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Synthesis and pharmacological evaluation of coumarin derivatives as cannabinoid receptor antagonists and inverse agonists

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ABSTRACT

In the present study we synthesized 36 coumarin and 2*H*-chromene derivatives applying a recently developed umpoled domino reaction using substituted salicylaldehyde and α,β-unsaturated aldehyde derivatives as starting compounds. In radioligand binding studies 5-substituted 3-benzylcoumarin derivatives showed affinity to cannabinoid CB_1 and CB_2 receptors and were identified as new lead structures. In further GTPγS binding studies selected compounds were shown to be antagonists or inverse agonists.

1. Introduction

Cannabinoid receptors are part of the endocannabinoid system, which consists of cannabinoid receptors, endogenous cannabinoids (endocannabinoids), and the enzymes that synthesize and degrade endocannabinoids.[1] The cloning of the cannabinoid CB_1 and CB_2 receptor subtypes[2,3] in the early 1990s followed by the discovery and characterization of their endogenous ligands—that is, anandamide (**3**)[4] and 2-arachidonoylglycerol[5]—offered new areas for therapeutic interventions.[6] Recently, the orphan receptor GPR55 has been described as a putative third cannabinoid receptor subtype, which appears to be also activated by other lipid metabolites including lysophosphatidic acid and sphingosine 1-phosphate.[7–10] Clinical trials involving either cannabinoid receptor ligands or *Cannabis sativa* extracts demonstrated pharmacological activities.[11] For instance, Δ^9-tetrahydrocannabinol (Δ^9-THC, **1**) was shown to be effective in the treatment of Tourette's syndrome tics[12] and in the relief of pain in case of multiple sclerosis and neuropathic pain.[1,13] It is further approved for the treatment of nausea and vomiting associated with chemotherapy and as an appetite stimulant for AIDS patients.[1] In animal models, cannabinoids have shown to reduce allergic contact dermatitis.[14]

Antagonists at cannabinoid CB_1 receptors also exhibit therapeutic potential while being devoid of severe psychotropic side effects such as addiction.[15,16] The CB_1 antagonist rimonabant (SR141716A, **5**) was approved as a novel therapeutic for the treatment of obesity[17] but meanwhile withdrawn from the market due to potential side effects; it may also be helpful for smoking cessation.[18] Another promising application for such drugs is the treatment of drug dependence (from alcohol, opioids, or cannabis) as indicated by both animal and human studies.[19] Furthermore, there is preliminary evidence for the usefulness of CB_1 antagonists in the treatment of Parkinson's and Huntington's diseases[20] probably due to a direct, physical, as well as an indirect, functional interaction between adenosine A_{2A} and cannabinoid CB_1 receptors in the brain striatum.[21]

The physiological role of CB_2 receptors has not been fully analyzed yet. CB_2 agonists are effective in chronic pain models[1] and possess potent peripheral analgesic activity.[22]

Both CB_1 and CB_2 cannabinoid receptors belong to the superfamily of G protein-coupled receptors (GPCRs). Both CB_1 and CB_2 receptors are coupled via $G_{i/o}$ proteins to inhibition of adenylate cyclase and activation of mitogen-activated protein (MAP) kinase.[23] G_s coupling of the CB_1 receptor has been described under certain conditions.[24] In contrast, GPR55 is coupled to $G_{\alpha 13}$ mediating activation of rhoA, cdc42, and rac1.[7,9] The highest density of the CB_1 cannabinoid receptor is found in the cerebellum, hippocampus, cerebral

Abbreviations: AIDS, acquired immune deficiency syndrome; BSA, bovine serum albumin; CB_1, cannabinoid receptor type 1; CB_2, cannabinoid receptor type 2; DABCO, 1,4-diazobicyclo[2.2.2]octane; DIC, diisopropylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DTT, dithiothreitol; EtOAc, ethylacetate; GPCR, G protein-coupled receptor; GPR55, G protein-coupled receptor 55; [^{35}S]GTPγS, guanosine 5′-(γ-^{35}S)-triphosphate; HEK, human embryonic kidney; HR-EIMS, high resolution mass spectrometry; IR, infrared; mp, melting point; MS, mass spectrometry; NMR, nuclear magnetic resonance; *n*-/*c*-Hex, *n*-/*cyclo*-hexane; R_f, ratio of fronts; THC, tetrahydrocannabinol.

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cortex, basal ganglia, substantia nigra pars compacta, and in some regions of the globus pallidus.[25] It is also present in peripheral organs such as the adrenal glands, bone marrow, lungs, testis, and uterus.[26] In contrast to the CB_1 cannabinoid receptor, the CB_2 receptor is limited essentially to cells associated with the immune system, like leukocytes, spleen, thymus, and tonsils.[27]

Due to the immense therapeutic potential of cannabinoid receptor ligands, considerable efforts have been undertaken to identify and optimize selective CB_1 and CB_2 receptor agonists and antagonists.[28,29] The cannabinoid receptor agonists are classified into four different groups. The classical cannabinoids consist of a tricyclic benzopyran-containing ring system like Δ^9-THC (**1**). A dihydrobenzopyran-type structure with a hydroxyl group at the C-1 aromatic position and an alkyl group on the C-3 aromatic position seems to be a requirement for high cannabinoid receptor affinity.[30] The length of the chain at C-3 is of major importance, but an all carbon side chain is not an absolute requirement, it may contain an ethereal oxygen.[31] The methyl group on C-9 and the double bond in the terpene ring is not required for activity.[32] The terpene ring may be exchanged for some heterocyclic systems.[33] Changes in the stereochemistry at various carbons of THC-type molecules may cause significant changes in pharmacological activity. Nonclassical cannabinoids lacking the dihydropyran ring of Δ^9-THC are represented by the bicyclic analogue CP55,940 (**2**).[2] Aminoalkylindoles possessing cannabimimetic properties, such as WIN55,212-2 (**4**), have also been described.[34] The prototypic member of the eicosanoid group of cannabinoid receptor agonists is anandamide (**3**), the first of five known endogenous cannabinoid receptor agonists. In addition to the biarylpyrazole derivatives, for example, rimonabant (**5**), several other heterocyclic classes of compounds such as triazoles,[35,36] thiazoles,[36] pyrazolines,[37] imidazoles[36,38], and pyridines[39] with antagonistic or inverse agonistic properties have been described.

Coumarins (chromene-2-ones, benzopyran-2-ones) are a naturally occurring class of compounds, which have been shown to possess a variety of pharmacological activities dependent on the substitution pattern.[40,41] Suitably substituted coumarin derivatives that show structural similarity to cannabinol, such as compounds **7** and **8,** have been described as highly selective CB_2 receptor agonists.[22,42]

In the present study, we synthesized a novel series of coumarin and related 2*H*-chromene derivatives and explored their potential as cannabinoid receptor ligands. The new compounds were investigated in radioligand binding studies to determine their affinity for cannabinoid CB_1 and CB_2 receptor subtypes. Furthermore, [^{35}S]GTPγS binding studies were performed in order to investigate the intrinsic activity (agonistic/antagonistic/inverse agonistic) of the new compounds.

2. Results and discussion

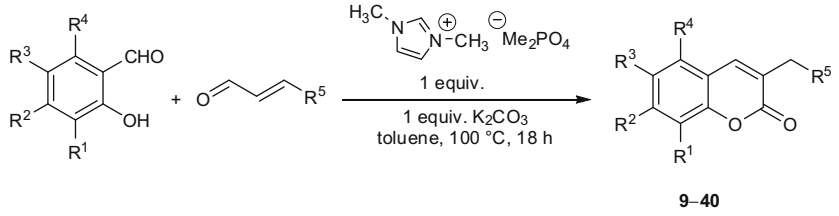
2.1. Chemistry

3-Alkylcoumarins **9**–**40** were synthesized from salicylaldehydes and α,β-unsaturated aldehydes utilizing nucleophilic carbenes in a recently developed umpoled domino reaction (Scheme 1).[43] While compounds **9**, **10**, **12**–**15**, **17**, **20**–**24**, **30**, **32**, and **37**–**40** were reported earlier without any biological data[43–50] all other compounds are new derivatives.

