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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis and in vitro evaluation of ferutinol aryl esters for estrogenic activity and affinity toward cannabinoid receptors

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Abstract Ferutinin (**1**), the major constituent of *Ferula hermonis* and other Ferula species, is a sesquiterpene ester with remarkable estrogenic activity, beside other valuable medicinal properties. To investigate the influence of chemical modification of the ferutinin structure on its estrogenic effect and binding affinity toward the cannabinoid CB1 and CB2 receptors, twelve derivatives of **1** were prepared and evaluated in vitro, together with the parent compound, for the respective bioactivities, based on the recent evidence for estrogen–endocannabinoid interaction. Nine of the prepared derivatives (**3**–**11**) are new semisynthetic esters of **1**. The parent compound ferutinin (**1**) exhibited the highest level of estrogenic activity (EC_{50} 0.3 μM and a percent maximal 17β-estradiol response of 90 % at 1 μM). Compound **6** was found to be a selective agonist for CB2 receptor (EC_{50} 0.051 μM, Ki 0.025 μM), with much less affinity for CB1 receptor (EC_{50} 97 μM, Ki 48.5 μM). Compound **8** was a selective agonist for CB1 (EC_{50} 62, Ki 0.031 μM) with no affinity toward CB2.

Keywords Ferutinin · *Ferula hermonis* · Semi-synthetic derivatives · Estrogenic activity · Cannabinoid receptors

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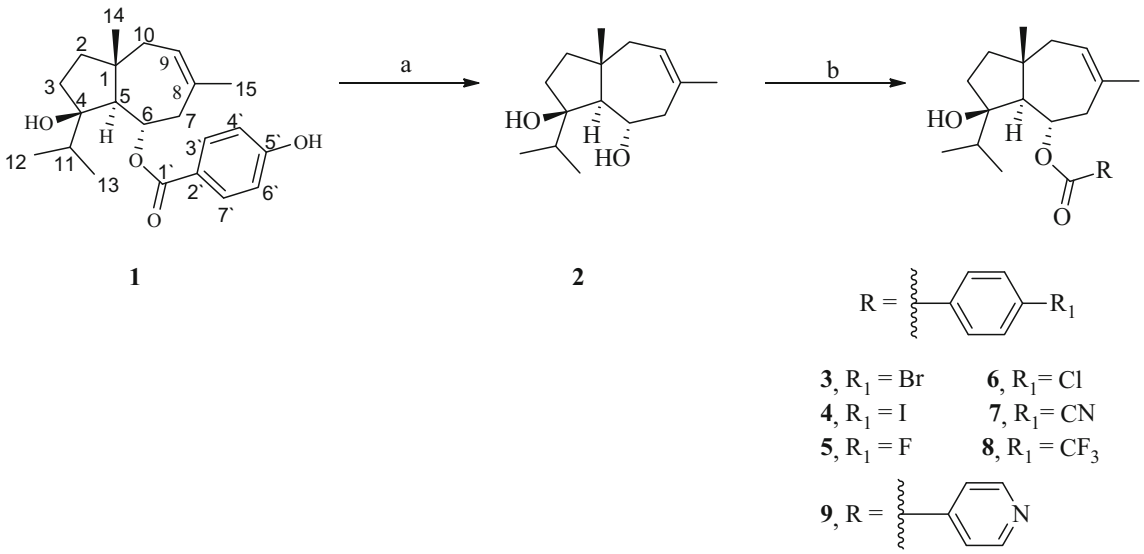
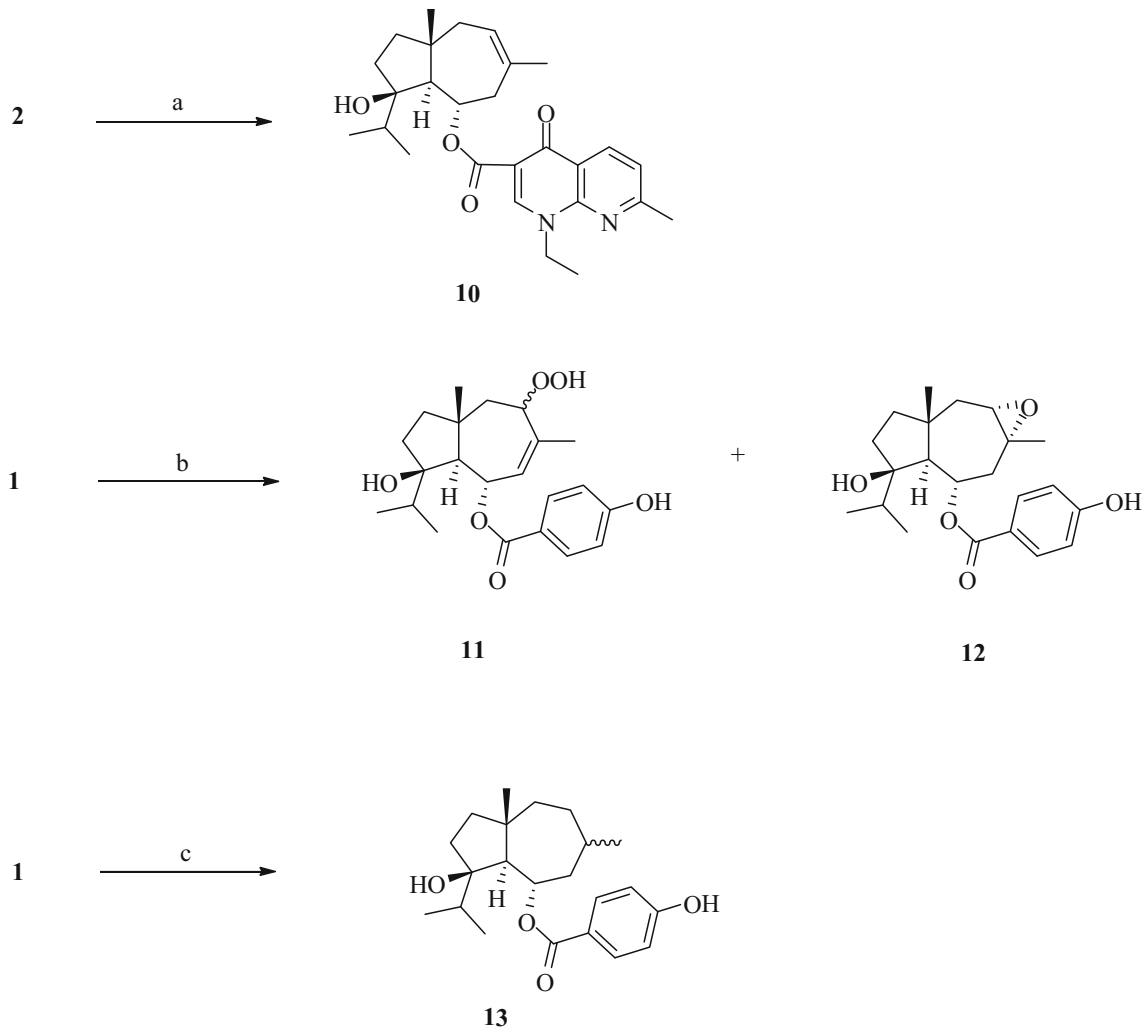
Introduction

