European Journal of Medicinal Chemistry 54 (2012) 952–958

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Preliminary communication

Synthesis and biological evaluation of novel flavonols as potential anti-prostate cancer agents☆

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ARTICLE INFO

Article history:
Received 29 March 2012
Received in revised form
7 June 2012
Accepted 14 June 2012
Available online 22 June 2012

Keywords:
Flavonols
AR signalling pathway
Prostate cancer management

ABSTRACT

A library of flavonol analogues was synthesised and evaluated as potential anticancer agents against a human prostate cancer cell line, 22rv1. Compounds **3**, **8** and **11** (IC_{50} 2.6, 3.3 and 4.0 μM respectively) showed potent cancer cell growth inhibition, comparable to the lead compound 3′,4′,5′-trimethoxyflavonol (**1**) (IC_{50} 3.1 μM) and superior to the naturally occurring flavonols quercetin (**16**) and fisetin (**22**) (both >15 μM). Results indicate that the 3′,4′,5′- arrangement of either hydroxy (OH) or methoxy (OMe) residues is important for growth arrest of these cells and that the OMe analogues may be superior to their OH counterparts. Compounds **1**, **3**, **8** and **11** warrant further investigation as potential agents for the management of prostate cancer.

1. Introduction

Prostate cancer is the most commonly diagnosed male cancer in the developed world with the incidence in Western Europe being 93 per 100,000 men compared to the global incidence of 28 cases per 100,000 and mortality being 12 and 7 per 100,000, respectively [1]. Other than surgical intervention and/or anti-androgen chemotherapy there are few alternatives for the treatment or prevention of prostate cancer.

Flavonoids are produced ubiquitously in plants, and many of them can be found in the diet or in traditional herbal medicines. A sub-class of these are the flavonols, which are characterized by a hydroxy group on C-3 of the flavone scaffold (Table 1). The naturally occurring flavonols fisetin (3′,4′,7-trihydroxyflavonol, **22**), and quercetin (3′,4′,5,7-tetrahydroxyflavonol, **16**), are produced ubiquitously in the plant kingdom and may be found in high concentrations in certain food plants, most notably grape, onion and cucumber, and tea, apple and onion, respectively. Both compounds have been demonstrated to possess anti-prostate cancer properties [2–8]. Fisetin caused apoptosis in LNCaP prostate cancer cells [2], and quercetin has been shown to attenuate proliferation and decrease androgen receptor (AR) expression [3] in 22rv1 cells. We have recently synthesized 3′,4′,5′-trimethoxyflavonol (TMFol (**1**)) and found it to possess colorectal cancer chemopreventive activity [9] in preclinical systems. In a mouse prostate tumour model TMFol displayed anti-prostate cancer activity which was comparable, if not superior, to that of the structurally related molecules quercetin (**16**) and fisetin (**22**) [10].

The normal development and maintenance of the prostate is dependent on the hormone androgen acting through the AR. Activation of the AR drives development of prostate cancer. Androgen ablation is an important therapeutic option for patients with primary androgen-dependent prostate cancer [11]. Androgen ablation is a type of hormone therapy that specifically removes testosterone from the body with the aim of controlling the progression of the prostate cancer. Two common types of androgen ablation are surgical castration or medical castration. However, almost all patients in this group become refractory to androgen ablation therapy [12]. In spite of this insensitivity, the AR and the AR target gene Prostate Specific Antigen (PSA) continues to be expressed in these patients, indicating that the AR signalling pathway remains active [13]. It has been suggested that agents which down-regulate AR can inhibit the growth of prostate cancer cells [12], and TMFol decreased AR and PSA expression in cells *in vitro* [11] significantly. We investigated a library of 27 flavonols

Abbreviations: AR, androgen receptor; PSA, Prostate Specific Antigen; TMFol, 3′,4′,5′-trimethoxyflavonol.

☆ Grant Support: Cancer Research UK (A325/A13101) and Prostate Action (G2009/25).

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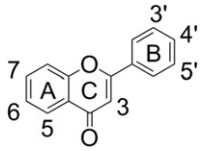
http://dx.doi.org/10.1016/j.ejmech.2012.06.031

Table 1
Cytotoxicity of flavonol analogues against 22Rv1 human prostate carcinoma cells and corresponding AR and PSA protein expression levels.



Compound	C		A			B		22rv1 IC_{50}[a]	AR	% Control[b]	PSA	% Control[b]
	3	5	6	7	3′	4′	5′	(μM)	10 μM	20 μM	10 μM	20 μM
1 (**TMFol**)	OH	H	H	H	OMe	OMe	OMe	3.1 ± 0.3	96(18)	**62**(23)[c]	**21**(15)[c]	**23**(15)[c]
2	OH	H	H	H	OH	OH	OH	7.7 ± 3.0	94(26)	89(47)	110(41)	109(40)
3	OMe	H	H	H	OMe	OMe	OMe	2.6 ± 0.5	94(47)	80(30)	167(99)	149(96)
4	OH	H	H	H	OMe	H	H	5.3 ± 0.4	64(36)	**23**(35)[c]	**27**(17)[c]	**13**(23)[c]
5	OH	H	H	H	H	OMe	H	30.5 ± 17.8	71(27)	**61**(22)[c]	127(37)	115(39)
6	OH	H	H	H	OMe	OMe	H	12.3 ± 3.5	63(46)	81(26)	**61**(21)[c]	**56**(22)[c]
7	OH	H	H	H	OMe	H	OMe	12.4 ± 1.7	74(20)	72(23)	133(48)	109(58)
8	OH	H	F	H	OMe	OMe	OMe	3.3 ± 0.8	91(2)	**75**(9)[c]	**14**(19)[c]	**5**(6)[c]
9	F	H	H	H	OMe	OMe	OMe	>40	110(4)	125(9)	**63**(13)[c]	63(35)
10 (**Robinetin**)	OH	H	H	OH	OH	OH	OH	7.1 ± 2.9	84(39)	102(68)	69(42)	111(48)
11	OH	H	H	F	OMe	OMe	OMe	4.0 ± 0.7	112(62)	75(43)	**37**(16)[c]	**22**(19)[c]
12	OH	H	H	F	OH	OH	OH	11.6 ± 6.9	100(38)	94(41)	111(58)	106(59)
13 (**Myricetin**)	OH	OH	H	OH	OH	OH	OH	13.1 ± 4.7	119(72)	114(69)	110(53)	121(40)
14	OH	F	H	F	OMe	OMe	OMe	5.9 ± 0.7	100(37)	103(55)	95(47)	68(25)
15	OH	F	H	F	OH	OH	OH	6.3 ± 0.8	118(52)	90(32)	134(71)	132(72)
16 (**Quercetin**)	OH	OH	H	OH	OH	OH	H	15.5 ± 6.2	114(52)	120(36)	**40**(23)[c]	**31**(23)[c]
17	OH	F	H	F	OMe	OMe	H	7.4 ± 1.3	110(42)	94(35)	78(14)	88(18)
18	OH	F	H	F	OH	OH	H	16.3 ± 11.5	116(14)	86(14)	**61**(12)[c]	**22**(19)[c]
19 (**Kaempferol**)	OH	OH	H	OH	H	OH	H	>40	128(30)	131(24)	**33**(13)[c]	**37**(15)[c]
20	OH	F	H	F	H	OMe	H	15.8 ± 6.6	115(14)	111(16)	85(13)	**66**(16)[c]
21	OH	F	H	F	H	OH	H	28.7 ± 18.4	136(36)	96(53)	77(27)	**33**(41)[c]
22 (**Fisetin**)	OH	H	H	OH	OH	OH	H	25.9 ± 10.8	109(17)	101(39)	**51**(6)[c]	**44**(20)[c]
23	OH	H	H	F	OMe	OMe	H	>40	129(31)	120(18)	78(23)	**70**(18)[c]
24	OH	H	H	F	OH	OH	H	9.2 ± 1.5	108(33)	59(43)	**21**(19)[c]	**18**(16)[c]
25	OH	H	H	OH	H	OH	H	30.2 ± 4.7	141(36)	125(31)	**47**(9)[c]	**39**(10)[c]
26	OH	H	H	F	H	OMe	H	7.1 ± 0.9	112(8)	104(15)	**70**(15)[c]	**62**(2)[c]
27	OH	H	H	F	H	OH	H	8.4 ± 1.3	129(45)	64(29)	**32**(14)[c]	**3**(6)[c]
Flavonol	OH	H	H	H	H	H	H	14.9 ± 8.3	100(38)	92(60)	124(38)	80(46)