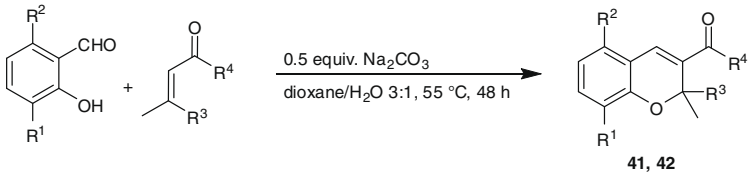
The chromene **41** was prepared as described earlier while the chromenes **42**–**44** were obtained according to procedures previously published by our group (see Schemes 2–4).[43] The starting compound for the preparation of **44** was obtained as described.[51]

2.2. Biological evaluation

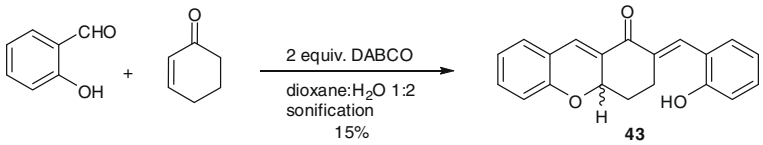
The new compounds were investigated in radioligand binding studies at CB_1 receptors using rat brain cortical membrane preparations, and at CB_2 receptors using commercially available membrane preparations of human embryonic kidney (HEK293) cells



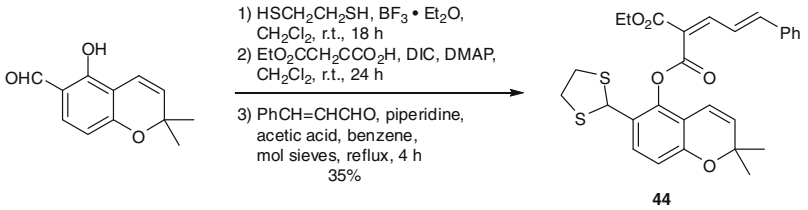
Scheme 1. One-pot synthesis of 3-alkylcoumarins.



Scheme 2. One-pot synthesis of 2*H*-chromenes **41** and **42** (for full structures see Table 1).



Scheme 3. Synthesis of tetrahydroxanthone **43**.



Scheme 4. Synthesis of 2*H*-chromene **44**.

recombinantly expressing the human CB_2 receptor. [^{3}H](−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940, **2**) was used as CB_1 and CB_2 radioligand. The assays were performed in a 96-well plate format to allow fast screening, and the assay conditions were optimized for that format (see Experimental). The evaluation of standard compounds—unlabeled CP55,940; WIN55,212-2; and AM281—confirmed that our assays produced results which were in accordance with published data[52,53] (see Table 1). Initially the compounds were tested a concentration of 10 μM. In cases where inhibition of radioligand binding was greater than 60%, full concentration–inhibition curves were determined using seven concentrations spanning three orders of magnitude in order to determine IC_{50} and to calculate K_i values. For all investigated compounds sigmoidal concentration–inhibition curves were obtained. Selected compounds of the present series were investigated for their functional properties in [^{35}S]GTPγS binding at rat brain cortical membranes and at HEK cells expressing the human CB_1 receptor.

2.3. Structure–activity relationships

A series of 32 coumarin derivatives (**9**–**40**) with variations in the 3-, 5-, 6-, 7-, and 8-position, as well as four 2*H*-chromene derivatives (**41**–**44**) were investigated in order to assess their potential to bind to cannabinoid CB_1 and/or CB_2 receptors and to study their structure–activity relationships. Selected concentration–inhibition curves from radioligand binding studies are shown in Figure 3.

In the 3-position of the coumarin scaffold, a methyl, benzyl, *o*-hydroxybenzyl, methoxybenzyl (*o*, *p*, *m*), *o*,*p*-dimethoxybenzyl, or chlorobenzyl (*o*, *p*) residue was present. None of the compounds with a methyl residue in the 3-position displayed significant binding neither towards CB_1 nor CB_2 receptors (**12**, **15**, **17**, **18**, **21**, **23**, **39** and **40**). In contrast, active compounds were identified among the benzyl-substituted derivatives (e.g., **10**, **24**–**36** and **38**). However, a 3-benzyl residue alone was not sufficient, since the 3-benzyl coumarin **9** was virtually inactive. Introduction of an additional, 5,6-annelated benzene ring into 3-benzylcoumarin (yielding the tricyclic compound **10**) was favorable, particularly with respect to CB_1 affinity (K_i 4.19 μM), while the *o*-methoxybenzyl derivative **11** was CB_2-selective (K_i 2.06 μM). The introduction of a methoxy group in the 5-position of 3-benzylcoumarin (**9**) resulting in compound **13** also led to an increase in CB_1 affinity (K_i 19.1 μM), but not CB_2 affinity. If a methoxy group was introduced into the 6-, 7-, or 8-position instead (compounds **14**, **20** and **22**) weakly active or inactive compounds were obtained. Residues other than methoxy in the 6-position (iodo, nitro) also resulted in only weakly active or inactive compounds (**16** and **19**). Careful analysis of the data appeared to show that a 6-methoxy group or a 6-iodo residue was somewhat better tolerated by the CB_2 receptor than by the CB_1 receptor.

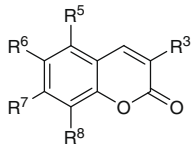
Additional introduction of a 7-methyl group into the best CB_1 ligand so far, 3-benzyl-5-methoxycoumarin (**13**), increased affinity for CB_1 receptors by almost sixfold (**24**, K_i 3.46 μM) without much effect on the CB_2 affinity thus yielding a CB_1-selective compound. This derivative was selected as a lead compound for further variation: a methyl, chloro, hydroxy, or methoxy group was introduced at various positions of the 3-benzyl residue (compounds **24**–**32** and **34**). These modifications were well tolerated by both receptor subtypes and yielded compounds with relatively high affinity and no or only weak selectivity. In general, substituents in the *ortho*-position were best tolerated; the rank order of affinity was as follows: *o*-methyl (**25**) > *p*-methyl (**26**); *o*-chloro (**27**) > *p*-chloro (**28**); *o*-methoxy (**30**) > *m*-methoxy (**31**) > *p*-methoxy (**32**) ⩾ *o*,*p*-dimethoxy (**34**). The *ortho*-substituted derivatives had similarly high affinity for CB_1 and CB_2 receptors and showed K_i values around 1 μM or even in the submicromolar range. The compound with the highest affinity was 3-*o*-methoxybenzyl-5-methoxy-7-methylcoumarin (**30**) with K_i values of 0.738 μM at CB_1 and 1.03 μM at CB_2 receptors. The corresponding 8-methyl isomer (3-*o*-methoxybenzyl-5-methoxy-8-methylcoumarin, **35**) showed a 17-fold lower K_i value at CB_1 and a fourfold lower affinity for CB_2 receptors. Also, combination of 3-benzyl-5-methoxycoumarin (**13**) with a bromine atom in the 8-position was unfavorable (compound **37**). Another trisubstituted coumarin derivative, 3-benzyl-5-isopropyl-8-methylcoumarin (**38**), showed micromolar affinity for both receptor subtypes (K_i CB_1 2.76 μM, CB_2 3.93 μM) indicating that an isopropyl residue in the 5-position of 3-benzylcoumarin may be favorable. However, in this case, *o*-methoxy-substitution of the 3-benzyl residue did not enhance affinity (compound **36**) possibly due to a different binding mode of the trisubstituted coumarin derivative. In the 7-position, a methyl group improved affinity by several-fold in comparison with a hydrogen atom as shown in a comparison of the lead compound **30** and its 7-unmethylated analogue **33**.

Two of the four investigated 2*H*-chromene derivatives, compounds **43** and **44**, also showed good affinity for CB_1, but not CB_2 receptors, with K_i values of 4.90 μM (**43**) and 1.37 μM (**44**), respectively. Thus it appears that the pyranone structure of coumarins is not required for interaction with the receptors, a partially hydrogenated benzopyran structure is sufficient. Again, a large, bulky, aromatic substituent is required in the 3-position of the benzopyran ring system (like in compound **43**) or nearby (**44**), while small substituents as in **41** and **42** result in virtually inactive or only slightly active compounds. Thus a bulky, aromatic group in that position appears to be essential.

