

Comparison of regioisomers **4a** and **5a** showed the same trend found for regioisomers **2** and **3** albeit enhanced with a lower redox potential for the isomer with the hydroxyl group in the *para* position relative to the acrylate side chain. Both compounds exhibited two redox potential values that may be ascribed to adsorbed and free forms.

## Antioxidant activity

The antioxidant capacity profiles for the synthesized compounds were evaluated by four representative assays. For the total antioxidant capacity (TAC) levels, we used an iron (III)-based TAC assay at a pH close to physiological values, as in the original ferricyanide method (FRAP).[43] Other methods that use acidic solutions may not reflect the true behaviour of physiologically important antioxidants in a cellular assay or *in vivo*. Radical scavenging capacity was first evaluated with the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay. Being these predominantly single electron transfer (SET)-based methods, they may be used to compare with the electrochemical data.[41] Compounds **4a-c** and **5a-c** were inactive in the FRAP and DPPH assays (see supporting information, Table S1).

To evaluate the effect of the isosteric replacement in a biological environment where the capacity to scavenge free

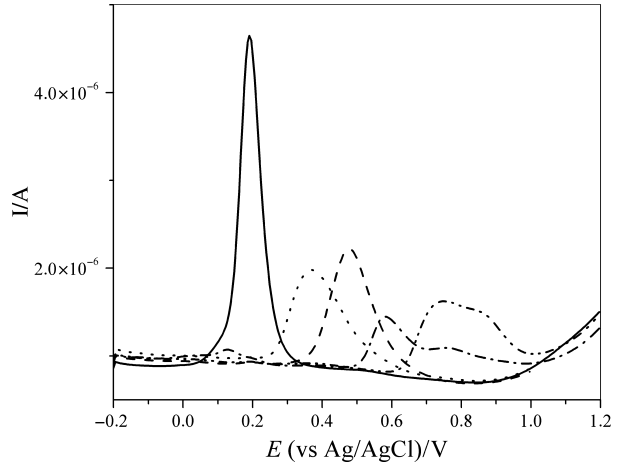



**Figure 4** Differential pulse voltammograms for 0.1 mM solutions of the tested compounds (scan rate: 5 mV/s): **1** (—) $E_p$ = 191 mV, **2** (. . . .) $E_p$ = 370 mV, **3** (—) $E_p$ = 478 mV, **4a** (-.-.-) $E_p$ = 582 and 761 mV and **5a** (-..-..-) $E_p$ = 748 and 836 mV. Phosphate buffer (pH 7.3) was used as the supporting electrolyte.

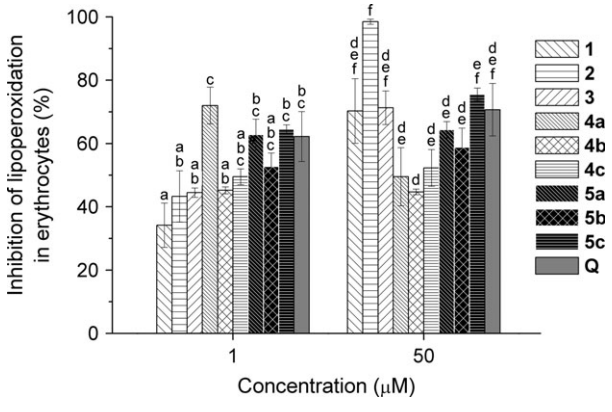



**Figure 5** Inhibition of lipid peroxidation in human erythrocytes at 1 and 50 μM of compounds **1-3**, **4a-c** and **5a-c**. Quercetin (**Q**) was used as positive control. Means ± SE from three independent experiments are shown. Differences were determined by one-way ANOVA followed by Tukey's multiple comparisons tests. At each concentration, bars with different superscript letters are significantly different from each other ($P < 0.05$). If one superscript letter is shared between bars, then no significant differences were found.