Ferutinin (**1**), the major constituent of *Ferula hermonis* Boiss. (family Apiaceae), is known to possess antioxidant (Ibraheim *et al.*, 2012), acetylcholinesterase inhibition (Dall'Acqua *et al.*, 2010), nitric oxide synthase enhancing activities (Colman-Saizarbitoria *et al.*, 2006), and in vitro induction of apoptosis in human RBCs (Gao *et al.*, 2013). Ferutinin also binds to estrogen receptors (ER*)* in vitro, acting as an agonist for ERα and an agonist/antagonist for ERβ, hence displaying estrogenic and antiestrogenic activity, respectively (Appendino *et al.*, 2004; Ikeda *et al.*, 2002; Zanoli *et al.*, 2005). The in vivo evaluation of ferutinin on sexual behavior of male rats revealed that it is able to stimulate sexual behavior in impotent male rats after acute ingestion. It also exerted a negative influence on the sexual capacity of potent male rats by reducing testosterone serum levels as well as negatively affecting appetitive and consummatory sexual behavior (Hadidi *et al.*, 2003; Zanoli *et al.*, 2003; Zanoli *et al.*, 2005). The effect of ferutinin on sexual behavior of female rats indicated that it did not have the capacity to alter sexual motivation. However, it significantly inhibited female receptivity, suggesting an important role for ferutinin in sexual behavior impairment of hormone-primed female rats (Zanoli *et al.*, 2009; Zavatti *et al.*, 2006). In addition, ferutinin was demonstrated to possess antiosteoporotic effect as potent as estradiol benzoate on bone mass, and in a number of cases, it was reported to be superior to the human estrogen (Palumbo *et al.*, 2009). A published molecular modeling and QSAR studies established that the estrogenic activity of ferutinin is attributed to the presence of the *p*-hydroxybenzoyl moiety as well as substitution of the daucane double bond (Rasulev *et al.*, 2007). Recent reports indicated that 17β-estradiol interacts with the endocannabinoid receptors (CB1 and CB2) and may, thus, have a modulating effect on colon cancer cell growth (CB1) or osteoblast differentiation (CB2) (Proto *et al.*, 2012), (Sophocleous *et al.*, 2011). Ligands for CB1 and CB2 receptors have emerged as lead compounds in developing therapeutic agents for a number of health disorders, such as diabetes, drug dependence, obesity, neurodegenerative diseases, including multiple sclerosis (Diaz *et al.*, 2008; Greineisen and Turner, 2010; Guindon and Hohmann, 2008; Malfitano *et al.*, 2013; Pryce *et al.*, 2014; Ribeiro *et al.*, 2013; Rivers and Ashton, 2010). The recent discovery of β-caryophyllene as a non-cannabinoid CB2 selective agonist ($Ki = 0.155$ μM) of plant origin warrants further investigation to discover natural products with better or comparable activity (Gertsch *et al.*, 2010). Based on the above-mentioned literature data, in particular the recently discovered relationship between estrogens and the endocannabinoid system, the goal of this work was to prepare a number of ferutinin derivatives, establish their identity via spectroscopic analysis, primarily 1D and 2D NMR, in addition to UV and HRESMS, and examine their estrogenic activity as well as their interaction with the cannabinoid receptors. The structural modification of ferutinin (**1**) was mainly targeting the aromatic core, since this part of the molecule has been proven to significantly contribute to the estrogenic activity (Rasulev *et al.*, 2007) and might also affect the affinity toward the cannabinoid receptors. Thus, compounds **3**–**9** (Scheme 1) were prepared with *p*-substituents conceivably capable of forming stronger hydrogen or halogen bonds with the estrogen/cannabinoid receptors in lieu of the *p*-hydroxyl group, which would presumably enhance their effect. Compound **10** (Scheme 2) was prepared to evaluate the effect of introducing a multifunctional aromatic core, capable of forming hydrogen bonds, in an attempt to enhance the binding affinity. Compounds **11** and **12** (Scheme 2) were also prepared to introduce oxygen-carrying



Scheme 1 Reagents and conditions: *a* KOH, reflux, 2 h; *b* toluene, acid chloride, Et_3N, DMAP, reflux



Scheme 2 Reagents and conditions: *a* CH_2Cl_2, nalidixic acid, 0 °C, Et_3N, ethylchloroformate, overnight stirring; *b* CH_2Cl_2/MeOH, *meso*-tetraphenylporphine, O_2, light, 13 °C, 3 h; *c* Pd(C)/MeOH, 24 h

functionalities in the daucane core and examining their effect on receptor binding affinity. Compound **13** (Scheme 2) was hitherto prepared and tested for estrogenic (Appendino *et al.*, 2004), but not for binding affinity at the cannabinoid CB1 and CB2 receptors. The daucane alcohol (hydrolysis product of **1**, ferutinol or jaeschkeanadiol **2**) was also evaluated along with the other derivatives.