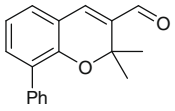
2.4. Species differences

The CB_1 cannabinoid receptor has been cloned from rat, mouse, and human tissues and it exhibits 97–99% amino acid sequence identity across species.[55–58] The CB_2 cannabinoid receptor shows ca. 48% homology with the CB_1 cannabinoid receptor.[3] The mouse CB_2 receptor has an 82% sequence identity to the human CB_2 receptor.[59] Sequence analysis of the CB_2 genomic clone indicates 93% amino acid identity between rat and mouse and 81% amino acid identity between rat and human.[42,60] Affinities and efficacies of several reference cannabinoid ligands were investigated at CB_1 and CB_2 receptors in three different species (rat, mouse, and human). The ligands follow the same ranking of affinity for the cannabinoid receptors in the mouse, rat, and human. In own competition experiments at the rat cannabinoid CB_1 recep-

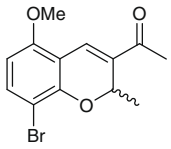
Table 1
Affinities of coumarin and 2*H*-chromene derivatives for cannabinoid receptor subtypes



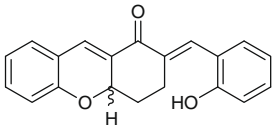
9–40



41



42



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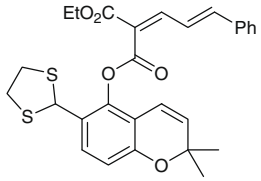

44

Compound	R^3	R^5	R^6	R^7	R^8	K_i ± SEM (μM) (% inhibition ± SEM)	
						Rat CB_1[a] versus [^{3}H]CP55,940	Human CB_2[a] versus [^{3}H]CP55,940
2 CP55,940 (agonist)	—	—	—	—	—	0.00124 ± 0.00058 (0.00137)[39]	0.000439 ± 0.000021 (0.00069)[38]
4 WIN55,212-2 (agonist)	—	—	—	—	—	0.00606 ± 0.00062 (0.0044,[54] 0.00994)[39]	0.00536 ± 0.00303 (0.0012)[40]
6 AM281 (inverse agonist)						0.0124 ± 0.0038 0.012[44]	4.87 ± 0.63 (n = 2) 4.2 [44][b]
9	Benzyl	H	H	H	H	>10 (18 ± 7%)[c]	>10 (28 ± 3%)[c]
10	Benzyl			H	H	4.19 ± 2.00	⩾10 (59 ± 9%)[c]
11	*o*-Methoxybenzyl			H	H	>10 (41 ± 3%)[c]	2.06 ± 1.19
12	Methyl			H	H	>10 (10 ± 4%)[c]	⩾10 (2 ± 1%)[c]
13	Benzyl	Methoxy	H	H	H	19.1 ± 4.0	>10 (28 ± 7%)[c]
14	Benzyl	H	Methoxy	H	H	⩾10 (8 ± 2%)[c]	>10 (37 ± 1%)[c]
15	Methyl	H	Methoxy	H	H	>10 (15 ± 10%)[c]	⩾10 (7 ± 8%)[c]
16	Benzyl	H	I	H	H	>10 (31 ± 7%)[c]	⩾10 (44 ± 2%)[c]
17	Methyl	H	I	H	H	>10 (12 ± 10%)[c]	>10 (10 ± 7%)[c]
18	Methyl	H	Cl	H	Br	⩾10 (8 ± 5%)[c]	⩾10 (3 ± 10%)[c]
19	Benzyl	H	Nitro	H	H	>10 (32 ± 15%)[c]	>10 (15 ± 5%)[c]
20	Benzyl	H	H	Methoxy	H	>10 (31 ± 13%)[c]	>10 (33 ± 4%)[c]
21	Methyl	H	H	Methoxy	H	⩾10 (1 ± 6%)[c]	⩾10 (2 ± 3%[e])[c]
22	Benzyl	H	H	H	Methoxy	⩾10 (6 ± 9%)[c]	>10 (25 ± 3%)[c]
23	Methyl	H	H	H	Methoxy	⩾10 (6 ± 3%)[c]	>10 (4 ± 2%)[c]
24	Benzyl	Methoxy	H	Methyl	H	3.46 ± 1.14	⩾10 (45 ± 3%)[c]
25	*o*-Methylbenzyl	Methoxy	H	Methyl	H	1.17 ± 0.32	2.16 ± 0.80
26	*p*-Methylbenzyl	Methoxy	H	Methyl	H	9.14 ± 1.87	4.76 ± 0.54
27	*o*-Chlorobenzyl	Methoxy	H	Methyl	H	0.978 ± 0.264	1.54 ± 0.39
28	*p*-Chlorobenzyl	Methoxy	H	Methyl	H	2.08 ± 0.40	9.39 ± 2.23
29	*o*-Hydroxybenzyl	Methoxy	H	Methyl	H	1.10 ± 0.12	5.24 ± 0.87
30	*o*-Methoxybenzyl	Methoxy	H	Methyl	H	0.738 ± 0.414	1.03 ± 0.22
31	*m*-Methoxybenzyl	Methoxy	H	Methyl	H	4.11 ± 1.38	3.37 ± 0.90
32	*p*-Methoxybenzyl	Methoxy	H	Methyl	H	6.14 ± 0.69	5.81 ± 2.39
33	*o*-Methoxybenzyl	Methoxy	H	H	H	9.57 ± 2.00	3.49 ± 0.33[d]
34	*o*,*p*-Dimethoxybenzyl	Methoxy	H	Methyl	H	6.23 ± 1.43	2.78 ± 1.22
35	*o*-Methoxy-benzyl	Methoxy	H	H	Methyl	12.3 ± 3.2	3.65 ± 1.20
36	*o*-Methoxy-benzyl	Isopropyl	H	H	Methyl	6.18 ± 0.96	5.67 ± 1.10
37	Benzyl	Methoxy	H	H	Br	>10 (39 ± 6%)[c]	>10 (29 ± 2%)[c]
38	Benzyl	Isopropyl	H	H	Methyl	2.76 ± 0.66	4.27 ± 0.10
39	Methyl	Isopropyl	H	H	Methyl	>10 (26 ± 11%)[c]	⩾10 (0 ± 2%)[c]
40	Methyl	H	Br	H	Methoxy	>10 (17 ± 6%)[c]	⩾10 (9 ± 7%)[c]
41	—	—	—	—	—	⩾10 (46 ± 7%)[c]	⩾10 (3 ± 1%)[c]
42	—	—	—	—	—	>10 (34 ± 8%)[c]	⩾10 (7 ± 7%)[c]
43	—	—	—	—	—	4.90 ± 1.11	>10 (37 ± 5%)[c]
44	—	—	—	—	—	1.37 ± 0.27	>10 (22 ± 3%)[c]

[a] Membrane preparations.
[b] Binding to rat cannabinoid CB_2 receptors on spleen tissue.
[c] Percent inhibition of radioligand binding at 10 μM.
[d] n = 2.

tors only very minor species differences were observed for the CB_1 receptors, for example, the determined K_i values for CP55,940 (**2**) at rat and human receptors were quite similar (1.24 nM at rat CB_1 and 0.71 nM at human CB_1 receptors; K. Atzler and C.E. Müller, unpublished data). Therefore, K_i values determined at rat CB_1 receptors should be highly predictive of K_i values at human CB_1 receptors.

2.5. Functional properties

The compounds of the present series that had shown the highest affinities were investigated in [^{35}S]GTPγS binding assays at rat brain CB_1 receptors and at human recombinant CB_1 receptors in order to study their intrinsic activity (see Fig. 4 and Table 2). GDP–GTP exchange is an early event in the signal transduc-

tion mechanism of a G protein-coupled receptor. Measuring the binding of [^{35}S]GTPγS—a radiolabeled non-hydrolyzable analogue of GTP—provides direct quantitative information on the interaction between the receptor and the G protein. Binding of an agonist to the GPCR will increase binding of the guanine nucleotide, while a neutral antagonist does not have any effects on the nucleotide binding, and an inverse agonist will decrease the [^{35}S]GTPγS binding to the G protein. The compounds were tested at a high concentration of 30 μM, and the determined effects were compared to those elicited by the full agonist CP55,940 (**2**) and the inverse agonist AM281 (**6**). As expected CP55,940 induced an increase in [^{35}S]GTPγS binding from a basal level of 100% to 169 ± 9% at CB_1 receptors natively expressed in rat brain cortical membranes, and to 228 ± 12% in membranes prepared from HEK293 cells expressing the human CB_1 receptor, respectively (Fig. 4). AM281 had no apparent effect on [^{35}S]GTPγS binding to CB_1 receptors in rat cortex, but induced a decrease in binding of the nucleotide to human recombinant CB_1 receptors expressed in HEK293 cells. AM281, previously described as full inverse agonist,[61] led to a reduction of GTPγS binding from a basal level of 100% to 73 ± 13% at CB_1 receptors in HEK cell membranes. The discrepant effect of the inverse agonist AM281 at native CB_1 receptors (neutral antagonistic effect) as compared to CB_1 receptors expressed in HEK293 cells (inverse agonistic effect) can be explained by the high density of CB_1 receptors in the recombinant cell system as compared to the lower density in cortex. Similar effects have been observed before, for example, for the antagonist/inverse agonist rimonabant.[62,63]

Figure 4 shows the effects of the coumarin derivatives **10**, **24**, **30**, **32**, and **38** on the binding of [^{35}S]GTPγS to HEK293 cell membranes expressing the human CB_1 receptor. In Table 2 the results of all [^{35}S]GTPγS assays are collected.