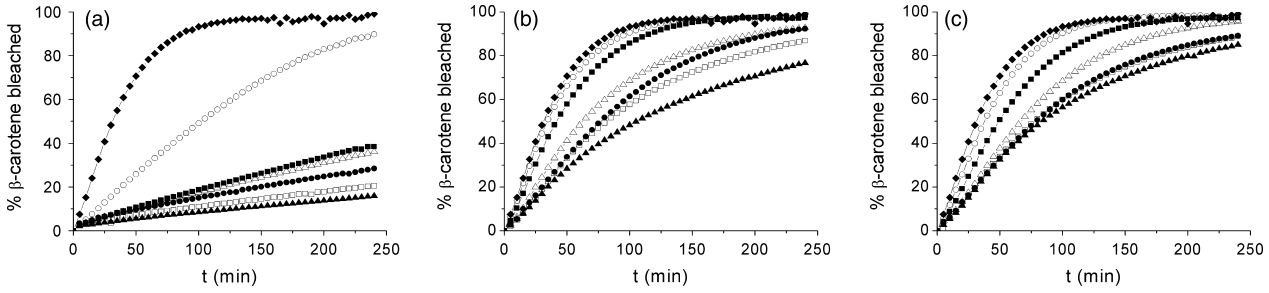



**Figure 6** Inhibition of β-carotene bleaching at 1 (○), 5 (■), 10 (△), 20 (●), 50 (□) and 100 (▲) μM of methanolic solutions of compounds **1** (a), **4a** (b) and **5a** (c). Methanol (♦) was used as control. All assays were carried out in duplicate.

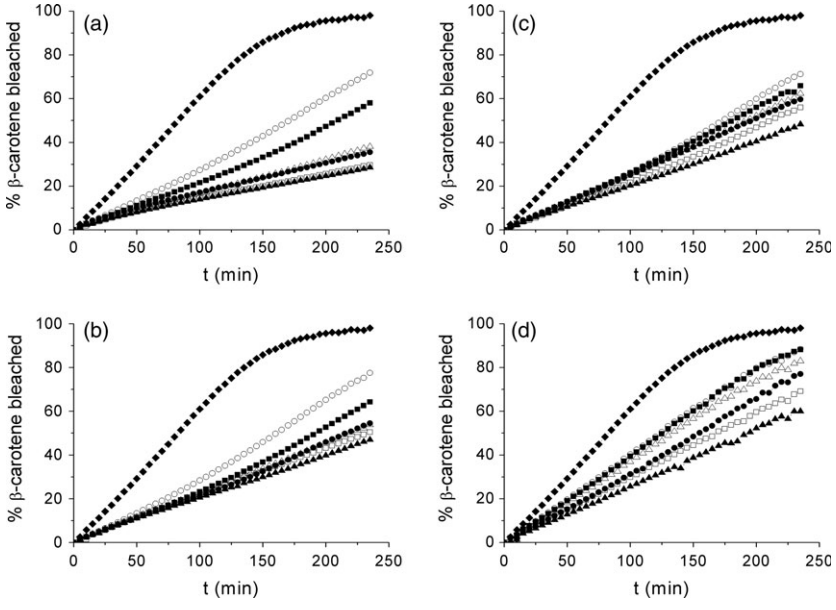



**Figure 7** Inhibition of β-carotene bleaching at 1 (○), 5 (■), 10 (△), 20 (●), 50 (□) and 100 (▲) μM of methanolic solutions of compounds **2** (a), **3** (b), **5b** (c) and **4b** (d). Methanol (♦) was used as control. All assays were carried out in duplicate.