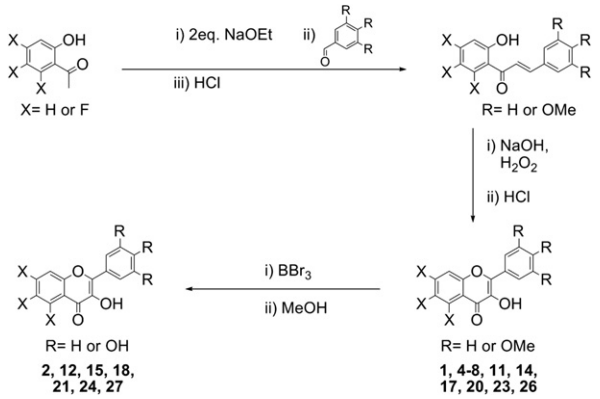
[a] IC_{50} calculated as the mean ± standard deviation of 3–6 independent experiments.
[b] Protein expression levels measured by densitometry and is the mean of 3–5 replicates. SD in brackets.
[c] Indicates data point is significant from vehicle control ($p < 0.05$).

(Table 1) including quercetin (**16**) and fisetin (**22**), two of the most widely studied flavonols for prostate cancer, to determine which flavonol structural features may contribute to their abilities to compromise prostate cancer cell growth and to modulate the expression of the AR and PSA using the human-derived prostate carcinoma cells, 22rv1 [14,15]. These cells mimic the transition between androgen-dependent and androgen-independent cancer types as they are androgen-independent but androgen-responsive, they also express PSA. Androgen-independence means that the cells are not reliant on androgens for growth however these cells will respond to them if present. The work aimed to identify agents which may warrant further development as potential anti-prostate cancer chemotherapeutic or chemopreventive drugs.

The synthetic strategy employed involved placement of OH, OMe or F in different positions on the flavonol scaffold generating a library of natural product analogues (Table 1). Replacement of OH groups with OMe moieties has been suggested to reduce the degree of metabolic removal of flavones whilst retaining anti-proliferative potency [16]. The use of fluorine as a bioisostere for C–H or C–OH has afforded many useful compounds, and about 20% of all pharmaceuticals and 30% of agrochemicals are now fluorinated [17]. Fluorine substituents can impart greater metabolic stability, increase lipophilicity, thus increasing bioavailability and they can also increase potency [18].

2. Chemistry

The library (Table 1) consisted of 27 flavonols, of which 7 occur in plants and can be purchased, whereas 20 have been synthesised, including 15 novel flavonols. The synthesis was performed using well established chemistry [9] with a maximum of 4 consecutive steps (Schemes 1, 3 and 4) and crystallisation purification. All starting materials could be purchased, apart from 4′,6′-difluoro-2′-hydroxyacetophenone (**28**), which was synthesised in high yield (89%) from 3,5-difluorophenol *via* *O*-acetylation followed by an $AlCl_3$ catalysed Fries rearrangement (Scheme 2). Methoxyflavonols were produced by the Claisen–Schmidt condensation of a 2′-hydroxyacetophenone with a benzaldehyde to afford the corresponding 2′-hydroxychalcones, which were then treated under Algar–Flynn–Oyamada conditions (H_2O_2, NaOH) to produce the desired flavonols with yields of 11%–78% over 2 steps following recrystallisation. Methoxyflavonols were then demethylated using BBr_3 to produce the corresponding hydroxyflavonols in good yields (76%–99%). 3,3′,4′,5′-Tetramethoxyflavone (**3**) was obtained by methylation of TMFol (**1**) using iodomethane (Scheme 3). 3-Fluoro-3′,4′,5′-trimethoxyflavone (**9**) was synthesised in 4 steps (Scheme 4). Initially, a condensation between 2′-hydroxyacetophenone and 3,4,5-trimethoxybenzoyl chloride produced 2-acetylphenyl 3,4,5-trimethoxybenzoate, which was then treated with base (KOH) to afford 3-hydroxy-1-(2′-



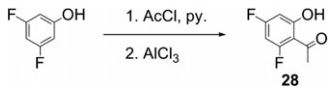
Scheme 1. Synthesis of methoxy and hydroxyflavonols.

hydroxyphenyl)-3-(3″,4″,5″-trimethoxyphenyl)prop-2-en-1-one (**29**) (47%, 2 steps) *via* a Baker–Venkataraman rearrangement. Compound **29** was fluorinated at the 2-position using *N*-fluorobenzenesulfonimide to give **30** (37%), and then cyclised and dehydrated under acidic conditions to yield **9** (96%).

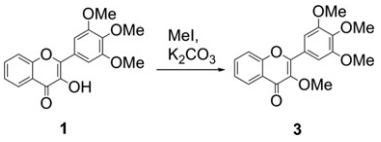
3. Results and discussion

Twenty-eight flavonoids (**1**–**27** plus flavonol) were tested for their anti-proliferative activity and their ability to modulate the AR signalling pathway in the human prostate cancer cell line 22rv1. The human-derived prostate cancer cell line 22rv1 was selected as it has been used previously to explore the ability of the flavonol fisetin to affect growth of cells in culture *in vitro* and in mice *in vivo*. It was therefore selected to screen the chosen library of flavonols. As 22rv1 cells express AR, produce PSA and are androgen responsive, they were considered a suitable paradigm for our study. TMFol (**1**) showed significant growth inhibition with an IC_{50} value of 3.1 μM (Table 1). Three of the 28 compounds tested, **3**, **8** and **11**, had comparable anti-proliferative activity, with IC_{50} values of 2.6, 3.3 and 4.0 μM respectively. Compounds **1**–**4**, **8**, **10**, **11**, **14**, **15**, **17**, **24**, **26** and **27** inhibited proliferation with IC_{50} values $\leq$10 μM and are more potent than fisetin (**22**) and quercetin (**16**), both of which have been under investigation pre-clinically as anticancer agents [2–8]. Of the thirteen compounds characterized by $IC_{50} \leq 10$ μM, seven are novel, and of those that had an $IC_{50} \leq 5$ μM (**1**, **3**, **8** and **11**), only TMFol (**1**) has been reported before [9]. The IC_{50} value of TMFol (**1**) was significantly lower ($p < 0.05$) than those of all the natural flavonols (**10**, **13**, **16**, **19**, **22** and **25**). The rank order for anti-proliferative activity of the natural products, as judged by IC_{50} value, was robinetin > myricetin > quercetin > fisetin > resokaempferol (**25**) > kaempferol.