Results and discussion

Chemistry

Alkaline hydrolysis of ferutinin (**1**) was exploited as the pivotal step toward the preparation of the new ester analogs of **1**. Coupling of the resulting daucane alcohol **2** to the relevant acyl chloride or nalidixic acid afforded compounds **3**–**10** in yields sufficient for full characterization and biological evaluation of these new compounds. Compound **10** was synthesized using nalidixic acid and ethylchloroformate activation to form a mixed carboxylic–carbonic anhydride which was then decomposed to afford **10**. Compounds **11** and **12** were synthesized via singlet oxygen photooxygenation of ferutinin, using *meso*-tetraphenylporphine as a photosensitizer and incandescent light to afford 9-hydroperoxyferutinin and 8,9-epoxyferutinin (Galal *et al.*, 2001; Wasserman *et al.*, 1986), respectively. The epoxy compound (**12**) was found to be identical with a natural one that has been previously isolated (Galal, 2000). The 8,9-dihydroferutinin (**13**) (Scheme 2) was prepared following a published procedure (Appendino *et al.*, 2004).

Bioassays

Compounds **1**–**13** were evaluated in vitro for estrogenic activity and binding to cannabinoid (subtype CB1 and

CB2) receptors. Compound **1** exhibited the highest estrogenic activity among all the tested compounds with an EC_{50} of 0.3 μM, which is in line with earlier results reported by Appendino *et al*. (Appendino *et al.*, 2004). Of the semisynthetic analogs, only **3**, **4**, and **11** exhibited weak activities (EC_{50} = 310.0, 300.0, and 16.0 μM, respectively, Table 1). The estrogenic activity of compound **12** was previously reported to be comparable to **1** (Appendino *et al.*, 2004). Overall, the estrogenic activities displayed by the compounds included in the current study were weaker than that of the human estrogen 17β-estradiol (EC_{50} 0.005 μM). Modifications at the aromatic *p*-position, removal of the aromatic system, and relocation of the daucane double bond all lead to significant reduction or loss of estrogenic activity of **1**. Compound **10** did not show any affinity despite possessing a heteroaromatic moiety with two nitrogen atoms, which was presumed to enhance the binding affinity through formation of hydrogen bonding between the aromatic system and the receptor site. This might be due to the existence of a bulk interaction between **10** and the receptor site that leads to failure of binding. In a recent report, Proto *et al*. (2012) demonstrated the interaction between 17β-estradiol and the CB1 receptor to suppress colon cancer cell proliferation. This finding, in conjunction with the fact that **1** possesses pronounced estrogenic activity, prompted us to evaluate parent compound **1** and the derivatives **2**–**13** for their binding affinity toward CB1 and CB2 receptors (Table 2). Compounds that showed sufficient binding affinity to the cannabinoid receptors were developed to the functional assays (Table 3). Compounds **6** and **8** showed significant and selective affinity to CB2 and CB1, with Ki 25 and 31 nM, respectively. In the functional assays, both **6** and **8** were shown to be agonists at CB2 and at CB1, respectively. The parent compound **1** exhibited much weaker binding affinity to both receptors, with Ki 7100 and 3634 nM at the CB1 and CB2, respectively. Ferutinin (**1**) and its 8,9-dihydroferutinin derivative (**13**) did not show any noticeable activity, indicating the insignificance of the double bond for the CB1 or CB2 binding. The binding affinity of the epoxide derivative (**12**) at CB1 when compared to that of ferutinin was found to be relatively weaker. Replacement of the hydroxyl group of the *p*-hydroxyphenyl residue with bromine or iodine, in an attempt to enhance the ability of formation of hydrogen/halogen bonds with the amino acid residues at the receptor site to increase binding affinity, resulted in marginal improvement. The fluoro-derivative exhibited weaker affinity at the CB2. Likewise, replacement of the hydroxyl with cyano group leads to a relative improvement in the binding affinity for CB1. Replacement of the *p*-hydroxyphenyl moiety of ferutinin with pyridine moiety marginally enhanced the binding affinity toward CB2 receptor. Compounds **6** and **8** displayed significant binding affinities at CB1 and CB2, respectively, and were found to be agonists for CB2 and CB1 receptors, respectively, as shown in (Table 3) and (Fig. 1). Compound **11** was not tested for binding affinity to the cannabinoid receptors owing to low yield and decomposition. In

Table 1 Estrogenic activity of compounds **1, 3, 4,** and **11**

Compound	Estrogenic activity	
	EC_{50} (μM)	% Maximal 17β-estradiol response
1	0.3	90 % at 1 μM
3	310.0	44 % at 500 μM
4	300.0	99 % at 500 μM
11	16.0	79 % at 125 μM
17β-estradiol	0.005	–

EC_{50}: half maximal effective concentration

% maximal response = (max sample absorbance/max 17β-estradiol absorbance) × 100

Compounds **2**, **5**, **6**, **7**, **8**, **9**, and **10** were inactive (No estrogenic effect up to 500 μM)

Table 2 Binding affinity of compounds **1–13** for CB1 and CB2 receptors expressed as Ki (nM)

Compound	CB1/Ki (μM)	CB2/Ki (μM)
1	7.08	3.63
2	130.80	52.00
3	2.50	0.78
4	1.59	0.12
5	NT[a]	4.30
6	0.33	4.35
7	4.05	NT
8	0.922	0.55
9	25.20	0.58
10	9.20	0.46
11	NT	NT
12	9.17	NT
13	11.86	4.46