None of the investigated compounds led to an increase in [^{35}S]GTPγS binding at rat brain CB_1 cannabinoid receptors thus showing that they were all antagonists. In HEK293 cells, which were stably transfected with the human CB_1 receptor, the coumarin derivatives induced a decrease in [^{35}S]GTPγS binding from a basal level of 100% down to between 72% and 90%. Compound **10** was as efficacious as the full inverse agonist AM281 (72 ± 6%). Thus, a condensed phenyl ring at the 5- and 6-position led to selective CB_1 receptor ligands with full inverse agonistic activity. The most potent compound of the present series, coumarin **30**, led to a reduction in [^{35}S]GTPγS binding from a basal level of 100% to 79 ± 2% indicating that it is also an efficacious inverse agonist. Compounds **24**, **32**, and **38** showed a less pronounced inhibitory effect on [^{35}S]GTPγS binding. This indicates that minor structural modifications may have a significant influence on the inverse agonistic efficacy of coumarin derivatives.

2.6. Structural considerations

A comparison of the coumarin-based agonists **7** and **8** (Fig. 2) with the 3-benzylcoumarin derivatives identified in the present study to be agonist/inverse agonists at CB_1 receptors reveals important differences. The agonists **7** and **8** feature a phenolic OH group and a long lipophilic alkyl side chain on the same phenyl ring. Comparing the structures of **7** and **8** with Δ^9-THC (**1**, Fig. 1) shows that this partial structure is found in both agonistic classes of compounds; however, it is lacking in the corresponding positions of the coumarin derivatives investigated in the present study (position 5 and position 7, for OH and the long alkyl chain, respectively). This may explain why the new compounds are CB receptor antagonists rather than agonists.

3. Conclusions

In conclusion, we have synthesized 32 coumarin (**9**–**40**) derivatives, 22 of which are new compounds not previously described in the literature, as well as four new 2*H*-chromene derivatives (**41**–**44**). The compounds were evaluated for their potency to bind to and activate cannabinoid receptors. 5-Substituted 3-benzylcoumarin derivatives were identified as new lead structures for the development of cannabinoid CB_1 and CB_2 receptor antagonists/inverse agonists, and initial structure activity relationships were analyzed. 3-*o*-Methoxybenzyl-5-methoxy-7-methylcoumarin (**30**) was the compound with the highest affinity of the present series exhibiting a K_i value of 0.738 μM at CB_1 and 0.944 μM at CB_2 receptors. Some of the compounds showed a certain degree of selectivity for CB_1 or CB_2 receptors. The new compounds will be used as novel lead structures for the development of more potent CB_1 or CB_2 antagonists/inverse agonists. In particular, the introduction of more bulky substituents in the 7-position in order to fill the lipophilic pocket which is, for example, occupied by the pentyl chain of Δ^9-THC may further increase affinity.

4. Experimental

4.1. Chemical synthesis

NMR spectra were recorded on a Varian XL-300 (^{1}H: 300 MHz, ^{13}C: 75 MHz) or a Bruker DRX 500 (^{1}H: 500 MHz, ^{13}C: 125 MHz). Deuterated $CDCl_3$ was used as a solvent unless otherwise noted. The chemical shifts of the remaining protons of the solvent were used as internal standard: ^{1}H, 7.26 ppm; ^{13}C, 77.0 ppm. All chemical shifts (δ) were expressed in ppm. Coupling constants (J) are given in Hertz (Hz). The reactions were monitored by thin layer

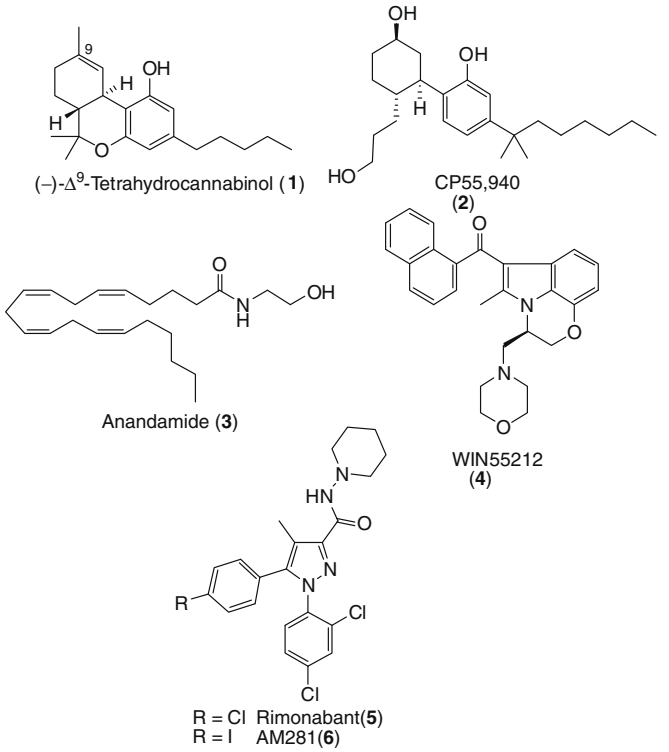

Figure 1. Structures of standard cannabinoid receptor ligands: Δ^9-THC (**1**, agonist), CP55,940 (**2**, agonist), anandamide (**3**, endogenous agonist), WIN55,212-2 (**4**, agonist), rimonabant (SR141716A, inverse agonist) (**5**), and AM281 (**6**, inverse agonist).

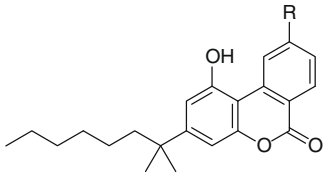

Figure 2. Structure of coumarin derivatives AM-1710 (**7**) and AM-1714 (**8**), two CB_2 receptor agonists (AM-1710: K_i 360 nM (rCB_1), 6.7 nM (mCB_2); AM-1714: K_i 400 nM (rCB_1), 0.82 nM (mCB_2)).[22]

chromatography (TLC) using aluminum sheets with silica gel 60 F_{254} (Merck). The melting points were determined on a Laboratory Devices Inc., model Mel-Temp II melting point apparatus, and are uncorrected. Elemental analyses were performed in the Institute of Organic Chemistry, University of Karlsruhe. Mass spectra were collected on an MS-50 A.E.I. (Manchester) mass spectrometer with an ionization energy of 70 eV. The coumarins **11**, **25–29**, **31**, and **33–36** were synthesized as described.[42]

4.1.1. General procedure

Under an atmosphere of argon, 0.602 mmol of substituted salicylaldehyde, 0.602 mmol of potassium carbonate, 1.51 mmol of α,β-unsaturated aldehyde and 0.602 mmol of 1,3-dimethylimidazolium dimethylphosphate were suspended in 2.00 ml of toluene. The reaction mixture was stirred at 100 °C for 24 h, after which it was allowed to cool to room temperature. The reaction was quenched by addition of 10 ml of water. The product was extracted with 2 × 10 ml of EtOAc and 1 × 10 ml of dichloromethane. The combined organic phases were dried over magnesium sulfate and the solvent was removed under reduced pressure. The product was purified by flash column chromatography.

4.1.2. 2-(2-Methoxybenzyl)-3*H*-benzo[*f*]chromen-3-one (11)

54.3 mg, 15% (Scale: 1.21 mmol). R_f (*n*-Hex/EtOAc 20:1) = 0.10. mp: 142–145 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.84 (s, 3H, OCH_3), 4.00 (s, 2H, CH_2), 6.92–6.96 (m, 1H, H_{ar}), 6.97–7.01 (m, 1H, H_{ar}), 7.29–7.36 (m, 2H, H_{ar}), 7.45 (d, ^3J = 9.0 Hz, 1H, H_{ar}), 7.52–7.54 (m, 1H, H_{ar}), 7.58–7.62 (m, 1H, H_{ar}), 7.86–7.91 (m, 2H, H_{ar}), 8.02 (d, ^3J = 8.3 Hz, 1H, H_{ar}), 8.08 (s, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 31.3 (s, CH_2), 55.4 (p, OCH_3), 110.6 (t, $C_{ar}H$), 113.6 (q, C_{ar}–CH), 116.8 (t, $C_{ar}H$), 120.8 (t, $C_{ar}H$), 121.4 (t, $C_{ar}H$), 125.7 (t, $C_{ar}H$), 126.0 (q, C–CH), 127.7 (q, C_{ar}–CH_2), 127.8 (t, $C_{ar}H$), 127.9 (q, C_{ar}), 128.4 (t, $C_{ar}H$), 128.9 (t, $C_{ar}H$), 130.2 (q, C_{ar}), 131.3 (t, $C_{ar}H$), 131.6 (t, CH), 134.7 (t, $C_{ar}H$), 152.3 (q, C_{ar}–OCO), 157.6 (q, C_{ar}–OCH_3), 161.9 (q, C=O). IR (KBr): 1713 (s, *v* C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 316 (100, M^+), 304 (31), 285 (15, $(M-CH_3)^+$). HR-EIMS ($C_{10}H_8O_2$): calcd 316.1099, found 316.1102. $C_{21}H_{16}O_3$ (316 g/mol): Calcd C, 79.73*; H, 5.10. Found: C, 78.45*; H, 5.14 (*deviation > 0.4%).