radicals by hydrogen atom transfer (HAT) plays an important role, we used a cellular assay based on the inhibition of lipid peroxidation in human erythrocytes.[30,44] All compounds were active in this assay displaying significant activity at 1 and 50 μM concentration (Figure 5). The activity of the fluorinated compounds **4a-c** and **5a-c** did not differ significantly from the reference compound quercetin (**Q**) at both concentrations. In the case of compounds **4a** and **5a** with a free phenolic hydroxyl, both compounds had a significantly better performance at 1 μM compared with their bioisostere methyl caffeate (**1**); however, there was no significant difference between the regioisomers. Isomers **2** and **3** with a phenolic hydroxyl and a methoxy substituent also had the same activity, although at 50 μM compound **2** showed a tendency to increase the lipid peroxidation inhibition consistent with the *para* position of the hydroxyl relative to the acrylate chain. The same tendency was observed between compounds **5b,c** and **4b,c**, suggesting a stabilization from the side chain although differences were not statistically significant. Thus, despite their poor performance in the single electron transfer-based methods, all difluoromethyl analogues equalled or bettered the activity of compounds **1**-**3** in the cell-based assay at the lower concentration. These results prompted us to determine the ability of the difluoromethyl analogues to scavenge free radicals by hydrogen donation in a direct chemical assay. The β-carotene bleaching assay specifically evaluates the antioxidant capacity by the hydrogen atom transfer (HAT) method, in a non-polar environment.[34,45] Figure 6 shows the dose–response effects of caffeic acid (**1**) and analogues **4a** and **5a**. Replacing either hydroxyl of **1** by a difluoromethyl reduced the antioxidant activity although **4a**, with

the hydroxyl in the *para* position relative to the acrylate side chain, was more active than its regioisomer. In the case of the methoxylated analogues **4b** and **5b**, both compounds were effective in inhibiting the β-carotene bleaching reaction although their activity was reduced compared to their hydroxyl bearing counterparts **3** and **2**, respectively (Figure 7). As expected, methyl ferulate (**2**) with the hydroxyl in the *para* position relative to the acrylate side chain was more active than methyl isoferulate (**3**). Compound **5b** with the difluoromethyl group *para* to the acrylate side chain followed the same trend, being more active than **4b** and comparable to compound **3**.

## Discussion

Current evidence supports the correlation of the antioxidant activity of HCAs with the presence of one or more phenolic hydroxyls and structural features capable of stabilizing the resulting phenoxyl radicals.[41] However, the correlation between redox potentials and antioxidant activity is not always evident when comparing different compounds. Furthermore, the influence of electron-withdrawing substituents on the redox potentials of HCAs has not been established.[9] As shown in Figure 4, the presence of the $CF_2H$ moiety *ortho* to the phenolic hydroxyl increased the redox potential as compared with **1-3**. In agreement with this increase in $E_p$ values, replacement of either hydroxyl group of methyl caffeate by a difluoromethyl moiety (compounds **4a** and **5a**) resulted in loss of activity in both SET-based assays (FRAP and DPPH scavenging). Compounds without a free phenolic hydroxyl (**4b,c** and **5b,c**) were also inactive in these assays as the difluoromethyl group cannot participate in electron transfer processes.

Interestingly, all the fluorinated compounds inhibited lipid peroxidation in the human erythrocytes assay, where even compounds **4b** and **5b** with non-hydrolysable methyl ethers achieved a high degree of cellular membrane protection in red blood cells. Cinnamate-like antioxidants without free phenolic hydroxyls are rare, and until now, their activity appeared to be restricted to whole cell systems. Li *et al.* reported a small but significant inhibition of AAPH-induced red blood cell haemolysis by 3,4-dimethoxycinnamic acid and this was ascribed to demethylation occurring in the erythrocytes.[13] In a similar assay of the schisandrins that contain several aromatic methoxy groups but no free phenolic hydroxyls, activity was lost upon inhibition of demethylases.[12] Other mechanisms not involving the phenolic hydroxyl have been proposed for a series of eugenol derivatives with antioxidant properties.[46] In our case, the behaviour of compounds **4b** and **5b** as chain-breaking antioxidants in the β-carotene bleaching assay supports the participation of the difluoromethyl group in a hydrogen atom transfer mechanism as opposed to a single electron transfer mechanism.[47] The capacity of the difluoromethyl group to generate a stable carbon radical in lipophilic environments was evidenced here on the higher activity of compound **5b**, in which this radical may be further stabilized by delocalization on the acrylate side chain.[22,23] The same trend (although not statistically significant) was observed for the inhibition of lipid peroxidation in erythrocytes, suggesting an active participation of the difluoromethyl moiety in the antioxidant process.