All compounds which inhibited cell growth with an $IC_{50} \leq 5$ μM (**1**, **3**, **8** and **11**) have methoxy moieties at the 3′,4′,5′ positions. Of the natural products that were tested, only those with hydroxy groups in positions 3′,4′,5′ (**10** and **13**) showed an $IC_{50} \leq 15$ μM. The results suggest that the 3′,4′,5′ arrangement of OH or OMe on the B ring of the flavonol scaffold is important for cell growth inhibition. This notion is borne out by the findings that compounds **1**–**3**, **8** and **10**–**15** were more potent than **4**–**7** and **16**–**27**, which lack the



Scheme 2. Synthesis of 4′,6′-difluoro-2′-hydroxyacetophenone (**28**).



Scheme 3. Synthesis of 3,3′,4′,5′-tetramethoxyflavone (**3**).

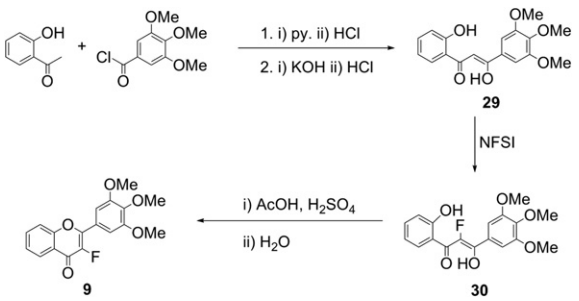
3′,4′,5′ OH/OMe substitution pattern. For compound **9** a concentration of 0.01 μM inhibited cell growth by 18% and 0.1 μM inhibited by 46%. A high concentration, 50 μM, caused cell growth inhibition of 48%. Therefore an accurate IC_{50} value could not be established, hence we state it is >40 μM. Compounds **8** and **11** both contain a fluorine on the A ring, **11** has a fluorine on C-7, which harbours OH or OMe in natural compounds such as fisetin (**22**) and robinetin (**10**), whereas the fluorine on compound **8** is on C-6, which is not a site substituted in naturally occurring compounds.

Several of the compounds tested altered expression levels of the AR or PSA proteins (Tables 1 and S63 of Supporting Information which shows western blot data for the expression of AR and PSA proteins in human 22rv1 prostate carcinoma cells against the full library of compounds). TMFol (**1**) and compounds **4**, **5** and **8** at 20 μM were able to significantly inhibit AR expression. None of these compounds contain OH or OMe on the A ring, whilst compound **8** has a fluorine at C-6.

Compounds **1**, **4**, **6**, **8**, **11**, **16**, **18**, **19**, **22** and **24**–**27** significantly reduced PSA expression at 10 and 20 μM, and compounds **20**, **21** and **23** at 20 μM. The most potent inhibitors of PSA expression were compounds **1**, **4**, **8**, **11** and **24**, three of which (**1**, **8** and **11**) were also among the more potent inhibitors of cell growth. Of the twelve compounds that significantly reduced PSA expression, eight (**11**, **16**, **19**, **22** and **24**–**27**) contain hydroxy, methoxy or fluorine at C-7 on the A ring, four (**11**, **24**, **26** and **27**) possess a fluorine at C-7 and compound **8** bears a fluorine on C-6.

Modulation of the AR signalling pathway may be one of the mechanisms by which these compounds exert their anti-proliferative activity. The most potent growth-inhibitory compound (**3**) was clearly a less potent modulator of the expression of either AR or PSA than compounds **1** and **8**. Assuming that modulation of AR and PSA contributes to the growth inhibition exerted by some of these flavonols, this finding hints at the possibility that compound **3** may inhibit cell growth by engaging mechanisms of action different from those operated by compounds **1** and **8**. In addition the low growth inhibitory potential of compound **5** may be related to its low ability to affect PSA expression. Whilst the AR system is possibly a target for some of these agents, we cannot make any inferences as to the ultimate role it may play in mediating the compounds growth inhibition.

Replacement of the OH residue with F did not appear to enhance, or interfere with, growth-inhibitory activity or the ability to modulate AR signalling. In contrast, substitution of the hydroxy group on C-7 of the A ring of the natural product 4′,7-



Scheme 4. Synthesis of 3-fluoro-3′,4′,5′-trimethoxyflavone (**9**).

dihydroxyflavonol (**25**) with fluorine yielding **27** increased growth-inhibitory potency and reduced PSA and AR expression when compared to **25**. A similar difference was observed for the pair compound **24** versus fisetin (**22**), where replacement of the hydroxyl on C-7 by fluorine imparted superior potency in terms of ability to affect PSA levels at 20 μM.

4. Conclusion

We synthesised a number of novel flavonol analogues and investigated their potential ability to inhibit cell growth and modulate components of the AR signalling pathway using the human-derived prostate cancer cell line 22rv1. TMFol(**1**), and novel compounds **3**, **8** and **11** showed potent growth inhibition. Compounds **1**, **4** and **8** inhibited the protein expression of AR and PSA proteins. The 3′,4′,5′ arrangement of either OH or OMe groups on the B ring of the flavonol structure may play an important role in inhibition of cell growth. It seems the inclusion of fluorine in the A ring failed to significantly alter the growth inhibition potency. Notably though, compounds **6** and **23** were exceptions, in that their growth-inhibitory ability was significantly lower than that of their non-fluorinated counterparts. We have identified novel flavonols which may possess anti-prostate cancer properties superior to those of fisetin (**22**) and quercetin (**16**).

5. Experimental section

5.1. General

NMR spectra were obtained on a Bruker DPX 300, Bruker DRX 400 or Bruker AV500 spectrometer. Chemical shifts (δ) are reported in ppm for a solution of the compound in specified deuterated solvent internally referenced to either TMS or residual protonated solvent and coupling constants (J) are quoted in hertz. Electrospray mass spectra were recorded on a Micromass Quattro. Dry solvents were obtained from an Innovative Technology, PureSolv solvent purification system, except pyridine, which was purchased from SigmaAldrich. All other chemicals were purchased from SigmaAldrich except compounds **10** and **25**, which were from Indofine Chemical Company. All compounds submitted for biological tests were confirmed to be ≥95% pure by HPLC analysis.