4.1.3. 3-Benzyl-6-iodochromen-2-one (16)

53.1 mg, 17% (Scale: 0.876 mmol). R_f (*c*-Hex/EtOAc 5:1) = 0.40. mp: 147–150 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.86 (br s, 2H, CH_2), 7.04 (d, 1H, ^3J = 8.6 Hz, C8–H), 7.11 (br s, 1H, C4–H), 7.24–7.35 (m, 8H, H_{ar}), 7.65 (d, 1H, ^4J = 2.0 Hz, C5–H), 7.68 (dd, 1H, ^4J = 2.0 Hz, ^3J = 8.7 Hz, C7–H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 36.6 (s, CH_2), 87.1 (q, C-6), 118.4 (t, C-7), 121.5 (q, C-4a), 127.1 (t, $C_{ar}H$), 128.9 (+, 2 × $C_{ar}H$), 129.5 (t, 2 × $C_{ar}H$), 130.7 (q, C-3), 135.8 (t, C-5), 137.2 (q, C_{ar}), 137.7 (t, C-7), 139.3 (t, C-4), 152.6 (q, C-8a), 161.0 (q, C-2). IR (KBr): 1726 (s, *v* C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 362 (100,

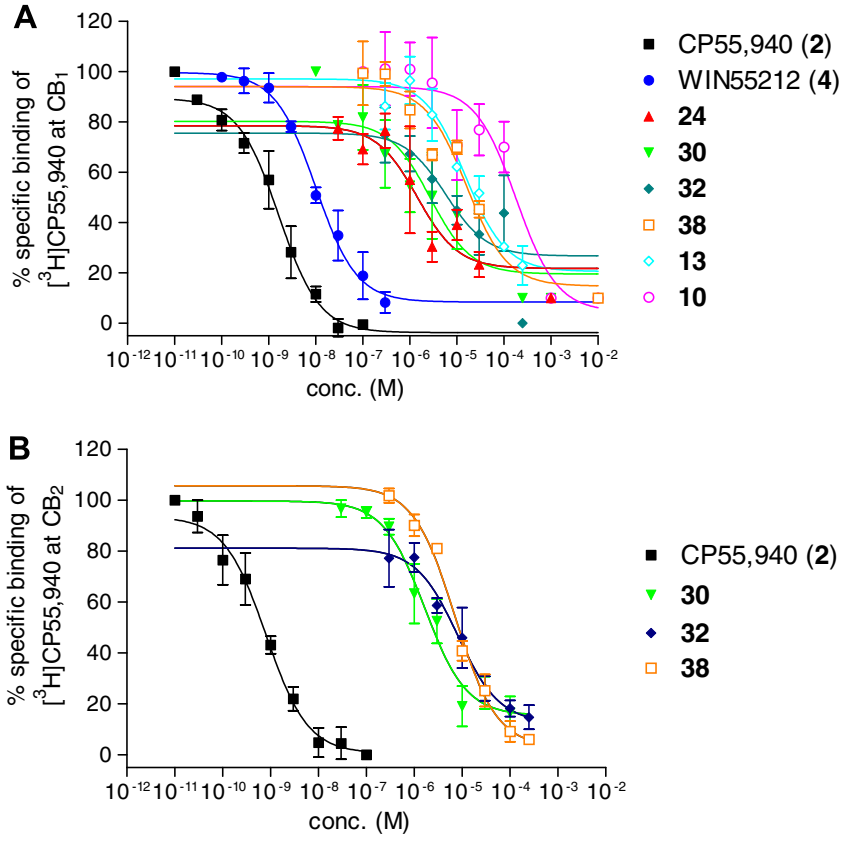

Figure 3. Competition curves of selected compounds versus 0.5 nM [^{3}H]CP55,940 at membrane preparations of rat brain cortical membranes (**A**), and at human embryonic kidney (HEK293) cells expressing the human CB_2 receptor (**B**). Data points represent means ± SEM of three independent experiments performed in triplicate.

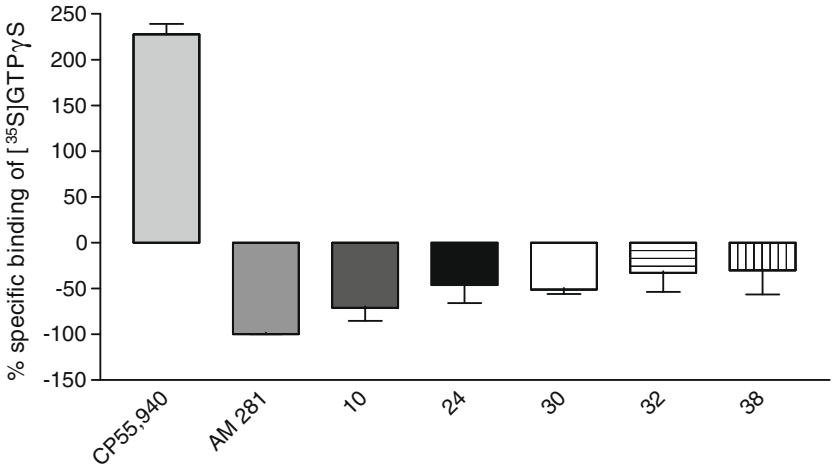

Figure 4. [^{35}S]GTPγS binding assay at human CB_1 cannabinoid receptors expressed in HEK293 cells. Data are expressed as means ± SEM of at least three separate experiments performed in triplicate. Obtained data were normalized with respect to the maximal effect of AM281 (set at −100%).

M^+), 333 (16). HR-EIMS ($C_{17}H_{14}O_3$): calcd 361.9804, found 361.9804. $C_{17}H_{14}O_3$ (266 g/mol): Calcd C, 53.06; H, 3.06. Found: C, 53.46; H, 3.16.

4.1.4. 8-Bromo-6-chloro-3-methylchromen-2-one (18)

28.1 mg, 17% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 5:1) = 0.29. mp: 147–150 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.24 (d, 3H, ^4J = 1.3 Hz, CH_3), 7.34 (d, 1H, ^4J = 2.3 Hz, C5-H), 7.40 (qt, 1H, ^4J = 1.4 Hz, C4-H), 7.66 (d, 1H, ^4J = 2.4 Hz, C7-H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.2 (p, *C*H$_3$), 110.7 (q, C-8), 121.3 (q, C-4a), 125.5 (t, C-5), 128.2 (q, C-3), 129.7 (q, C-6), 133.4 (t, C-4), 137.6 (t, C-7), 148.7 (q, C-8a), 160.6 (q, C-2). IR (KBr): 1718 (s, *v* C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 272/274 (100/98, M^+), 243/245 (40/38, M^+−CO). HR-EIMS ($C_{10}H_6BrClO_2$): calcd 271.9240, found 271.9246. $C_{10}H_6BrClO_2$ (272 g/mol): Calcd C, 43.91*; H, 2.21. Found: C, 44.55*; H, 2.59 (*deviation > 0.4%).

4.1.5. 3-Benzyl-6-nitrochromen-2-one (19)

23.8 mg, 5% (Scale: 1.64 mmol). R_f (cHex/EtOAc 5:1) = 0.35. mp: 167–171 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.92 (s, 2H, CH_2), 7.27–7.45 (m, 7H, C_{ar}H, C4–H), 8.29 (d, 1H, ^4J = 2.3 Hz, C5–H), 8.32 (dd, 1H, ^4J = 2.6 Hz, ^3J = 8.9 Hz, C7–H). ^{13}C NMR (75 MHz, $CDCl_3$): δ = 36.7 (s, *C*H$_2$), 117.6 (t, C-8), 119.5 (q, C-4a), 123.3 (t, C-5), 125.6 (t, C-7), 127.3 (t, C_{ar}H), 129.0 (t, 2 × C_{ar}H), 129.5 (t, 2 × C_{ar}H), 132.2 (q, C-3), 136.6 (q, C_{ar}), 137.8 (t, C-4), 144.0 (q, C-6), 156.5 (q, C-8a), 160.1 (q, C-2). IR (KBr): 1735 (s, *v* C=O), 1529 (s, v_{as} NO_2), 1342 (s, v_{sym} NO_2) cm^{-1}. MS (EI): *m*/*z* (%) = 281 (100, M^+), 252 (16), 178 (12). HR-EIMS ($C_{16}H_{11}NO_4$): calcd 281.0688, found 281.0689. $C_{16}H_{11}NO_4$ (281 g/mol): Calcd C, 68.32; H, 3.94; N, 4.98. Found: C, 68.54; H, 4.11; N, 4.70.