[a] Not tested

Table 3 Results of functional assays of compounds **3**, **4**, **6**, **8**, and **9** for CB1 and CB2 receptors

Compound	CB1/Ki (μM)	CB2/Ki (μM)
3	1.24	NA[a]
4	27.96	NA
6	48.50	0.025
8	0.03	NA
9	1.10	0.499
CP-55,940	0.0008	0.0021

[a] No activity

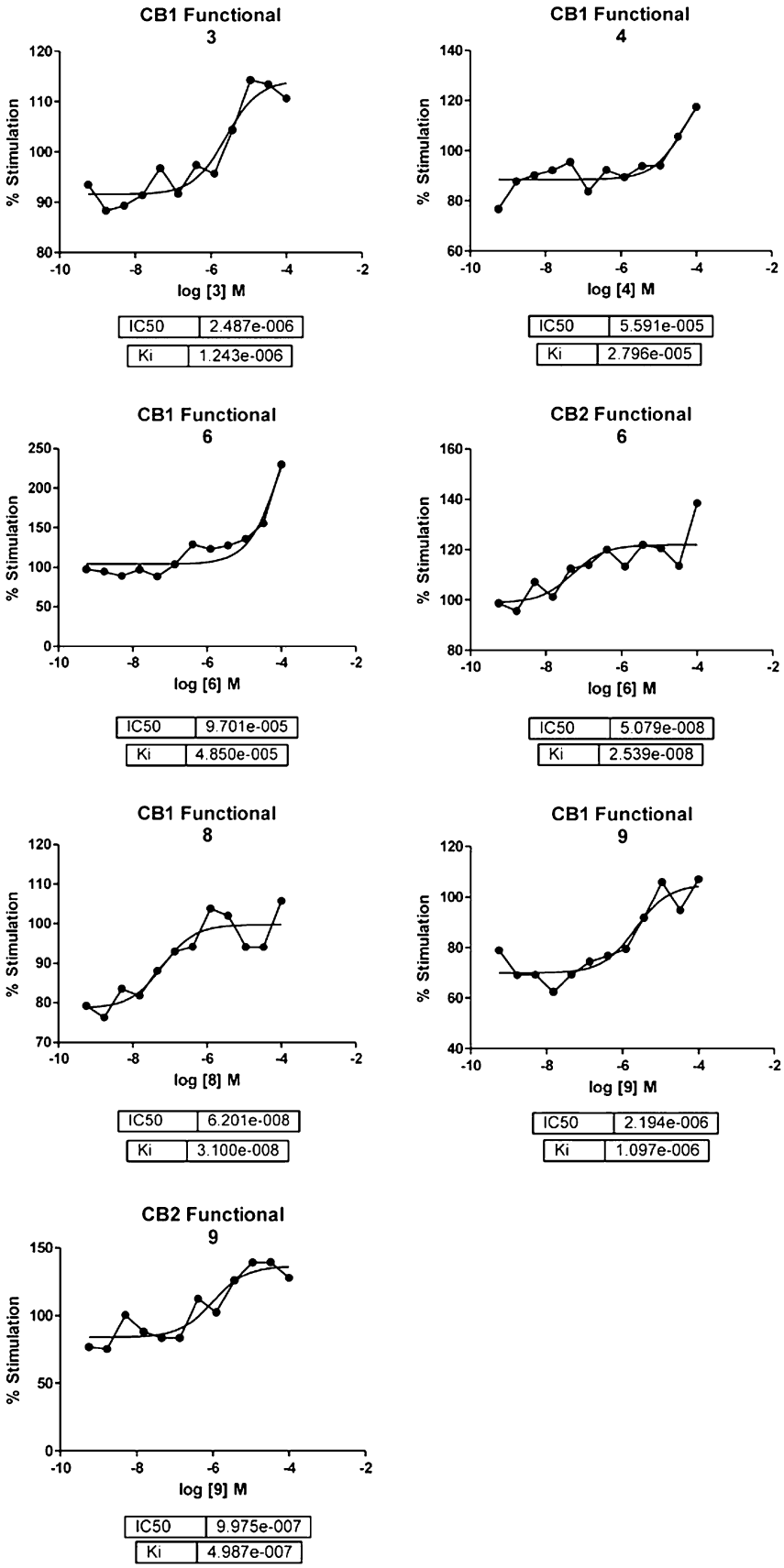


Fig. 1 Dose–response curves of compounds **3**, **4**, **6**, **8**, and **9** at cannabinoid receptors (CB1 and/or CB2)

conclusion, these results warrant further investigation of compounds **6** and **8,** as cannabinoid agonists, for possible development as potential therapeutic candidates. Further modifications are thus needed to characterize the structural parameters of this series of ferutinin derivatives necessary for binding to the cannabinoid receptors. The results of binding assays are displayed in (Table 2), whereas the results of functional assays are shown in (Table 3) and (Fig. 1).

Conclusion

Compounds **3**–**13** were synthesized from readily available starting materials and utilizing straightforward reactions. The new compounds in addition to **1** and **2** were evaluated for their in vitro estrogenic activities as well as affinity to cannabinoid receptors. Compounds **2**–**11** exhibited estrogenic activities that were weaker than the parent compound **1**. Nevertheless, compounds **6** and **8** showed significant binding affinities at the cannabinoid receptors and were found to be agonists for CB2 and CB1, respectively, as shown in (Table 3) and (Fig. 1). The current results necessitate further investigation for possible development of compounds **6** and **8** as potential therapeutic candidates.