Based on the behaviour of other resonance-stabilized carbon-centred radicals, dioxygen addition to the difluoromethyl radicals is expected to be reversible. Thus, their effectiveness as chain-breaking antioxidants should be enhanced at low oxygen partial pressure (e.g. in mammalian tissues).[47] The higher lipophilicity of the difluoromethyl-substituted analogues compared to the phenolic parent compounds (see supporting information, Table S2) should give an improved membrane permeability and also play a crucial role due to the 'polar paradox', by which lipophilic antioxidants tend to concentrate in the lipid environment.[48] These properties would explain at least in part, why these analogues exhibited either equal or better inhibition of lipid peroxidation in the cellular assay, compared to their hydroxylated counterparts.

## Conclusions

The radical scavenging activity of the difluoromethyl derivatives described here was manifested only in the β-carotene bleaching assay and the lipid peroxidation cellular assay, strongly suggesting that the difluoromethyl group actively participates in the radical scavenging process by a hydrogen atom transfer mechanism. Our results show that as a bioisostere of the phenolic hydroxyl, the lipophilic difluoromethyl group retains not only the H-bond donor properties, but also radical scavenging capacities that prove useful to confer enhanced antioxidant properties in lipophilic conditions. This bioisosteric replacement may thus provide a strategy for modifying polar phenolic antioxidants rendering them more effective in lipophilic environments and biological systems.

## Declarations

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1** Antioxidant efficacy (DPPH and FRAP) and redox potentials ($E_p$).

**Table S2** Calculated partition coefficients for compounds **1-3**, **4a-c**, **5a-c**.

**Appendix S1** Synthesis of intermediates **6b**, **6c**, **9b** and **9c**.

**Appendix S2** $^1$H (500.13 MHz) and $^{13}$C (125.72 MHz) NMR spectra of compounds **4a-c** and **5a-c**.

## Appendix

## Physical and spectroscopic data

### Methyl 3-(3-formyl-4-hydroxyphenyl)-(E)-propenoate (6a)

White solid, mp 104°C (lit.[49] 100–101°C); $^1$H NMR ($CDCl_3$) δ: 11.21 (s, 1H, ArO**H**), 9.93 (d, J = 0.5 Hz, 1H, ArC**H**O), 7.73 (dd, J = 2.3, 8.6 Hz, 1H, 6'-H), 7.71 (d, J = 2.2 Hz, 1H, 2'-H), 7.66 (d, J = 16.0 Hz, 3-H), 7.03 (d, J = 8.5 Hz, 1H, 5'-H), 6.37 (d, J = 16.0 Hz, 1H, 2-H), 3.82 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 196.4 (Ar**C**HO), 167.4 (1-C), 163.2 (4'-C), 142.9 (3-C), 135.8 (6'-C), 134.0 (2'-C), 126.8 (1'-C), 120.7 (3'-C), 118.8 (5'-C), 117.1 (2-C), 51.9 (**C**H$_3$O); HRMS: calcd for $C_{11}H_{11}O_4^+$ $[M+H]^+$: 207.0652, found: 207.0658.