5.1.1. 4′,6′-Difluoro-2′-hydroxyacetophenone (*28*)

To a solution of 3,5-difluorophenol (10 g, 76.9 mmol) and dry pyridine (9.32 mL, 115 mmol) in dry CH_2Cl_2 (100 mL) was slowly added acetyl chloride (7.11 mL, 100 mmol) and the reaction was stirred at room temperature for 30 min $NaHCO_3$ (sat. aq.) (50 mL) was added and the product extracted with CH_2Cl_2, washed with H_2O (2 × 50 mL), dried ($MgSO_4$) and evaporated to yield 3,5-difluorophenyl acetate which was used without further purification. To 3,5-difluorophenyl acetate (13.2 g, 76.9 mmol) was added $AlCl_3$ (13.35 g, 100 mmol) and the mixture was heated (neat) to 150 °C with stirring for 10 min. The reaction mixture was cooled to room temperature and dissolved in ethyl acetate (100 mL) before careful quenching with H_2O (100 mL). The product was extracted with ethyl acetate, washed with H_2O, dried ($MgSO_4$) and evaporated to afford an oil that crystallised on standing (may be recrystallised from ethanol) (11.2 g, 85% (from phenol)). ESI-MS m/z 171 $(M-H)^-$; δ_H (300 MHz, $CDCl_3$) 2.59 (3H, d, J 7.3, CH_3), 6.30 (1H, ddd, J 11.8, 9.1, 2.6, C(5′)H), 6.41 (1H, ddd, J 10.1, 1.7, 2.6, C(3′)H); δ_C (75 MHz, $CDCl_3$) 32.10 (d, J 11.2), 95.9 (dd, J 28.2, 27.1), 101.33 (dd, J 23.6, 3.7), 107.35 (dd, J 14.4, 2.9), 164.11 (dd, J 169.8, 17.1), 165.91 (dd, J 16.6, 7.2), 167.51 (dd, J 168.5, 17.2), 202.06 (d, J 3.6); $\delta_{F\{H\}}$ (282 MHz, $CDCl_3$) −97.5 (d, J 13.6), −101.1 (d, J 13.6).

5.2. Synthesis of methoxyflavonols

5.2.1. General method

5.2.1.1. 5,7-Difluoro-3′,4′-dimethoxyflavonol. Sodium ethoxide (1.19 g, 17.44 mmol) was dissolved in ethanol (20 mL) and cooled to room temperature. To this solution was added 4′,6′-difluoro-2′-hydroxyacetophenone (1.0 g, 5.81 mmol) and this was stirred at room temperature for 1 h. 3,4-Dimethoxybenzaldehyde (0.96 g, 5.81 mmol) was then added and stirred at room temperature overnight. The resulting yellow suspension was poured into H_2O (50 mL) and acidified to pH 1 with HCl (10% aq.). The yellow precipitate was filtered, washed with H_2O and dried. Conversion and product verification was by TLC (SiO_2, ethyl acetate/Pet. ether (40–60 °C) (1:2)) and ESI-MS. This chalcone (1.4 g, 4.37 mmol) was suspended in methanol (50 mL), and NaOH (3 M aq.) (7.5 mL) was added to produce a yellow solution, which was cooled to 0 °C. To this was added H_2O_2 (30% aq.) (1.90 mL, 16.76 mmol) and the solution stirred at 0 °C for 3 h then overnight at room temperature if the reaction was not complete (by TLC). The resulting suspension was poured into HCl (10% aq.) (50 mL), extracted with $CHCl_3$ or CH_2Cl_2 (3 × 30 mL), dried ($MgSO_4$) and evaporated to yield a yellow solid which was recrystallised from ethanol or ethanol/methanol to yield yellow crystals of 5,7-difluoro-3′,4′-dimethoxyflavonol (372 mg, 26% (from benzaldehyde)).

*5.2.1.2. 3′,4′,5′-Trimethoxyflavonol (**1**).* 38% Yield; ESI-MS m/z 329 $(M+H)^+$, 361 $(M+Na)^+$; δ_H (400 MHz; $CDCl_3$) 3.92 (3H, s, OCH_3), 3.93 (6H, s, 2 × OCH_3), 7.37 (1H, ddd, J 8.0, 7.1, 0.7, C(6)H), 7.50 (2H, s, C(2′,6′)H), 7.54 (1H, dd, J 8.6, 0.7, C(8)H), 7.66 (1H, ddd, J 8.6, 7.1, 1.6, C(7)H), 8.20 (1H, dd, J 8.0, 1.6, C(5)H); δ_c (75 MHz; $CDCl_3$) 56.13, 60.84, 105.26, 118.03, 120.45, 124.39, 125.26, 126.07, 133.41, 138.08, 139.80, 144.61, 153.00, 155.03, 173.10; HRMS (EI) 328.09420 (M^+ $C_{18}H_{16}O_6$ requires 328.09429).

*5.2.1.3. 3′-Methoxyflavonol (**4**).* 21% Yield; ESI-MS m/z 269 $(M+H)^+$, 267 $(M-H)^-$; δ_H (300 MHz, $CDCl_3$) 3.89 (3H, s, OCH_3), 7.02 (1H, s, OH), 7.05 (1H, dd, J 2.6, 0.8, C(4′)H), 7.39-7.49 (2H, m, C(5′,6)H), 7.60 (1H, dd, J 8.5, 0.5, C(8)H), 7.72 (1H, ddd, J 8.5, 7.0, 1.6, C(7)H), 7.82-7.89 (2H, m, C(2′,6′)H), 8.26 (1H, dd, J 8.0, 1.6, C(5)H); δ_c (100 MHz; $CDCl_3$) 55.42, 113.25, 115.97, 118.30, 120.26, 120.63, 124.52, 125.47, 129.62, 132.33, 133.67, 138.59, 144.66, 155.41, 159.68, 173.50.

*5.2.1.4. 4′-Methoxyflavonol (**5**).* 11% Yield; ESI-MS m/z 269 $(M+H)^+$, 267 $(M-H)^-$; δ_H (300 MHz, $CDCl_3$) 3.81 (3H, s, OCH_3), 6.87 (1H, s, OH), 6.99 (2H, d, J 9.0, C(2′,6′)H), 7.34 (1H, ddd, J 8.1, 7.0, 1.7, C(7)H), 7.52 (1H, d, J 8.1, C(8)H), 7.63 (1H, ddd, J 7.8, 7.0, 1.0, C(6)H), 8.15–8.20 (3H, m, C(3′,5′,5)H); δ_c (100 MHz; *d*6-DMSO) 55.35, 114.02, 118.29, 121.33, 123.56, 124.45, 124.70, 129.38, 133.46, 138.13, 145.57, 154.42, 160.42, 172.61.