4.1.6. 5-Methoxy-7-methyl-3-(2-methylbenzyl)-2*H*-chromen-2-one (25)

71.8 mg, 41% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 30:1) = 0.04. mp: 132–135 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.28 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 3.86 (d, ^4J = 1.1 Hz, 2H, CH_2), 6.47 (s, 1H, H_{ar}), 6.73 (s, 1H, H_{ar}), 7.19–7.22 (m, 4H, H_{ar}), 7.71 (d, ^4J = 1.1 Hz, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 17.0 19.5 (p, *C*H$_3$), 22.3 (p, *C*H$_3$), 33.9 (s, *C*H$_2$), 55.7 (p, OCH$_3$), 106.2 (t, C_{ar}H), 107.7 (q, C_{ar}–CH), 109.0 (t, C_{ar}H), 125.0 (q, *C*–CH$_2$), 126.2 (t, C_{ar}H), 126.9 (t, C_{ar}H), 130.2 (t, C_{ar}H), 130.5 (t, C_{ar}H), 134.0 (t, CH), 136.1 (q, C_{ar}–CH$_2$), 136.8 (q, C_{ar}–CH$_3$), 142.5 (q, C_{ar}–CH$_3$), 154.0 (q, C_{ar}–O–CO), 155.3 (q, C_{ar}–OCH$_3$), 162.1 (q, *C*=O). IR (KBr): 1714 (s, *v* C=O) cm^{-1} MS (EI): *m*/*z* (%) = 294 (100, M^+), 276 (18), 203 (31, $(C_{12}H_{11}O_3)^+$). HR-EIMS ($C_{10}H_8O_2$): calcd 294.1256, found 294.1258. $C_{19}H_{18}O_3$ (294 g/mol): Calcd C, 77.53*; H, 6.16. Found C, 76.89*; H, 6.17 (*deviation > 0.4%).

4.1.7. 5-Methoxy-7-methyl-3-(4-methylbenzyl)-2*H*-chromen-2-one (26)

70.2 mg, 35% (Scale:0.602 mmol). R_f (*c*-Hex/EtOAc 20:1) = 0.12. mp: 120–123 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.33 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.82 (s, 2H, CH_2), 3.86 (s, 3H, OCH_3), 6.48 (s, 1H, H_{ar}), 6.71 (s, 1H, H_{ar}), 7.13 (d, ^3J = 7.9 Hz, 2H, H_{ar}), 7.20 (d,

Table 2
Results from [^{35}S]GTPγS binding studies of selected compounds at native rat CB_1 receptors and human CB_1 receptors expressed in HEK cells in comparison with K_i values from competition binding studies

Compd.	CB_1 rat brain cortical membranes versus [^{3}H]CP55,940 K_i (μM)	Effect on [^{35}S]GTPγS binding at rat brain cortical membranes in relation to the full agonist CP55,940 set at 100% (±SEM)	Effect on [^{35}S]GTPγS binding at membranes from HEK cells expressing the human CB_1 receptor in relation to the full agonist CP55,940 set at 100% (±SEM)
CP55,940	0.00124	100 ± 0[a]	100 ± 0[a]
AM281	0.012[64]	2 ± 11[b]	−19 ± 9[c]
10	4.19	15 ± 3	−21 ± 4
24	3.46	3 ± 9	−8 ± 7
30	0.738	−2 ± 7	−16 ± 1
32	6.14	13 ± 4	−10 ± 7
38	2.76	19 ± 12	−9 ± 8

[a] The full agonist CP55,940 led to a maximal stimulation of 169 ± 6% (n = 3) at native rat CB_1 receptors in brain cortical membranes, and 228 ± 12% at human CB_1 receptors expressed in HEK cells (n = 2) over basal (=100%).
[b] The full inverse agonist AM281 had no apparent effect on [^{35}S]GTPγS binding in rat cortical membranes: 102 ± 7% (n = 3), basal (=100%).
[c] The full inverse agonist AM281 reduced [^{35}S]GTPγS binding in CB_1-transfected HEK293 cells from 100% basal to 73 ± 13% (n = 2).

^3J = 7.9 Hz,, 2H, H_{ar}), 7.72 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 21.0 (p, CH_3), 22.3 (p, CH_3), 36.3 (s, CH_2), 55.8 (p, OCH_3), 106.2 (t, $C_{ar}H$), 107.8 (q, C_{ar}), 109.1 (t, $C_{ar}H$), 126.1 (q, C–H), 129.0 (t, 2 × $C_{ar}H$), 129.3 (t, 2 × $C_{ar}H$), 134.4 (t, CH), 135.3 (q, C_{ar}–CH_2), 136.0 (q, C_{ar}–CH_3), 142.4 (q, C_{ar}–CH_3), 154.2 (q, C_{ar}–O–CO), 155.4 (q, C_{ar}–OCH_3), 162.0 (q, C=O). IR (KBr): 1711 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 294 (100, M^+), 279 (24, $(M-CH_3)^+$), 265 (11). HR-EIMS ($C_{10}H_8O_2$): calcd 294.1256, found 294.1254. $C_{19}H_{18}O_3$ (294 g/mol): Calcd C, 77.53; H, 6.16. Found: C, 77.51; H, 6.12.

4.1.8. 3-(2-Chlorobenzyl)-5-methoxy-7-methyl-2*H*-chromen-2-one (27)

39.7 mg, 21% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 40:1) = 0.03. mp: 138–144 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.40 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 3.99 (s, 2H, CH_2), 6.48 (s, 1H, H_{ar}),6.72 (s, 1H, H_{ar}), 7.19–7.25 (m, 2H, H_{ar}), 7.36–7.41 (m, 2H, H_{ar}), 7.64 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.3 (p, CH_3), 34.2 (s, CH_2), 55.8 (p, OCH_3), 106.2 (t, $C_{ar}H$), 107.7 (q, C_{ar}–CH), 109.0 (t, $C_{ar}H$), 123.8 (q, C–CH_2), 127.0 (t, $C_{ar}H$), 128.2 (t, $C_{ar}H$), 129.6 (t, $C_{ar}H$), 131.6 (t, $C_{ar}H$), 134.4 (q, C_{ar}–Cl), 134.8 (t, CH), 135.8 (q, C_{ar}–CH_2), 142.7 (q, C_{ar}–CH_3), 154.1 (q, C_{ar}–O–CO), 155.4 (q, C_{ar}–OCH_3), 161.9 (q, C=O). IR (KBr): 1707 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 314 (8, M^+), 279 (100, $(M-Cl)^+$). HR-EIMS ($C_{10}H_8O_2$): calcd 314.0710, found 314.0706. $C_{18}H_{15}ClO_3$ (314 g/mol): Calcd C, 68.68; H, 4.80. Found: C, 68.43; H, 4.85.

4.1.9. 3-(4-Chlorobenzyl)-5-methoxy-7-methyl-2*H*-chromen-2-one (28)

50.3 mg, 26% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 20:1) = 0.04. mp: 143–147 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.40 (s, 3H, CH_3), 3.82 (s, 2H, CH_2), 3.86 (s, 3H, OCH_3), 6.49 (s, 1H, H_{ar}), 6.70 (s, 1H, H_{ar}), 7.22–7.29 (m, 4H, H_{ar}), 7.71 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.3 (p, CH_3), 36.2 (s, CH_2), 55.8 (p, OCH_3), 106.2 (t, $C_{ar}H$), 107.6 (q, C_{ar}), 109.1 (t, $C_{ar}H$), 125.2 (q, C–CH), 128.7 (t, 2 × $C_{ar}H$), 130.5 (t, 2 × $C_{ar}H$), 132.3 (q, C_{ar}–CH_2), 134.7 (t, CH), 136.8 (q, C_{ar}–CH_3), 142.8 (q, C_{ar}–CH_3), 154.2 (q, C_{ar}–O–CO), 155.4 (q, C_{ar}–OCl), 161.8 (q, C=O). IR (KBr): 1711 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 314 (100, M^+), 279 (80, $(M-Cl)^+$), 125 (18). HR-EIMS ($C_{10}H_8O_2$): calcd 314.0710, found 314.0707. $C_{18}H_{15}ClO_3$ (314 g/mol): Calcd C, 68.68; H, 4.80. Found: C, 68.58; H, 4.83.