### Methyl 3-(3-formyl-4-methoxyphenyl)-(E)-propenoate (6b)

White solid, mp 123–124°C; $^1$H NMR ($CDCl_3$) δ: 10.46 (s, 1H, ArC**H**O), 8.01 (d, J = 2.4 Hz, 1H, 2'-H), 7.71 (dd, J = 2.4, 8.7 Hz, 1H, 6'-H), 7.65 (d, J = 16.0 Hz, 1H, 3-H), 7.03 (d, J = 8.7 Hz, 1H, 5'-H), 6.40 (d, J = 16.0 Hz, 1H, 2-H), 3.98 (s, 3H, C**H$_3$**OAr), 3.80 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 189.3 (Ar**C**HO), 167.5 (1-C), 163.1 (4'-C), 143.3 (3-C), 135.5 (6'-C), 128.3 (2'-C), 127.4 (1'-C), 125.1 (3'-C), 117.3 (2-C), 112.3 (5'-C), 56.1 (**C**H$_3$OAr), 51.9 (**C**H$_3$O); HRMS: calcd for $C_{12}H_{13}O_4^+$ $[M+H]^+$: 221.0808, found: 221.0805.

### Methyl 3-(4-acetyloxy-3-formylphenyl)-(E)-propenoate (6c)

White needles, mp 141–142°C; $^1$H NMR ($CDCl_3$) δ: 10.13 (bs, 1H, ArC**H**O), 8.02 (d, J = 2.3 Hz, 1H, 2'-H), 7.77 (ddd, J = 0.4, 2.3, 8.4 Hz, 1H, 6'-H), 7.70 (d, J = 16.0 Hz, 1H, 3-H), 7.25 (d, J = 8.4 Hz, 1H, 5'-H), 6.49 (d, J = 16.0 Hz, 1H, 2-H), 3.83 (s, 3H, C**H$_3$**O), 2.41 (s, 3H, C**H$_3$**C(O)); $^{13}$C NMR ($CDCl_3$) δ: 188.2 (Ar**C**HO), 169.1 ($CH_3$**C**(O)), 167.0 (1-C), 152.7 (4'-C), 142.4 (3-C), 134.3 (6'-C), 133.1 (1'-C), 130.5 (2'-C), 128.4 (3'-C), 124.4 (5'-C), 119.9 (2-C), 51.9 (**C**H$_3$O), 21.0 (**C**H$_3$C(O)); HRMS: calcd for $C_{13}H_{12}NaO_5^+$ $[M+Na]^+$: 271.0577, found: 271.0580.

### Methyl 3-[(3-(difluoromethyl)-4-methoxyphenyl)]-(E)-propenoate (4b)

White solid, mp 77–78°C; $^1$H NMR ($CDCl_3$) δ: 7.76–7.75 (m, 1H, 2'-H), 7.66 (d, J = 16.0 Hz, 1H, 3-H), 7.60–7.58 (m, 1H, 6'-H), 6.95 – 6.91 (m, 1H, 5'-H), 6.93 (t, J = 55.5 Hz, 1H, $CF_2$**H**), 6.37 (d, J = 16.0 Hz, 1H, 2-H),

3.91 (s, 3H, C**H$_3$**OAr), 3.80 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 167.6 (1-C), 158.9 (t, *J* = 6 Hz, 4'-C), 143.7 (3-C), 132.2 (6'-C), 127.3 (1'-C), 126.1 (t, *J* = 6 Hz, 2'-C), 123.4 (t, *J* = 23 Hz, 3'-C), 116.8 (2-C), 111.4 (5'-C), 111.2 (t, *J* = 237 Hz, **C**F$_2$H), 56.0 (**C**H$_3$OAr), 51.9 (**C**H$_3$O); HRMS: calcd for $C_{12}H_{12}F_2NaO_3^+$ $[M+Na]^+$: 265.0647, found: 265.0656.