*5.2.1.5. 3′,4′-Dimethoxyflavonol (**6**).* 22% Yield; ESI-MS m/z 299 $(M+H)^+$, 321 $(M+Na)^+$; δ_H (300 MHz, $CDCl_3$) 3.95 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 6.98 (1H, br s, OH), 7.05 (1H, dd, J 8.2, 2.0, C(6′)H), 7.42 (1H, ddd, J 7.5, 7.0, 1.6, C(6)H), 7.61 (1H, d, J 8.2, C(5′)H), 7.71 (1H, ddd, J 9.1, 7.0, 1.5, C(7)H), 7.86 (1H, d, J 2.0, C(2′)H), 7.91 (1H, dd, J 9.1, 1.6, C(8)H), 8.25 (1H, dd, J 7.5, 1.5, C(5)H); δ_c (100 MHz; *d*6-DMSO) 55.57, 55.63, 111.01, 111.51, 118.36, 121.27, 121.50, 123.63, 124.43, 124.68, 133.41, 138.26, 145.45, 148.39, 150.32, 154.38, 172.58.

*5.2.1.6. 3′,5′-Dimethoxyflavonol (**7**).* 21% Yield; ESI-MS m/z 299 $(M+H)^+$, 321 $(M+Na)^+$; δ_H (300 MHz, $CDCl_3$) 3.81 (6H, s, 2 × OCH_3), 6.52 (1H, t, J 2.3, C(4′)H), 6.98 (1H, br s, OH), 7.37 (1H, ddd, J 8.4, 7.1, 1.5, C(7)H), 7.40 (2H, d, J 2.3, C(2′,6′)H), 7.52 (1H, dd, J 8.4, 1.6, C(8)H), 7.65 (1H, ddd, J 8.4, 7.1, 1.6, C(6)H), 8.19 (1H, dd, J 8.4, 1.5, C(5)H); δ_c

(100 MHz; *d*6-DMSO) 56.37, 102.50, 106.99, 119.49, 122.15, 125.54, 125.72, 133.91, 134.72, 140.28, 145.65, 155.47, 161.36, 173.98.

*5.2.1.7. 6-Fluoro-3′,4′,5′-trimethoxyflavonol (**8**).* 29% Yield; ESI-MS *m*/*z* 347 $(M + H)^+$, 346 $(M - H)^-$; δ_H (300 MHz, $CDCl_3$) 3.88 (3H, s, OCH_3), 3.90 (6H, s, 2 × OCH_3), 6.90 (1H, s, OH), 7.39 (1H, ddd, *J* 9.2, 7.7, 3.1, C(7)H), 7.46 (2H, s, C(2′,6′)H), 7.57 (1H, dd, *J* 9.2, 4.1, C(8)H), 7.82 (1H, dd, *J* 8.0, 3.1, C(5)H); δ_c (100 MHz; *d*6-DMSO) 56.31, 60.99, 105.53, 109.89 (d, *J* 23.8), 120.36 (d, *J* 8.3), 121.55 (d, *J* 8.1), 122.10 (d, *J* 25.8), 125.90, 137.94, 140.22, 145.22, 151.51, 153.23, 159.06 (d, *J* 246.6), 172.59 (d, *J* 2.4); $\delta_{F\{H\}}$ (282 MHz, $CDCl_3$) −116.60 (s); HRMS (FAB) 347.09274 ($M + H^+$ $C_{18}H_{16}O_6F$ requires 347.09269).

*5.2.1.8. 7-Fluoro-3′,4′,5′-trimethoxyflavonol (**11**).* 78% Yield; ESI-MS m/*z* 347 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.79 (3H, s, OCH_3), 3.89 (6H, s, 2 × OCH_3), 7.29 (1H, td, *J* 8.8, 2.4, C(6)H), 7.55 (2H, s, C(2′,6′)H), 7.66 (1H, dd, *J* 9.7, 2.4, C(8)H), 8.16 (1H, dd, *J* 8.8, 6.4, C(5)H), 9.52 (1H, s, C(3)OH); δ_c (100 MHz; *d*6-DMSO) 55.98, 60.11, 104.92 (d, *J* 25.7), 105.45, 113.34 (d, *J* 23.5), 118.33, 127.44 (d, *J* 11.0), 138.70, 139.13, 145.15, 152.63, 155.30 (d, *J* 14.3), 164.71 (d, *J* 251.2), 172.10; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.53 (s); HRMS (FAB) 347.09259 ($M + H^+$ $C_{18}H_{16}O_6F$ requires 347.09269).

*5.2.1.9. 5,7-Difluoro-3′,4′,5′-trimethoxyflavonol (**14**).* 25% Yield; ESI-MS m/*z* 365 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.78 (3H, s, OCH_3), 3.87 (6H, s, 2 × OCH_3), 7.25 (1H, ddd, *J* 11.5, 9.5, 2.4, C(6)H), 7.52 (2H, s, C(2′,6′)H), 7.60 (1H, br d, *J* 9.5, C(8)H), 9.60 (1H, s, C(3)OH); δ_c (100 MHz; *d*6-DMSO) 56.00, 60.13, 101.03 (t, *J* 25.9), 101.63 (dd, *J* 25.9, 4.1), 105.32, 109.07 (d, *J* 12.9), 125.79, 138.96, 139.18, 143.93, 152.67, 156.02 (dd, *J* 16.8, 6.3), 160.47 (dd, *J* 263.3, 15.8), 163.92 (dd, *J* 250.8, 15.6), 170.44; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.06 (d, *J* 11.2), −102.03 (d, *J* 11.2).

*5.2.1.10. 5,7-Difluoro-3′,4′,-dimethoxyflavonol (**17**).* 26% Yield; ESI-MS *m*/*z* 335 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.86 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 7.11 (1H, d, *J* 8.6, C(5′)H), 7.22 (1H, ddd, *J* 11.5, 9.5, 2.3, C(6)H), 7.55 (1H, br d, *J* 9.5, C(8)H), 7.77 (1H, d, *J* 2.1, C(2′)H), 7.85 (1H, dd, *J* 8.6, 2.1, C(6′)H), 9.44 (1H, s, C(3)OH); δ_C (75 MHz, DMSO) 55.51, 55.63, 101.00 (t, *J* 25.8), 102.07 (dd, *J* 26.1, 4.1), 109.61 (dd, *J* 11.2, 1.8), 110.59, 111.40, 121.33, 122.88, 138.36, 144.54, 148.40, 150.31, 156.52 (dd, *J* 16.2, 6.8) 161.04 (dd, *J* 256.7, 13.3), 164.37 (dd, *J* 246.1, 14.7), 170.33; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.15 (d, *J* 11.0), −102.26 (d, *J* 11.0).

*5.2.1.11. 5,7-Difluoro-4′,-methoxyflavonol (**20**).* 37% Yield; ESI-MS m/*z* 305 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.86 (3H, s, OCH_3), 7.09 (2H, d, *J* 9.0, C(3′,5′)H), 7.22 (1H, ddd, *J* 11.5, 9.5, 2.3, C(6)H), 7.49 (1H, br d, *J* 9.5, C(8)H), 8.17 (2H, d, *J* 9.0, C(2′,6′)H), 9.42 (1H, s, C(3)OH); $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.10 (d, *J* 10.8), −102.14 (d, *J* 10.8).