4.1.10. 3-(2-Hydroxybenzyl)-5-methoxy-7-methyl-2*H*-chromen-2-one (29)

187 mg, 42% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 20:1) = 0.18. mp: 173–177 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.41 (s, 3H, CH_3), 3.83 (s, 2H, CH_2), 3.92 (s, 3H, OCH_3), 6.53 (s, 1H, H_{ar}), 6.72 (s, 1H, H_{ar}), 6.87 (ddd, ^3J = 7.5 Hz, ^4J = 1.2 Hz, 1H, H_{ar}), 6.96 (dd, ^3J = 8.1 Hz, ^4J = 1.2 Hz, 1H, H_{ar}), 7.14 (ddd, ^3J = 8.1 Hz, ^4J = 1.7 Hz, 1H, H_{ar}), 7.22 (dd, ^3J = 7.5 Hz, ^4J = 1.7 Hz, 1H, H_{ar}), 8.08 (s, 1H, C*H*), 8.19 (s, 1H, O*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.4 (p, CH_3), 32.3 (s, CH_2), 55.9 (p, OCH_3), 106.7 (t, $C_{ar}H$), 108.0 (q, C_{ar}), 109.1 (t, $C_{ar}H$), 118.2 (t, $C_{ar}H$), 120.8 (t, $C_{ar}H$), 125.0 (q, C–CH_2), 125.3 (q, C_{ar}–CH_2), 128.6 (t, $C_{ar}H$), 130.5 (t, $C_{ar}H$), 136.0 (t, CH), 143.4 (q, C_{ar}–CH_3), 153.9 (q, C_{ar}–O–CO), 154.7 (q, C_{ar}–OH), 155.4 (q, C_{ar}–OCH_3), 165.0 (q, C=O). IR (KBr): 1725 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 296 (100, M^+), 190 (35), 159 (19). HR-EIMS ($C_{10}H_8O_2$): calcd 296.1049, found 296.1046. $C_{18}H_{16}O_4$ (296 g/mol): Calcd C, 72.96*; H, 5.44. Found: C, 72.49*; H, 5.48 (*deviation > 0.4%).

4.1.11. 5-Methoxy-3-(3-methoxybenzyl)-7-methyl-2*H*-chromen-2-one (31)

84.1 mg, 45% (Scale: 0.602 mmol). R_f (*c*-Hex/EtOAc 20:1) = 0.04. mp: 108–112 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.39 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 3.84 (s, 2H, CH_2), 3.85 (s, 3H, OCH_3), 6.48 (s, 1H, H_{ar}), 6.70 (s, 1H, H_{ar}), 6.79 (dd, ^3J = 8.2 Hz, ^4J = 2.5 Hz, 1H, H_{ar}), 6.85–6.87 (m, 1H, H_{ar}), 6.89 (d, ^3J = 7.6 Hz, 1H, H_{ar}), 7.22–7.26 (m, 1H, H_{ar}), 7.72 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.9 (p, CH_3), 36.7 (s, CH_2), 55.1 (p, OCH_3), 55.7 (p, OCH_3), 106.2 (t, $C_{ar}H$), 107.7 (q, C_{ar}––CH), 109.0 (t, $C_{ar}H$), 111.9 (t, $C_{ar}H$), 114.8 (t, $C_{ar}H$), 121.5 (t, $C_{ar}H$), 125.6 (q, C–CH_2), 129.5 (t, $C_{ar}H$), 134.7 (t, CH), 139.9 (q, C_{ar}–CH_2), 142.5 (q, C_{ar}–CH_3), 154.1 (q, C_{ar}–OCH_3), 155.3 (q, C_{ar}–OCH_3), 159.7 (q, C_{ar}–O–CO), 162.0 (q, C=O). IR (KBr): 1701 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 310 (100, M^+), 279 (10, $(M-OCH_3)^+$), 203 (13). HR-EIMS ($C_{10}H_8O_2$): calcd 310.1205, found 310.1208. $C_{19}H_{18}O_4$ (310 g/mol): Calcd C, 73.53*; H, 5.85. Found: C, 73.03*; H, 5.93 (*deviation > 0.4%).

4.1.12. 5-Methoxy-3-(2-methoxybenzyl)-2*H*-chromen-2-one (33)

57.8 mg, 30% (Scale: 0.789 mmol). R_f (*c*-Hex/EtOAc 40:1) = 0.05. mp: 147–150 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 3.81 (s, 3H, OCH_3), 3.85 (s, 3H, OCH_3), 3.88 (s, 2H, CH_2), 6.65 (d, ^3J = 8.1 Hz, 1H, H_{ar}), 6.88–6.91 (m, 2H, H_{ar}), 6.92–6.96 (m, 1H, H_{ar}), 7.24–7.28 (m, 2H, H_{ar}), 7.32–7.36 (m, 1H, H_{ar}), 7.69 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 31.0 (s, CH_2), 55.3 (p, OCH_3), 55.8 (p, OCH_3), 104.8 (t, $C_{ar}H$), 108.8 (t, $C_{ar}H$), 110.2 (q, C_{ar}–CH), 110.5 (t, $C_{ar}H$), 120.6 (t, $C_{ar}H$), 126.3 (q, C_{ar}–CH_2), 126.5 (q, C_{ar}–CH_2), 128.0 (t, $C_{ar}H$), 130.8 (t, $C_{ar}H$), 131.0 (t, $C_{ar}H$), 134.0 (t, CH), 154.0 (q, C_{ar}–O–CO), 155.6 (q, C_{ar}–OCH_3), 157.6 (q, C_{ar}–OCH_3), 161.9 (q, C=O). IR (KBr): 1723 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 296 (15, M^+), 189 (11). HR-EIMS ($C_{10}H_8O_2$): calcd 296.1049, found 296.1050. $C_{18}H_{16}O_4$ (296 g/mol): Calcd C, 72.96; H, 5.44. Found: C, 72.99; H, 5.54.

4.1.13. 3-(2,4-Dimethoxybenzyl)-5-methoxy-7-methyl-2*H*-chromen-2-one (34)

27.3 mg, 21% (Scale: 0.602 mmol) R_f (*c*-Hex/EtOAc 5:1) = 0.12. mp: 161–165 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.39 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 3.79 (s, 2H, CH_2), 3.81 (s, 3H, OCH_3), 3.84 (s, 3H, OCH_3), 6.45–6.48 (m, 3H, H_{ar}), 6.71 (s, 1H, H_{ar}), 7.16 (d, ^3J = 8.2 Hz, 1H, H_{ar}), 7.61 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 22.3 (p, CH_3), 30.3 (s, CH_2), 55.3 (p, OCH_3), 55.4 (p, OCH_3), 55.8 (p, OCH_3), 98.7 (t, $C_{ar}H$), 104.1 (t, $C_{ar}H$), 106.1 (t, $C_{ar}H$), 108.0 (q, C_{ar}–CH), 109.0 (t, $C_{ar}H$), 118.9 (q, C_{ar}–CH_2), 125.6 (q, C–CH_2), 131.3 (t, $C_{ar}H$), 133.9 (t, CH), 142.0 (q, C_{ar}–CH_3), 154.0 (q, C_{ar}–O–CO), 155.3 (q, C_{ar}–OCH_3), 158.5 (q, C_{ar}–OCH_3), 159.8 (q, C_{ar}–OCH_3), 162.2 (q, C=O). IR (KBr): 1724 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 340 (100, M^+), 325 (17, $(M-CH_3)^+$), 309 (18, $(M-OCH_3)^+$). HR-EIMS ($C_{10}H_8O_2$): calcd 340.1311, found 340.1314. $C_{20}H_{20}O_5$ (340 g/mol): Calcd C, 70.57; H, 5.92. Found: C, 70.38; H, 6.05.