## Methyl 3-[4-acetyloxy-(3-(difluoromethyl) phenyl)]-(E)-propenoate (4c)

White solid, mp 101–102°C; $^1$H NMR ($CDCl_3$) δ: 7.77–7.75 (m, 1H, 2'-H), 7.69 (d, J = 16.0 Hz, 1H, 3-H), 7.66–7.62 (m, 1H, 6'-H), 7.25–7.23 (m, 1H, 5'-H), 6.76 (t, J = 55.2 Hz, 1H, CF$_2$**H**), 6.45 (d, J = 16.0 Hz, 1H, 2-H), 3.82 (s, 3H, C**H$_3$**O), 2.35 (s, 3H, C**H$_3$**C(O)); $^{13}$C NMR ($CDCl_3$) δ: 168.6 (CH$_3$**C**(O)), 167.1 (1-C), 149.7 (t, J = 5 Hz, 4'-C), 142.8 (3-C), 132.7 (1'-C), 131.2 (t, J = 2 Hz, 6'-C), 127.1 (t, J = 23 Hz, 3'-C), 126.3 (t, J = 6 Hz, 2'-C), 124.0 (5'-C), 119.4 (2-C), 111.5 (t, J = 239 Hz, **C**F$_2$H), 52.0 (**C**H$_3$O), 21.0 (**C**H$_3$C(O)); HRMS: calcd for $C_{13}H_{12}F_2NaO_4^+$ $[M+Na]^+$: 293.0596, found: 293.0592.

## Methyl 3-[(3-(difluoromethyl)-4-hydroxyphenyl)]-(E)-propenoate (4a)

Light yellow solid, mp 132–133°C; $^1$H NMR ($CDCl_3$) δ: 7.64 (d, J = 16.0 Hz, 1H, 3-H), 7.65–7.63 (m, 1H, 2'-H), 7.53–7.48 (m, 1H, 6'-H), 6.93–6.90 (m, 1H, 5'-H), 6.90 (t, J = 55.3 Hz, 1H, CF$_2$**H**), 6.45 (bs, 1H, ArO**H**), 6.35 (d, J = 16.0 Hz, 1H, 2-H), 3.82 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 167.8 (1-C), 155.8 (t, J = 4.6 Hz, 4'-C), 143.8 (3-C), 132.0 (6'-C), 127.5 (1'-C), 127.2 (t, J = 6 Hz, 2'-C), 121.3 (t, J = 22 Hz, 3'-C), 117.3 (5'-C), 116.7 (2-C), 112.8 (t, J = 237 Hz, **C**F$_2$H), 52.0 (**C**H$_3$O); HRMS: calcd for $C_{11}H_{10}F_2NaO_3^+$ $[M+Na]^+$: 251.0490, found: 251.0500.

## 2-Hydroxymethyl-5-iodophenol (7)

White solid, mp 138°C; $^1$H NMR ($CD_3OD$) δ: 7.16 (dd, J = 1.7, 7.9 Hz, 1H, 5-H), 7.12 (d, J = 1.6 Hz, 1H, 2-H), 7.03 (d, J = 7.9 Hz, 1H, 6-H), 4.58 (s, 2H, ArC**H$_2$**OH); $^{13}$C NMR ($CD_3OD$) δ: 157.1 (2-C), 130.7 (6-C), 129.5 (5-C), 128.9 (1-C), 124.7 (3-C), 93.0 (4-C), 60.4 (Ar**C**H$_2$OH); HRMS: calcd for $C_7H_7INaO_2^+$ $(M+Na)^+$: 272.9383, found: 272.9394.

## 2-Hydroxy-4-iodo-benzaldehyde (8)

White solid, mp 85–86°C (lit.[50] 87°C); $^1$H NMR ($CDCl_3$) δ: 11.02 (s, 1H, ArO**H**), 9.85 (d, J = 0.6 Hz, 1H, ArC**H**O), 7.45–7.43 (m, 1H, 3-H), 7.40 (dd, J = 1.5, 8.1 Hz, 1H, 5-H), 7.24 (d, J = 8.1 Hz, 1H, 6-H); $^{13}$C NMR ($CDCl_3$) δ: 196.2 (Ar**C**HO), 161.5 (2-C), 134.4 (6-C), 129.6 (5-C), 127.4 (3-C), 120.1 (1-C), 105.3 (4-C).