Experimental

Chemistry

General experimental procedures 1D and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker Avance DPX-400 spectrometer and on a Varian AS 400 spectrometer. HRESIMS was obtained using a Bruker Bioapex FTMS in ESI mode. LRESIMS was obtained using a 3200 Q Trap LC/MS/MS (Applied Biosystems MDS Sciex, Foster City, CA). TLC was carried out on aluminum-backed plates precoated with silica gel F_{254} (20 × 20 cm, 200 µM, 60 Å, Merck). Visualization was accomplished by spraying with *p*-anisaldehyde [0.5 mL in glacial acetic acid (50 mL) and H_2SO_4 (97 %, 1 mL)] spray reagent followed by heating. Flash silica gel (40–63 µM, 60 Å, Silicycle) was used for column chromatography. Ferutinin (**1**) was prepared and characterized as described elsewhere (Galal *et al.*, 2001).

*General procedure for synthesis of ferutinol esters (**3–9**)* The sesquiterpenediol starting material, **2**, was prepared via alkaline hydrolysis of **1** by refluxing with 10 % KOH for 2 h. The liberated alcohol was subsequently extracted with ether followed by vacuum removal of solvent to obtain **2**. The identity of **2** was confirmed by comparison of its NMR data with the literature. Ferutinol **2** (75 mg, 0. 315 mmol) was dissolved in dry toluene (3 mL), 0.5 mL of triethylamine added, and the reaction mixture was stirred until clear solution was obtained. To this solution was added each of the following acid chlorides (two equivalents): *p*-chlorobenzoyl chloride, *p*-bromobenzoyl chloride, *p*-iodobenzoyl chloride, *p*-fluorobenzoyl chloride, *p*-cyanobenzoyl chloride, *p*-trifluoromethylbenzoyl chloride, and isonicotinoyl chloride hydrochloride plus a catalytic amount of 4-dimethylaminopyridine (DMAP). The reaction mixture was heated at 95–100 °C for 2–12 h, during which time the progress of the reaction was monitored by TLC. The reaction was quenched when TLC showed the disappearance or the presence of persistent traces of the starting material. The reaction mixture was then purified on silica gel column or by preparative TLC to isolate the target compounds. Isonicotinoyl chloride hydrochloride was dissolved in triethylamine, since it is insoluble in toluene.

Ferutinyl 6-p-bromobenzoate (**3**) Yield, 26 %, oil; $R_f = 0.7$ (Hexanes-EtOAc, 80:20); UV (MeOH) λ_{max} 235 sh, 250 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, d, $J = 6.8$ Hz, H-12), 0.95 (3H, d, $J = 7.2$ Hz, H-13), 1.10 (3H, s, H-15), 1.81 (3H, s, H-14), 2.06 (1H, d, $J = 8.8$ Hz, H-10a), 2.24 (1H, d, $J = 14.0$ Hz, H-10b), 2.52 (1H, m, H-11), 2.28 (1H, d, $J = 14.0$ Hz, H-5) 2.49 (2H, m, H-7), 1.50–1.67 (4H, m, H-2, H-3), 5.29 (1H, dt, $J = 10.0$, 2.8 Hz, H-6), 5.54 (1H, t, $J = 6.2$ Hz, H-9), 7.57 (2H, d, $J = 8.4$ Hz, H-3′, H-7′), 7.85 (2H, d, $J = 8.4$ Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.3 (C-1), 32.1 (C-2), 41.2 (C-3), 86.5 (C-4), 60.1 (C-5), 72.0 (C-6), 44.3 (C-7), 133.5 (C-8), 125.5 (C-9), 41.5 (C-10), 37.5 (C-11), 18.7 (C-12), 17.7 (C-13), 26.7 (C 14), 20.4 (C-15), 166.0 (C-1′), 129.6 (C-2′), 131.3 (C-3′), 132.0 (C-4′), 128.5 (C-5′), 132.0 (C-6′), 131.3 (C-7′); HRESIMS: *m/z* 443.1211 $[M + Na]^+$ (calcd for $C_{22}H_{29}BrNaO_3$, 443.1198).

Ferutinyl 6-p-iodobenzoate (**4**) Yield 27.1 %, white amorphous solid; $R_f = 0.76$ (Hexanes- EtOAc, 83:17); UV (MeOH) λ_{max} 255 nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.83 (3H, d, $J = 6.8$ Hz, H-12), 0.94 (3H, d, $J = 7.2$ Hz, H-13), 1.10 (3H, s, H-15), 1.80 (3H, s, H-14), 2.06 (1H, d, $J = 8.8$ Hz, H-10a), 2.24 (1H, d, $J = 14.0$ Hz, H-10b), 2.50 (1H, m, H-11), 2.27 (1H, d, $J = 14.0$ Hz, H-5) 2.47 (2H, m, H-7), 1.50–1.66 (4H, m, H-2, H-3), 5.27 (1H, dt, $J = 10.0$, 2.8 Hz, H-6), 5.53 (1H, bt, $J = 6.2$ Hz, H-9), 7.70 (2H, d, $J = 8.4$ Hz, H-3′, H-7′), 7.77 (2H, d, $J = 8.4$ Hz, H-4′, H-6′); ^{13}C NMR (100 MHz, $CDCl_3$): δ 44.3 (C-1), 32.2 (C-2), 41.2 (C-3), 86.5 (C-4), 60.1 (C-5), 72.0 (C-6), 44.3 (C-7), 133.5 (C-8), 125.5 (C-9), 41.5 (C-10), 37.5 (C-11), 18.7 (C-12), 17.7 (C-13), 26.7 (C 14), 20.4 (C-15), 166.7 (C-1′), 130.2 (C-2′), 131.9 (C-3′), 138.0 (C-4′), 101.3 (C-5′), 138.0 (C-6′), 131.9 (C-7′); HRESIMS: *m/z* 491.1063 $[M + Na]^+$ (calcd for $C_{22}H_{29}INaO_3$, 491.1059).