*5.2.1.12. 7-Fluoro-3′,4′-dimethoxyflavonol (**23**).* 77% Yield; ESI-MS *m*/*z* 317 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.87 (6H, s, 2 × OCH_3), 7.12 (1H, d, *J* 8.6, C(5′)H), 7.30 (1H, td, *J* 8.8, 2.4, C(6)H), 7.66 (1H, dd, *J* 9.8, 2.4, C(8)H), 7.81 (1H, d, *J* 2.0, C(2′)H), 7.88 (1H, dd, *J* 8.6, 2.0, C(6′)H), 8.16 (1H, dd, *J* 8.8, 6.4, C(5)H), 9.42 (1H, s, C(3)OH); δ_C (75 MHz, DMSO) 55.49, 55.61, 105.37 (d, *J* 25.7), 110.80, 111.54, 113.32 (d, *J* 23.2), 118.52, 121.46, 123.44, 127.38 (d, *J* 10.7), 138.21, 145.79, 148.37, 150.33, 155.31 (d, *J* 14.1), 164.67 (d, *J* 250.7), 172.01; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.75 (s).

*5.2.1.13. 7-Fluoro-4′-methoxyflavonol (**26**).* 75% Yield; ESI-MS *m*/*z* 287 $(M + H)^+$; δ_H (400 MHz, *d*6-DMSO/$CDCl_3$) 3.86 (3H, s, OCH_3), 7.08 (2H, d, *J* 9.1, C(3′,5′)H), 7.27 (1H, td, *J* 8.8, 2.4, C(6)H), 7.55 (1H, dd, *J* 9.7, 2.4, C(8)H), 8.16 (1H, dd, *J* 8.8, 6.4, C(5)H), 8.19 (2H, d, *J* 9.1, C(2′,6′)H), 9.35 (1H, br s, C(3)OH); δ_C (100 MHz, DMSO) 55.33, 104.82 (d, *J* 25.6), 113.35 (d, *J* 23.5), 113.99, 118.55, 123.32, 127.51 (d, *J* 11.0), 129.28, 138.05, 145.95, 155.37 (d, *J* 14.3), 160.44, 164.66 (d, *J* 251.1), 172.00; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.66 (s).

*5.2.1.14. 3,3′,4′,5′-Tetramethoxyflavone (**3**).* 3′,4′,5′-Trimethoxyflavonol (0.21 g, 0.642 mmol) was dissolved in acetone (20 ml), this was followed by addition of K_2CO_3 (1.97 g, 14.3 mmol), and subsequent addition of iodomethane (1.8 ml, 29 mmol). This was refluxed at 60 °C for 2 days. The reaction was cooled to room temperature, excess K_2CO_3 was filtered out, washed with acetone (15 ml), and the acetone was evaporated. The residue was dissolved in CH_2Cl_2 (15 ml), washed with H_2O (20 ml), dried ($MgSO_4$) and the solvent was removed under reduced pressure. The product was recrystallised from hot ethanol and water and dried under reduced pressure over P_2O_5 to give **3** as a white solid (0.137 g, 62%). ESI-MS *m*/*z* 343 $(M + H)^+$; δ_H (400 MHz, $CDCl_3$) 3.83 (3H, s, OCH_3), 3.89 (9H, s, 3 × OCH_3), 7.34 (1H, ddd, *J* 8.0, 7.0, 0.9, C(6)H), 7.35 (2H, s, C(2′,6′)H), 7.48 (1H, dd, *J* 8.4, 0.9, C(8)H), 7.62 (1H, ddd, *J* 8.4, 7.0, 1.5, C(7)H), 8.21 (1H, dd, *J* 8.0, 1.5, C(5)H); δ_c (100 MHz; $CDCl_3$) 56.34, 60.10, 60.99, 106.21, 117.97, 124.16, 124.73, 125.80, 125.96, 133.46, 140.46, 141.32, 153.10, 155.13, 155.26, 174.95; HRMS (FAB) 343.11775 ($M + H^+$ $C_{19}H_{19}O_6$ requires 343.11769).

5.3. Demethylation

5.3.1. Method A

5, 7-Difluoro-3′,4′-dimethoxyflavonol (200 mg, 0.60 mmol) was suspended in dry CH_2Cl_2 (5 mL) and cooled to −78 °C before BBr_3 (1.0 M in CH_2Cl_2) (1.20 mL, 1.20 mmol) was added dropwise. The reaction was allowed to warm to 0 °C and stirred for a further 3 h. Methanol (5 mL) was added dropwise and stirred at room temperature for 30 min then the volatiles removed *in vacuo*. The resulting yellow solid was crystallised from ethanol to yield 5, 7-difluoro-3′,4′-dihydroxyflavonol (164 mg, 89%).

5.3.2. Method B

5, 7-Difluoro-4′-methoxyflavonol (210 mg, 0.69 mmol) was suspended in dry toluene (5 mL) and cooled to 0 °C before BBr_3 (1.0 M in CH_2Cl_2) (1.5 mL, 1.50 mmol) was added dropwise. The reaction was warmed to room temperature and stirred for 1 h before being heated to reflux (110 °C) for 2 h. After cooling to 0 °C, methanol (5 mL) was added dropwise and stirred at room temperature for 30 min then the volatiles removed *in vacuo*. The resulting yellow solid was crystallised from ethanol to yield 5, 7-difluoro-4′-hydroxyflavonol (200 mg, 99%).

*5.3.2.1. 3′,4′,5′-Trihydroxyflavonol (**2**). Method A.* 20% yield; ESI-MS *m*/*z* 287 $(M + H)^+$, 285 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 7.31 (2H, s, C(2′,6′)H), 7.45 (1H, ddd, *J* 8.2, 7.0, 1.4, C(7)H), 7.67 (1H, dd, *J* 8.2, 1.6, C(8)H), 7.29 (1H, ddd, *J* 8.3, 7.0, 1.6, C(6)H), 8.10 (1H, dd, *J* 8.3, 1.4, C(5)H), 9.12—9.29 (3H, br s, 3 × OH); δ_c (100 MHz; *d*6-DMSO) 107.29, 118.00, 121.11, 121.22, 124.32, 124.68, 133.35, 135.76, 137.97, 145.72, 146.14, 154.27, 172.37.

*5.3.2.2. **7**-Fluoro-3′,4′,5′-trihydroxyflavonol (**12**). Method A.* 99% yield; ESI-MS *m*/*z* 303 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 7.28 (2H, s, C(2′,6′)H), 7.33 (1H, td, *J* 8.8, 2.4, C(6)H), 7.60 (1H, dd, *J* 9.8, 2.4, C(8)H), 8.14 (1H, dd, *J* 8.8, 6.4, C(5)H); δ_c (100 MHz; *d*6-DMSO) 104.57 (d, *J* 25.5), 107.29, 113.23 (d, *J* 23.4), 118.45, 120.89, 127.47 (d, *J* 11.0), 135.83, 137.87, 145.71, 146.63, 155.23 (d, *J* 14.0), 164.62 (d, *J* 251.0), 171.77; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.77 (s).