## Methyl 3-(4-formyl-3-hydroxyphenyl)-(E)-propenoate (9a)

White solid, mp 87–88°C; $^1$H NMR ($CDCl_3$) δ: 11.03 (bs, 1H, ArO**H**), 9.91 (d, J = 0.6 Hz, 1H, ArC**H**O), 7.63 (d, J = 16.0 Hz, 3-H), 7.59 (d, J = 8.0 Hz, 1H, 5'-H), 7.17 (dd, J = 1.5, 8.0 Hz, 1H, 6'-H), 7.12 (d, J = 1.2 Hz, 1H, 2'-H), 6.53 (d, J = 16.0 Hz, 1H, 2-H), 3.83 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 196.1 (Ar**C**HO), 166.8 (1-C), 161.9 (3'-C), 143.0 (3-C), 142.6 (1'-C), 134.2 (5'-C), 122.1 (2-C), 121.4 (4'-C), 119.4 (6'-C), 117.1 (2'-C), 51.2 (**C**H$_3$O); HRMS: calcd for $C_{11}H_{11}O_4^+$ $[M+H]^+$: 207.0652, found: 207.0659.

## Methyl 3-(4-formyl-3-methoxyphenyl)-(E)-propenoate (9b)

White solid, mp 145°C; $^1$H NMR ($CDCl_3$) δ: 10.5 (d, J = 0.8 Hz, 1H, ArC**H**O), 7.84 (d, J = 7.9 Hz, 1H, 5'-H), 7.68 (d, J = 16.0 Hz, 1H, 3-H), 7.23–7.17 (m, 1H, 6'-H), 7.09 (d, J = 1.4 Hz, 1H, 2'-H), 6.53 (d, J = 16.0 Hz, 1H, 2-H), 3.97 (s, 3H, C**H$_3$**OAr), 3.83 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 189.2 (Ar**C**HO), 166.9 (1-C), 162.0 (3'-C), 143.6 (3-C), 141.6 (1'-C), 129.2 (5'-C), 125.9 (4'-C), 121.0 (2-C), 120.4 (6'-C), 111.1 (2'-C), 55.9 (**C**H$_3$OAr), 55.1 (**C**H$_3$O); HRMS: calcd for $C_{12}H_{12}NaO_4^+$ $[M+Na]^+$: 243.0628, found: 243.0624.

## Methyl 3-(3-acetyloxy-4-formylphenyl)-(E)-propenoate (9c)

White solid, mp 106–107°C; $^1$H NMR ($CDCl_3$) δ: 10.10 (bs, 1H, ArC**H**O), 7.90 (d, J = 8.0 Hz, 1H, 5'-H), 7.67 (d, J = 16.0 Hz, 1H, 3-H), 7.53 (dd, J = 1.5, 8.0 Hz, 1H, 6'-H), 7.33 (d, J = 1.5 Hz, 1H, 2'-H), 6.53 (d, J = 16.0 Hz, 1H, 2-H), 3.83 (s, 3H, C**H$_3$**O), 2.42 (s, 3H, C**H$_3$**C(O)); $^{13}$C NMR ($CDCl_3$) δ: 188.1 (Ar**C**HO), 169.2 (CH$_3$**C**(O)), 166.7 (1-C), 151.9 (3'-C), 142.3 (3-C), 141.4 (1'-C), 131.9 (5'-C), 128.8 (4'-C), 126.0 (6'-C), 122.8 (2'-C), 122.0 (2-C), 52.2 (**C**H$_3$O), 21.0 (**C**H$_3$C(O)); HRMS: calcd for $C_{13}H_{12}NaO_5^+$ $[M+Na]^+$: 271.0577, found: 271.0568.