Ferutinyl 6-p-fluorobenzoate (**5**) Yield 33.4 %, white amorphous solid; $R_f = 0.62$ (Hexanes- EtOAc, 85:15); UV

(MeOH) λ_{max} 225 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, d, J = 6.8 Hz, H-12), 0.95 (3H, d, J = 7.2 Hz, H-13), 1.10 (3H, s, H-15), 1.81 (3H, s, H-14), 2.06 (1H, d, J = 8.8 Hz, H-10a), 2.24 (1H, d, J = 14.0 Hz, H-10b), 2.52 (1H, m, H-11), 2.28 (1H, d, J = 14.0 Hz, H-5) 2.49 (2H, m, H-7), 1.50–1.67 (4H, m, H-2, H-3), 5.29 (1H, dt, J = 10.0, 2.8 Hz, H-6), 5.54 (1H, t, J = 6.2 Hz, H-9), 8.53 (2H, d, J = 8.4 Hz, H-3′, H-7′), 7.13 (2H, d, J = 8.4 Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.3 (C-1), 32.1 (C-2), 41.2 (C-3), 86.5 (C-4), 60.1 (C-5), 72.0 (C-6), 44.3 (C-7), 133.6 (C-8), 125.5 (C-9), 41.5 (C-10), 37.5 (C-11), 18.7 (C-12), 17.7 (C-13), 26.7 (C 14), 20.4 (C-15), 165.8 (C-1′), 126.9 (C-2′), 132.3 (C-3′), 116.6 (C-4′), 161.4 (C-5′), 116.6 (C-6′), 132.3 (C-7′); HRESIMS: *m/z* 383.2001 $[M + Na]^+$ (calcd for $C_{22}H_{29}FNaO_3$, 383.1998).

*Ferutinyl 6-**p**-chlorobenzoate* **(6)** Yield 48 %, white amorphous solid; R_f = 0.7 (Hexanes- EtOAc, 85:15); UV (MeOH) λ_{max} 245 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (3H, d, J = 6.8 Hz, H-12), 0.95 (3H, d, J = 7.2 Hz, H-13), 1.10 (3H, s, H-15), 1.82 (3H, s, H-14), 2.06 (1H, d, J = 8.8 Hz, H-10a), 2.24 (1H, d, J = 14.0 Hz, H-10b), 2.52 (1H, m, H-11), 2.28 (1H, d, J = 14.0 Hz, H-5) 2.49 (2H, m, H-7), 1.50–1.67 (4H, m, H-2, H-3), 5.30 (1H, dt, J = 10.0, 2.8 Hz, H-6), 5.54 (1H, t, J = 6.2 Hz, H-9), 7.42 (2H, d, J = 8.4 Hz, H-3′, H-7′), 7.94 (2H, d, J = 8.4 Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.0 (C-1), 32.0 (C-2), 41.2 (C-3), 86.3 (C-4), 60.0 (C-5), 71.7 (C-6), 44.0 (C-7), 133.3 (C-8), 125.3 (C-9), 41.3 (C-10), 37.2 (C-11), 18.5 (C-12), 17.4 (C-13), 26.4 (C 14), 20.2 (C-15), 165.6 (C-1′), 129.0 (C-2′), 129.0 (C-3′), 132.0 (C-4′), 128.8 (C-5′), 132.0 (C-6′), 129.0 (C-7′); HRESIMS: *m/z* 399.1710 $[M + Na]^+$ (calcd for $C_{22}H_{29}ClNaO_3$, 399.1703).

*Ferutinyl 6-**p**-cyanobenzoate* **(7)** Yield 30.1 %, white amorphous solid; R_f = 0.48 (Hexanes- EtOAc, 80:20); UV (MeOH) λ_{max} 245 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.80 (3H, d, J = 6.8 Hz, H-12), 0.94 (3H, d, J = 7.2 Hz, H-13), 1.04 (3H, s, H-15), 1.80 (3H, s, H-14), 2.06 (1H, d, J = 8.8 Hz, H-10a), 2.24 (1H, d, J = 14.0 Hz, H-10b), 2.52 (1H, m, H-11), 2.28 (1H, d, J = 14.0 Hz, H-5) 2.49 (2H, m, H-7), 1.50–1.67 (4H, m, H-2, H-3), 5.28 (1H, dt, J = 10.0, 2.8 Hz, H-6), 5.51 (1H, t, J = 6.2 Hz, H-9), 8.07 (2H, d, J = 8.4 Hz, H-3′, H-7′), 7.71 (2H, d, J = 8.4 Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.2 (C-1), 32.1 (C-2), 41.2 (C-3), 86.5 (C-4), 60.0 (C-5), 72.5 (C-6), 44.2 (C-7), 133.4 (C-8), 125.6 (C-9), 41.5 (C-10), 37.5 (C-11), 18.7 (C-12), 17.7 (C-13), 26.7 (C 14), 20.3 (C-15), 164.9 (C-1′), 134.6 (C-2′), 130.2(C-3′), 132.5 (C-4′), 116.5 (C-5′), 132.5 (C-6′), 130.2 (C-7′), 118.1 (CN); HRESIMS: *m/z* 390.2039 $[M + H]^+$ (calcd for $C_{23}H_{29}NNaO_3$, 390.2045).