4.1.14. 5-Methoxy-3-(2-methoxybenzyl)-8-methyl-2*H*-chromen-2-one (35)

50.4 mg, 26% (Scale: 1.50 mmol). R_f (*c*-Hex/EtOAc 10:1) = 0.11. mp: 124–126 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.35 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.89 (s, 2H, CH_2), 6.56 (d, ^3J = 8.3 Hz, 1H, H_{ar}), 6.90 (d, ^3J = 8.3 Hz, 1H, H_{ar}), 6.92–6.96 (m, 1H, H_{ar}), 7.18 (d, ^3J = 8.4 Hz, 1H, H_{ar}), 7.23–7.28 (m, 2H, H_{ar}), 7.70 (s, 1H, C*H*). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.8 (p, CH_3), 30.9 (s, CH_2), 55.3 (p, OCH_3), 55.7 (p, OCH_3), 104.3 (t, $C_{ar}H$), 110.0 (q, C_{ar}–CH), 110.5 (t, $C_{ar}H$), 117.5 (q, C_{ar}–CH_3), 120.6 (t, $C_{ar}H$), 126.0 (q, C_{ar}–CH_2), 126.4 (q, C–CH), 128.0 (t, $C_{ar}H$), 131.0 (t, $C_{ar}H$), 131.7 (t, $C_{ar}H$), 134.4 (t, CH), 152.0 (q, C_{ar}–O–CO), 153.8 (q, C_{ar}–OCH_3), 157.6 (q, C_{ar}–OCH_3), 162.0 (q, C=O). IR (KBr): 1711 (s, ν C=O) cm^{-1}. MS (EI): *m*/*z* (%) = 310 (100, M^+), 279 (10, $(M-OCH_3)^+$), 203 (13). HR-EIMS ($C_{10}H_8O_2$): calcd 310.1205, found 310.1208. $C_{19}H_{18}O_4$ (310 g/mol): Calcd C, 73.53; H, 5.85. Found: C, 73.13; H, 5.81.

4.1.15. 5-Isopropyl-3-(2-methoxybenzyl)-7-methyl-2*H*-chromen-2-one (36)

130 mg, 55% (Scale: 0.729 mmol). R_f (*c*-Hex/EtOAc 80:1) = 0.15. mp: 107–111 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.21 (d, ^3J = 6.8 Hz,

6 H, 2 × ^{i}Pr–CH_3), 2.41 (s, 3H, CH_3), 3.17 (sept, ^3J = 6.8 Hz, 1H, ^{i}Pr–CH), 3.81 (s, 3H, OCH_3), 3.91 (s, 2H, CH_2), 6.89–6.97 (m, 2H, H_{ar}), 7.02–7.04 (m, 1H, H_{ar}), 7.23–7.33 (m, 3H, H_{ar}), 7.64 (s, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 15.4 (p, CH_3), 23.4 (t, ^{i}Pr–CH), 28.4 (p, 2 × ^{i}Pr–CH_3), 31.4 (s, CH_2), 55.2 (p, OCH_3), 110.3 (t, C_{ar}H), 116.9 (q, C_{ar}–CH), 119.9 (t, C_{ar}H), 120.7 (t, C_{ar}H), 123.2 (q, C_{ar}–CH_2), 126.2 (q, C–CH), 126.9 (q, C_{ar}–CH_3), 128.2 (t, C_{ar}H), 131.2 (t, C_{ar}H), 131.7 (t, C_{ar}H), 135.9 (t, CH), 143.2 (q, C_{ar}-^{i}Pr), 151.9 (q, C_{ar}–O–CO), 157.5 (q, C_{ar}–OCH_3), 161.7 (q, C=O). IR (KBr): 1718 (s, ν C=O) cm^{-1}. MS (EI): m/z (%) = 322 (6, M^+), 310 (100), 91 (20). HR-EIMS ($C_{10}H_8O_2$): calcd 322.1569, found 322.1570. $C_{21}H_{22}O_3$ (322 g/mol): Calcd C, 78.23; H, 6.88. Found: C, 78.13; H, 6.89.

4.2. Biological assays

4.2.1. Materials

GTPγS, EDTA, GDP, AM281, and cell culture materials were obtained from Sigma–Aldrich (Steinheim, Germany). Tris and BSA were from Carl Roth GmbH (Karlsruhe, Germany), HCl and DTT from Applichem (Darmstadt, Germany), $MgCl_2$ and sucrose from Fluka (Buchs, Switzerland), NaCl from KMF Optichem (Lohmar, Germany), DMSO from Riedel-de Haën (Seelze, Germany), and CP55,940 and WIN55,212-2 from Tocris (Ellisville, MO, USA). [^{3}H]CP55,940 was obtained from PerkinElmer Life Sciences, Rodgau-Jügesheim, Germany (158 Ci/mmol; 5.85 TBq/mmol).

4.2.2. Membrane preparations

Frozen rat brains were purchased from Pel Freez®, Rogers, AR, USA. Rat brain cortical membrane preparations were obtained as previously described[65] with minor modifications. Brains were thawed and then kept on ice while the cortex was dissected. The cortex was subsequently homogenized in ice-cold 0.32 M sucrose solution using an Ultra-Turrax® T25 basic homogenizer, IKA Labortechnik for 10 s at setting 3. The suspension was centrifuged at 1000*g* for 5 min (4 °C), the pellet was discarded and the supernatant was subsequently centrifuged at 37,000*g* for 60 min (4 °C). The supernatant was discarded and the pellet was resuspended in ice-cold water, homogenized with the Ultra Turrax® and recentrifuged at 37,000*g* for 60 min (4 °C). The resulting membrane pellet was resuspended in Tris–HCl buffer, 50 mM, pH 7.4, and stored at −80 °C. Protein concentration was determined by the method of Lowry.[66] Membrane preparations of human embryonic kidney (HEK) 293 EBNA cells expressing the human CB_1 or the human CB_2 receptor, respectively, were obtained from Perkin Elmer, Boston, USA.

4.2.3. Radioligand binding assays

Competition experiments were performed versus the cannabinoid receptor agonist radioligand [^{3}H]CP55,940 as described before[67] with some minor modifications. Stock solutions of compounds were prepared in DMSO. The final DMSO concentration in the assays was 2.5%. Inhibition curves were determined using seven different concentrations of test compound spanning three orders of magnitude. Experiments were performed using 0.5 nM [^{3}H]CP55,940 at rt in 96-well microtest plates (0.5 ml, Costar® Corning Inc., USA). Each well contained 15 μl of test compound dissolved in DMSO, 465 μl of buffer solution (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.1% BSA, pH 7.4), 60 μl radioligand solution and 60 μl of rat brain cortical membrane suspension, or commercially available membrane preparation containing the human CB_2 receptor (50 μg of protein per well) to give a final volume of 600 μl.

Total binding was determined in the presence of 2.5% DMSO and nonspecific binding in the presence of 10 μM of unlabeled CP55,940. In binding studies at rat brain cortical membranes at least three separate experiments were performed each in triplicates. Binding studies at human CB_2 receptors were performed in duplicates and at least three separate experiments were performed unless otherwise noted. The incubation was started by adding the protein, and terminated after 2 h by rapid filtration using a Brandel 96-channel cell harvester (Brandel, Gaithersburgh, Maryland, USA) through GF/C glass fiber filters (Perkin Elmer, Boston, USA). Filters were rinsed three times with 2 ml of ice-cold washing buffer each, containing 50 mM Tris–HCl, pH 7.4, and 0.1% bovine serum albumin (BSA). Radioactivity of filter plates, which had been dried for 90 min at 50 °C, was measured in a Topcount® microplate scintillation and luminescence counter after 10 h of preincubation with 0.05 ml of Microscint™20 scintillation cocktail (Perkin Elmer, Boston, USA).

4.2.4. [^{35}S]GTPγS binding assays

Assays were performed essentially as previously described.[68,69] Membrane preparations of rat brain cortical membranes, or commercially available membrane preparations expressing the human CB_1 receptor, respectively, 5 μg per tube, were incubated with 0.1 nM [^{35}S]GTPγS (46.3 TBq/mmol, PerkinElmer Life Sciences, Roddgau-Jügesheim, Germany) in a total volume of 200 μl of Tris–HCl buffer, 50 mM, pH 7.4, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 30 μM GDP, 100 mM NaCl, 0.5% BSA, and test compound dissolved in DMSO. The final DMSO concentration in the assay was 1%. Nonspecific binding was determined in the presence of 30 μM of unlabeled GTPγS. Incubations were terminated after 60 min of incubation at rt by the addition of 2 ml of ice-cold buffer containing 50 mM Tris–HCl, pH 7.4, and 5 mM $MgCl_2$, and rapid filtration through GF/B glass fiber filter (Schleicher and Schuell, Germany) on a Brandel® 48-channel harvester, followed by two washing steps with ice-cold buffer, 2 ml each. Radioactivity on the wet filters was measured by liquid scintillation counting after punching out the filters and incubating them for 9 h in 2.5 ml of scintillation cocktail (Ready Safe™, Beckman Coulter, USA).

4.2.5. Data analysis

Data were analyzed using Graph Pad PRISM Version 3.0 (San Diego, CA, USA). For the calculation of K_i values by nonlinear regression analysis, the Cheng Prusoff equation and K_D values of 0.58 nM (rCB_1) and 0.7 (hCB_2) for [^{3}H]CP55,940 were used.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.02.027.

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