*5.3.2.3. 5,7-Difluoro-3′,4′,5′-trihydroxyflavonol (**15**). Method A.* 76% yield; ESI-MS *m*/*z* 321 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 7.26 (2H, s, C(2′,6′)H), 7.31 (1H, ddd, *J* 11.7, 9.7, 2.4, C(6)H), 7.48 (1H, dt, *J* 9.6,

1.6, C(8)H); δ_C (100 MHz; *d*6-DMSO) 100.92 (t, *J* 25.8), 101.22 (dd, *J* 25.2, 4.0), 107.18, 109.11 (dd, *J* 10.7, 2.1), 120.44, 135.88, 138.10, 145.88, 145.73, 155.97 (dd, *J* 16.6, 6.3), 160.51 (dd, *J* 263.1, 15.9), 163.78 (dd, *J* 251.0, 15.4), 170.14; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.18 (d, *J* 11.0), −102.26 (d, *J* 11.0).

*5.3.2.4. 5,7-Difluoro-3′,4′,-dihydroxyflavonol (**18**). Method A.* 89% yield; ESI-MS *m*/*z* 305 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 6.90 (1H, d, *J* 8.5, C(5′)H), 7.31 (1H, ddd, *J* 11.7, 9.6, 2.4, C(6)H), 7.53 (1H, m, C(8)H), 7.55 (1H, dd, *J* 8.5, 2.2, C(6′)H), 7.72 (1H, d, *J* 2.2, C(2′)H), 9.27 (1H, br s, ArOH), 9.36 (1H, s, C(3)OH), 9.60 (1H, br s, ArOH); δ_C (125 MHz, *d*6-DMSO) 100.88 (t, *J* 26.0), 101.27 (dd, *J* 25.3, 2.8), 109.10 (d, *J* 10.0), 115.22, 115.57, 119.75, 121.62, 137.98, 145.08, 145.26, 147.10, 155.98 (dd, *J* 16.3, 5.9), 160.50 (dd, *J* 263.5, 15.6), 163.77 (dd, *J* 251.2, 15.1), 170.16; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.20 (d, *J* 11.0), −102.30 (d, *J* 11.0).

*5.3.2.5. 5,7-Difluoro-4′,-hydroxyflavonol (**21**). Method B.* 99% yield; ESI-MS *m*/*z* 289 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 6.94 (2H, d, *J* 9.0, C(3′,5′)H), 7.31 (1H, ddd, *J* 11.7, 9.6, 2.4, C(6)H), 7.56 (1H, dt, *J* 9.6, 1.9, C(8)H), 8.07 (2H, d, *J* 9.0, C(2′,6′)H), 9.40 (1H, br s, OH), 10.10 (1H, br s, OH); δ_C (100 MHz, *d*6-DMSO) 100.91 (t, *J* 24.8), 101.35 (dd, *J* 25.8, 4.4), 109.16 (dd, *J* 10.9, 2.1), 115.39, 121.24, 129.31, 137.89, 145.17, 156.00 (dd, *J* 16.6, 6.4), 159.17, 160.48 (dd, *J* 263.1, 15.6), 163.74 (dd, *J* 251.0, 15.3), 170.19; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −109.18 (d, *J* 11.0), −102.30 (d, *J* 11.0).

*5.3.2.6. 7-Fluoro-3′,4′-dihydroxyflavonol (**24**). Method A.* 87% yield; ESI-MS *m*/*z* 287 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 6.90 (1H, d, *J* 8.5, C(5′)H), 7.33 (1H, td, *J* 8.8, 2.3, C(6)H), 7.58 (1H, dd, *J* 8.5, 2.3, C(6′)H), 7.65 (1H, dd, *J* 9.8, 2.3, C(8)H), 7.74 (1H, d, *J* 2.3, C(2′)H), 8.14 (1H, dd, *J* 8.8, 6.4, C(5)H), 9.39 (3H, br s, 3 × OH); δ_C (100 MHz, *d*6-DMSO) 104.65 (d, *J* 25.4), 113.22 (d, *J* 23.4), 115.40 (d, *J* 25.6), 118.48, 119.84, 122.03, 127.44 (d, *J* 10.9), 137.72, 145.03, 146.50, 147.63, 155.26 (d, *J* 14.1), 164.59 (d, *J* 251.0), 171.81; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.81 (s).

*5.3.2.7. 7-Fluoro-4′-hydroxyflavonol (**27**). Method B.* 84% yield; ESI-MS *m*/*z* 271 $(M - H)^-$; δ_H (400 MHz, *d*6-DMSO) 6.94 (2H, d, *J* 8.9, C(3′,5′)H), 7.34 (1H, td, *J* 8.7, 2.4, C(6)H), 7.68 (1H, dd, *J* 9.8, 2.4, C(8)H), 8.10 (2H, d, *J* 8.9, C(2′,6′)H), 8.15 (1H, dd, *J* 8.9, 6.4, C(5)H), 9.40 (1H, br s, OH), 10.10 (1H, br s, OH); δ_C (100 MHz, *d*6-DMSO) 104.72 (d, *J* 25.6), 113.24 (d, *J* 23.4), 115.39, 118.53, 121.71, 127.43 (d, *J* 11.0), 129.44, 137.67, 146.45, 155.28 (d, *J* 14.2), 159.14, 164.58 (d, *J* 250.9), 171.85; $\delta_{F\{H\}}$ (376 MHz, *d*6-DMSO) −104.81 (s).

5.4. Synthesis of 3-fluoro-3′,4′,5′-trimethoxyflavone (**9**)

5.4.1. 3-Hydroxy-1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trimethoxy phenyl)prop-2-en-1-one (**29**)

2′-Hydroxyacetophenone (2.68 g, 19.71 mmol) was dissolved in dry pyridine (70 mL) and 3,4,5-trimethoxybenzoyl chloride (5.0 g, 21.68 mmol) was added. The reaction was heated to 70 °C and stirred overnight. The reaction mixture was then cooled to room temperature and acidified to pH 1 by addition of HCl (10% aq.). The precipitate was filtered, washed with H_2O and dried. This white solid was dissolved in dry pyridine (100 mL) and KOH powder (1.6 g) was added. The reaction was heated to 65 °C with stirring for 5 h then cooled in an ice bath before cold HCl was added to neutralise to pH 7. The precipitate was filtered and recrystallised from ethanol to yield **29** as a yellow solid (3.07 g, 47% from 2′-hydroxyacetophenone). ESI-MS *m*/*z* 329 $(M - H)^-$; δ_H (400 MHz, $CDCl_3$) 3.94 (3H, s, OCH_3), 3.96 (6H, s, 2 × OCH_3), 6.75 (1H, s, C(2)H), 6.93 (1H, ddd, *J* 8.2, 7.3, 1.1, C(5′)H), 7.01 (1H, dd, *J* 8.5, 1.1, C(3′)H), 7.17 (2H, s, C(2″,6″)H), 7.47 (1H, ddd, *J* 8.5, 7.3, 1.6, C(4′)H), 7.77 (1H, dd, *J* 8.2, 1.6, C(6′)H), 11.97 (1H, C(3)OH); δ_C (75 MHz, $CDCl_3$) 56.38, 61.03, 91.94, 104.32, 106.49, 118.83, 119.07, 128.42, 128.78, 135.80, 142.08, 153.32, 162.39, 177.52, 195.13.