Ferutinyl 6-p-trifluoromethylbenzoate **(8)** Yield 44.4 %, wax; R_f = 0.66 (Hexanes-EtOAc, 80:20); UV (MeOH) λ_{max} 215, 225, 275 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.93 (3H, d, J = 6.8 Hz, H-12), 0.98 (3H, d, J = 7.2 Hz, H-13), 1.13 (3H, s, H-15), 1.83 (3H, s, H-14), 2.12 (1H, d, J = 8.8 Hz, H-10a), 2.24 (1H, d, J = 14.0 Hz, H-10b), 2.60 (1H, m, H-11), 2.33 (1H, d, J = 14.0 Hz, H-5), 2.32 (2H, m, H-7), 1.25–1.60 (4H, m, H-2, H-3), 5.40 (1H, dt, J = 10.0, 2.8 Hz, H-6), 5.60 (1H, t, J = 6.2 Hz, H-9), 8.15 (2H, d, J = 8.4 Hz, H-3′, H-7′), 7.75 (2H, d, J = 8.4 Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.0 (C-1), 32.1 (C-2), 41.3 (C-3), 86.3 (C-4), 60.1 (C-5), 72.3(C-6), 41.3 (C-7), 133.7 (C-8), 125.7 (C-9), 41.4 (C-10), 37.3 (C-11), 18.5 (C-12), 17.3 (C-13), 26.5 (C 14), 20.5 (C-15), 165.2 (C-1′), 133.3 (C-2′), 130.1 (C-3′), 125.8 (C-4′), 135.2 (C-5′), 125.8 (C-6′), 130.1 (C-7′), 124.9 (CF_3); HRESIMS: *m/z* 409.1996 $[M-H]^-$ (calcd for $C_{23}H_{28}F_3O_3$, 409.1991).

Ferutinyl 6-isonicotinate **(9)** Yield 40 %; white solid; R_f = 0.15 (Hexanes-EtOAc, 80:20); UV (MeOH) λ_{max} 255, 335 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (3H, d, J = 6.8 Hz, H-12), 0.95 (3H, d, J = 7.2 Hz, H-13), 1.10 (3H, s, H-15), 1.82 (3H, s, H-14), 2.06 (1H, d, J = 8.8 Hz, H-10a), 2.24 (1H, d, J = 14.0 Hz, H-10b), 2.52 (1H, m, H-11), 2.28 (1H, d, J = 14.0 Hz, H-5) 2.49 (2H, m, H-7), 1.50–1.67 (4H, m, H-2, H-3), 5.30 (1H, dt, J = 10.0, 2.8 Hz, H-6), 5.54 (1H, t, J = 6.2 Hz, H-9), 8.04 (2H, d, J = 8.4 Hz, H-3′, H-7′), 8.90 (2H, d, J = 8.4 Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.0 (C-1), 33.0 (C-2), 41.2 (C-3), 86.3 (C-4), 60.0 (C-5), 73.6 (C-6), 44.0 (C-7), 133.0 (C-8), 125.3 (C-9), 41.3 (C-10), 37.3 (C-11), 18.6 (C-12), 17.5 (C-13), 26.7 (C-14), 20.3 (C-15), 163.2 (C-1′), 142.4 (C-2′), 125.5 (C-3′), 146.7 (C-4′), 146.7 (C-6′), 125.5 (C-7′); HRESIMS: *m/z* 344.2243 $[M + H]^+$ (calcd for $C_{21}H_{30}NO_3$, 344.2226).

*Synthesis of ferutinyl 6-nalidixate (**10**)* Nalidixic acid (1. 0 mmol) was suspended in dichloromethane (20 mL) and stirred at 0 °C. To the stirred suspension, triethyl amine (1. 1 mmol) was added followed by drop wise addition of ethylchloroformate (1.1 mmol) and the stirring continued for 20 min. The resulting clear solution was then added to a stirred solution of ferutinol (1.1 mmol) in dichloromethane and stirring continued overnight. Solvent was evaporated under reduced pressure, and the product was purified on silica gel column.

Ferutinyl 6-nalidixate **(10)** Yield 90 %; white solid; R_f = 0.59 (Hexanes–acetone, 70:30); UV (MeOH) λ_{max} 250 sh, 255, 325 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, d, J = 6.8, H-12), 0.98 (3H, d, J = 7.2, H-13), 1.10 (3H, s, H-15), 1.81 (3H, s, H-14), 2.04 (1H, d, J = 8.8, H-10a), 2.11 (1H, d, J = 14.0, H-10b), 2.52 (1H, m, H-11), 2.11 (1H, d, J = 14.0, H-5) 2.41 (2H, m, H-7), 1.50–1.62 (4H, m, H-2, H-3), 5.01 (1H, dt, J = 10.0, 2.8, H-6), 5.50 (1H, t, J = 6.2, H-9), 8.34 (1H, s, H-2′), 8.53 (1H, d,

$J = 7.6$ Hz, H-5′), 7.20 (1H, d, $J = 6.7$ Hz, H-6′), 2.61 (3H, s, H-10′), 4.36, 4.47 (2H, q, $J = 7.2$, H-11′), 1.44 (3H, t, $J = 7.2$, H-12′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.3 (C-1), 32.2 (C-2), 41.8 (C-3), 86.1 (C-4), 60.8 (C-5), 73.4 (C-6), 44.3 (C-7), 133.9 (C-8), 125.1 (C-9), 41.9 (C-10), 37.1 (C-11), 19.0 (C-12), 17.8 (C-13), 27.1 (C 14), 20.2 (C-15), 147.2 (C-2′), 114.1 (C-3′), 174.5 (C-4′), 137.0 (C-5′), 121.3 (C-6′), 163.1 (C-7′), 148.8 (C-8′), 121.2 (C-9′), 25.3 (C-10′), 46.7 (C-11′), 15.4 (C-12′), 166.6 (C-13′); HRESIMS: *m/z* 453.2747 $[M + H]^+$ (calcd for $C_{27}H_{37}N_2O_4$, 453.2753).

*Synthesis of 9-hydroperoxyferutinin (**11**) and 8,9-epoxyferutinin (**12**)* Ferutinol (100 mg) was dissolved in 25 mL of a mixture of dichloromethane/ethanol (1:1) in a Dudley tube fitted in a cooling bath and then was added the photosensitization dye *meso*-tetraphenylporphine (~2.0 mg). The wine-red reaction mixture was then subjected to 500 W incandescent light, while oxygen was gently bubbled through, and the temperature of the bath was maintained at 13 °C. After 3 h of irradiation, the reaction was complete (disappearance of ferutinol on TLC) (Wasserman *et al.*, 1986). The solvent was distilled off from the reaction mixture to leave an olive green residue which was purified by normal phase preparative scale TLC to afford 9-hydroperoxyferutinin (**11**) and the known 8,9-epoxyferutinin (**12**).