## Methyl 3-[(4-(difluoromethyl)-3-methoxyphenyl)]-(E)-propenoate (5b)

White solid, mp 108–109°C; $^1$H NMR ($CDCl_3$) δ: 7.68 (d, J = 16.0 Hz, 1H, 3-H), 7.58 (d, J = 7.9 Hz, 1H, 5'-H), 7.20 (d, J = 8.0 Hz, 1H, 6'-H), 7.06–7.03 (m, 1H, 2'-H), 6.93 (t, J = 55.6 Hz, 1H, CF$_2$**H**), 6.48 (d, J = 16.0 Hz, 1H, 2-H), 3.91 (s, 3H, C**H$_3$**OAr), 3.83 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 167.2 (1-C), 157.7 (t, J = 6 Hz, 3'-C), 144.0

(3-C), 138.2 (t, J = 2 Hz, 1'-C), 126.9 (t, J = 6 Hz, 5'-C), 124.5 (t, J = 22.3 Hz, 4'-C), 120.7 (6'-C), 119.7 (2-C), 111.3 (t, J = 236 Hz, **C**$F_2$H), 110.0 (2-C), 55.8 (**C**$H_3$OAr), 52.0 (**C**$H_3$O); HRMS: calcd for $C_{12}H_{12}F_2NaO_3^+$ $[M+Na]^+$: 265.0647, found: 265.0653.

## Methyl 3-[3-acetyloxy-4-(difluoromethyl) phenyl)]-(E)-propenoate (5c)

White solid, mp 73–74°C; $^1$H NMR ($CDCl_3$) δ: 7.65 (d, J = 16.0 Hz, 1H, 3-H), 7.63 (d, J = 8.2 Hz, 1H, 5'-H), 7.47 (d, J = 8.2 Hz, 1H, 6'-H), 7.34 (bs, 1H, 2'-H), 6.74 (t, J = 55.1 Hz, 1H, $CF_2$**H**), 6.47 (d, J = 16.0 Hz, 1H, 2-H), 3.82 (s, 3H, C**H$_3$**O), 2.36 (s, 3H, C**H$_3$**C(O)); $^{13}$C NMR ($CDCl_3$) δ: 168.8 ($CH_3$**C**(O)), 166.9 (1-C), 148.9 (t, J = 5.3 Hz, 3'-C), 142.7 (3-C), 138.3 (t, J = 2 Hz, 1'-C), 127.8 (t, J = 23 Hz, 4'-C), 127.3 (t, J = 6.4 Hz, 5'-C), 125.8 (6'-C), 122.5 (2'-C), 120.7 (2-C), 111.6 (t, J = 2383 Hz, **C**$F_2$H), 52.1 (**C**$H_3$O), 20.9 (**C**$H_3$C(O)); HRMS: calcd for $C_{13}H_{13}F_2O_4^+$ $(M+H)^+$: 271.0776, found: 271.0776.

## Methyl-3-[(4-(difluoromethyl)-3-hydroxyphenyl)]-(E)-propenoate (5a)

White solid, mp 129–130°C; $^1$H NMR ($CDCl_3$) δ: 7.64 (d, J = 16.0 Hz, 1H, 3-H), 7.48 (d, J = 8.0 Hz, 1H, 5'-H), 7.17 (d, J = 8.0 Hz, 1H, 6'-H), 7.10–7.06 (m, 1H, 2'-H), 6.90 (t, J = 55.3 Hz, 1H, $CF_2$**H**), 6.46 (d, J = 16.0 Hz, 1H, 2-H), 6.21 (t, J = 1.6 Hz, 1H, ArO**H**), 3.84 (s, 3H, C**H$_3$**O); $^{13}$C NMR ($CDCl_3$) δ: 167.6 (1-C), 154.5 (t, J = 5.0 Hz, 3'-C), 143.9 (3-C), 138.2 (t, J = 1.7 Hz, 1'-C), 127.7 (t, J = 6 Hz, 5'-C), 122.5 (t, J = 22 Hz, 4'-C), 120.5 (6'-C), 119.7 (2-C), 116.0 (2'-C), 112.8 (t, J = 236 Hz, **C**$F_2$H), 52.2 (**C**$H_3$O); HRMS: calcd for $C_{11}H_{10}F_2NaO_3^+$ $[M+Na]^+$: 251.0490, found: 251.0493.