5.4.2. 2-Fluoro-3-hydroxy-1-(2′-hydroxyphenyl)-3-(3″,4″,5″-trime thoxyphenyl)prop-2-en-1-one (**30**)

To a solution of **29** (1.57 g, 4.76 mmol) in dry CH_2Cl_2 (20 mL) and dry CH_3CN (70 mL) was added *N*-fluorobenzenesulfonimide (1.80 g, 5.71 mmol) and the reaction was stirred at room temperature for 7 days. The volatiles were removed *in vacuo* and the crude mixture was purified by FC(SiO_2, ethyl acetate/hexanes (3:7)) to afford **30** as a white solid (0.62 g, 37%). δ_H (400 MHz, $CDCl_3$) 3.95 (3H, s, OCH_3), 3.96 (6H, s, 2 × OCH_3), 7.27 (2H, s, C(2″,6″)H), 7.45 (1H, ddd, *J* 8.0, 7.1, 0.6, C(5′)H), 7.60 (1H, dd, *J* 8.6, 0.6, C(3′)H), 7.73 (1H, ddd, *J* 8.6, 7.1, 1.7, C(4′)H), 8.30 (1H, dd, *J* 8.0, 1.7, C(6′)H); $\delta_{F\{H\}}$ (282 MHz, $CDCl_3$) −160.31 (s).

5.4.3. 3-Fluoro-3′,4′,5′-trimethoxyflavone (**9**)

30 (0.46 g, 1.32 mmol) was suspended in acetic acid (20 mL) and H_2SO_4 (conc.) (0.2 mL) was added. The mixture was heated to reflux (125 °C) for 10 min then cooled to room temperature before cold H_2O (100 mL) was added. The precipitate was collected by filtration and air dried to afford **9** as a white solid (0.42 g, 96%). ESI-MS *m*/*z* 331 $(M + H)^+$; δ_H (400 MHz, $CDCl_3$) 3.96 (3H, s, OCH_3), 3.96 (6H, s, 2× OCH_3), 7.28 (2H, s, C(2′,6′)H), 7.46 (1H, ddd, *J* 8.0, 7.1, 0.5, C(6)H), 7.60 (1H, dd, *J* 8.5, 0.5, C(8)H), 7.74 (1H, ddd, *J* 8.5, 7.1, 1.6, C(7)H), 8.31 (1H, dd, *J* 8.0, 1.6, C(5)H); δ_C (75 MHz, $CDCl_3$) 56.20, 60.87, 105.48, 105.60, 117.98, 123.59 (d, *J* 5.1), 123.97 (d, *J* 7.1), 125.00, 125.65 (d, *J* 3.2), 133.84, 140.88, 144.19, 148.90 (d, *J* 213.7), 150.62, 153.21, 154.82, 170.56 (d, *J* 17.0); $\delta_{F\{H\}}$ (282 MHz, $CDCl_3$) −160.33 (s).

5.5. HPLC

HPLC analyses for purity determination were performed using a Varian Prostar HPLC system (Varian Ltd) consisting of a Varian ProStar 230 solvent delivery system, a ProStar 325 UV detector, and a 410 Varian autosampler. Separation was achieved on a Gemini C18 column (4.6 mm × 150 mm, 3 μm, Phenomenex Ltd) at a flow rate of 0.75 mL/min, using an isocratic mobile phase of 69% methanol in 0.1 M ammonium acetate buffer (pH 5.1). UV absorbance at 352 nm was used to detect compounds.

5.6. Cell proliferation assay

22rv1 cells were kindly provided by Dr. Imran Ahmad from the Beatson Institute for Cancer Research (Glasgow, UK). Cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (Gibco, Paisley, UK) and harvested by a 5 min treatment with trypsin–EDTA solution (Gibco, Paisley, UK). Cells were seeded (5×10^3) onto 24 well plates and allowed to adhere overnight, then agents dissolved in DMSO were added and cells incubated for periods of up to 144 h to furnish a final DMSO concentration of <0.1%. Cells were washed with phosphate buffered saline (PBS), harvested by trypsinisation and resuspended in cell culture media (1 ml). Aliquots (1 ml) were diluted 10-fold (Isoton II buffer) and 500 μl was analysed using a Z2 Coulter Particle Count and Size Analyser (Beckman Coulter, UK). IC_{50} values were calculated from the plot of cell number as percentage of DMSO control versus agent log concentration at 144 h, at which time cells were still in linear growth phase. When the data points were plotted a line of best fit was added and the equation of the straight line was used to calculate the IC_{50} value. The number generated from the equation was converted back from the $\log_{10}$ concentration to give the actual IC_{50} value. Values are the mean of 3–6 separate experiments.

5.7. Western blot analysis

22rv1 Cells were seeded at 2×10^5 cells per well into 6 well dishes and left to adhere overnight. Cells were then exposed for 72 h to TMFol and its analogues at 10 and 20 μM. Cells were centrifuged (5 min, 10000 × g, 4 °C), and the cell pellet was suspended in lysis buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, with protease inhibitors PMSF 1 mM, aprotinin 2 μg/ml, leupeptin 5 μg/ml and phosphatase inhibitors sodium vanadate 1 mM and sodium fluoride 1 mM). The homogenate was placed on ice (15 min) and centrifuged (20 min, 10000 × g, 4 °C). Protein concentration in cell lysate was determined using the Biorad protein assay (Biorad, Hemel Hempstead, UK) with bovine serum albumin as standard. An aliquot of protein lysate (15 μg) was separated by SDS–PAGE and transferred for 1 h onto a nitrocellulose membrane (Schleicher & Schuell, NH, USA). Blots were blocked for 2 h with 5% skimmed milk in PBS/Tween 0.05% (PBS-T), and probed with a specific primary antiserum (AR (1:1000) and Actin (1:2000); Santa Cruz Biotechnology; PSA (1:1000), DAKO) in PBS containing 0.05% PBS-T and 5% non-fat dry milk (4 °C, overnight). After washing (PBS-T), blots were treated with horseradish peroxidase-conjugated secondary antibody in PBS containing 0.05% PBS-T and 5% non-fat dry milk (1:2000) for 1 h and washed (5× for 5 min) in PBS-T. Proteins were detected by the enhanced chemiluminescence system (Geneflow, Staffordshire, UK). Protein densitometry data were collected using a Syngene (Cambridge, Cambs., UK) GeneGnome gel documentation system and protein expression was normalised to actin levels.

5.8. Statistical analyses

Values shown in the results are the mean ± SD. Statistical significance was evaluated using the Statistical Package for the Social Sciences (SPSS) version 18 programme (Windows XP). Effect of flavonols on AR and PSA expression were subjected to one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. P values <0.05 were considered significant.

Acknowledgement

This work was funded by a programme grant from Cancer Research UK (C325/A13101) and a grant from Prostate Action (G2009/25). Thanks to Dr A. Stuart for proof reading.

Appendix A. Supporting information

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.ejmech.2012.06.031.

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