9-Hydroperoxyferutinin **(11)** Yield 11.4 %; oil; $R_f = 0.33$ (Hexanes–EtOAc, 70:30); UV (MeOH) λ_{max} 255 nm; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.83 (3H, d, $J = 6.8$ Hz, H-12), 0.89 (3H, d, $J = 7.2$ Hz, H-13), 1.14 (3H, s, H-15), 1.88 (3H, s, H-14), 2.06 (1H, d, $J = 8.8$ Hz, H-10a), 2.11 (1H, d, $J = 14.0$ Hz, H-10b), 2.40 (1H, m, H-11), 2.11 (1H, d, $J = 14.0$ Hz, H-5) 1.50–1.67 (4H, m, H-2, H-3), 5.50 (1H, bs, H-6), 4.45 (1H, bs, H-9), 5.83 (1H, d, $J = 10.4$ Hz, H-7), 7.90 (2H, d, $J = 8.4$ Hz, H-3′, H-7′), 6.86 (2H, d, $J = 8.4$ Hz, H-4′, H-6′); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 44.5 (C-1), 32.0 (C-2), 42.1 (C-3), 87.1 (C-4), 54.1 (C-5), 73.0 (C-6), 128.0 (C-7), 137.2 (C-8), 83.5 (C-9), 42.4 (C-10), 37.1 (C-11), 18.6 (C-12), 17.7 (C-13), 24.5 (C 14), 20.2 (C-15), 167.5 (C-1′), 121.8 (C-2′), 132.4 (C-3′), 115.7 (C-4′), 161.5 (C-5′), 115.7 (C-6′), 132.4 (C-7′); HRESIMS: *m/z* 413.1958 $[M + H]^+$ (calcd for $C_{22}H_{30}NaO_6$, 413.1940).

In vitro biological assays

Estrogenic assay The estrogenic activity was determined by the YES (yeast estrogen screen) assay as described earlier with some modifications (Beresford *et al.*, 2000; Tabanca *et al.*, 2004).

Saccharomyces cerevisiae cells expressing the estrogen receptor alpha (ER-α) were cultured at 30 °C for 24 h. For the assay, 1.5 mL of the culture and 250 µL of chlorophenol red β-D-galactopyranoside (CPRG, 10 mg/mL) were added to 25 mL of growth medium. Test compounds (10 mM) or 17β-estradiol (1 mM, positive control) were dissolved in DMSO. Serial dilutions were made, and 10 µL of the diluted samples were transferred from the dilution plate to the assay plate and mixed with 190 µL of yeast cell suspension containing CPRG. The highest DMSO concentration was 5 %. The plate was incubated at 30 °C for 48 h, and the absorbance was measured at a dual wavelength of 540/630 nm. Dose curves were generated, and half maximal effective concentration (EC_{50}) values were obtained. Highest effective dose was determined for each compound showing estrogenic response, and the percent maximal response at the highest effective dose was calculated in comparison to 17β-estradiol.

Cell culture HEK293 cells (ATCC #CRC-1573) were stably transfected via electroporation with full-length human recombinant cDNA for cannabinoid receptor subtypes 1 and 2 (obtained from Origene). These cells were maintained in a Dulbecco's modified Eagles's medium/F-12 (50/50) nutrient mixture supplemented with 10 % fetal bovine serum, 1 % penicillin/streptomycin, and either 1 % G418 sulfate (Geneticin), depending on the cell line. Percentages are based on a total media volume of 500 mL. Both cannabinoid cell lines were kept at 37 °C and 5 % CO_2. Membranes were prepared by scraping the cells in a 50 mM Tris–HCl buffer, homogenized via sonication, and centrifuged for 40 min at 13,650 rpm at 4 °C. These were kept at 80 °C until used for binding and functional assays. Protein concentration was determined via Bio-Rad protein assay (Bradford, 1976).

Radioligand binding for cannabinoid receptor subtypes In the primary bioassay screen, compounds were tested at a final concentration of 10 µM for competitive binding to the respective receptor. For the cannabinoid receptor assays, test compounds were added into a 96-well plate followed by 0.6 nM [3H]CP-55,940 and 10 µg of cannabinoid membrane resuspended in 50 mM Tris (pH 7.4), 154 mM NaCl, and 20 mM Di-Na-EDTA supplemented with 0.02 % BSA. The cannabinoid assay was allowed to incubate at 37 °C for 90 min. The reaction was then terminated by rapid filtration using GF/C (presoaked in 0.3 % BSA) and washed with the buffer. Dried filters were then covered with scintillant and measured for the amount of radioligand retained using a PerkinElmer Topcount (PerkinElmer Life Sciences Inc., Boston, MA, USA). Nonspecific binding, which was determined in the presence of 1 µM CP-55,940 for cannabinoid receptors, was subtracted from the total binding to yield the specific-binding values. Compounds showing competitive inhibition of the

labeled ligand to bind to the receptor at 50 % or greater were tested in a dose–response curve with concentrations of the test compound ranging from 300 μM to 1.7 nM. Ki and IC_{50} values were calculated using GraphPad Prism 5.

[^{35}S]-GTP-γS binding In the functional assay for cannabinoid receptor binding, membranes (20 μg/well) were incubated with 0.5 nM [^{35}S]-GTP-γS and test compound in 50 mM Tris–HCl, 0.2 mM EGTA, 9 mM $MgCl_2$, 150 mM NaCl, 50 μM GDP, and 1.4 mg mL^{-1} BSA. The reaction incubated for 2 h at 30 °C and was terminated by rapid vacuum filtration with cold 10 mM Tris–HCl in a Perkin-Elmer harvester through GF/B filters. Nonspecific binding was determined by 40 μM of GTP-γS.

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