7-Alkyl-3-benzylcoumarins: A Versatile Scaffold for the Development of Potent and Selective Cannabinoid Receptor Agonists and Antagonists

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ⓢ *Supporting Information*
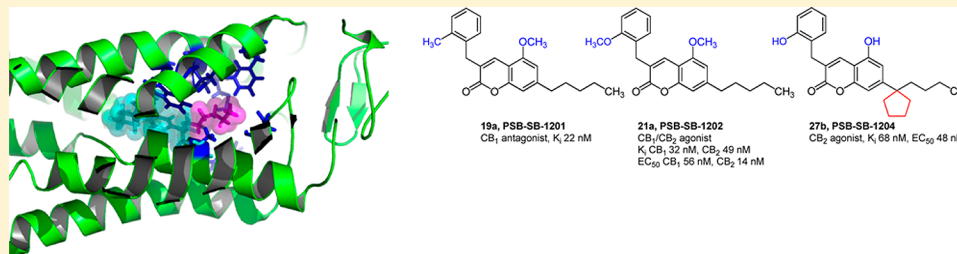
ABSTRACT: A series of 7-alkyl-3-benzylcoumarins was designed, synthesized, and tested at cannabinoid CB_1 and CB_2 receptors in radioligand binding and cAMP accumulation studies. 7-Alkyl-3-benzylcoumarins were found to constitute a versatile scaffold for obtaining potent CB receptor ligands with high potency at either CB_1 or CB_2 and a broad spectrum of efficacies. Fine-tuning of compound properties was achieved by small modifications of the substitution pattern. The most potent compounds of the present series include 5-methoxy-3-(2-methylbenzyl)-7-pentyl-2H-chromen-2-one (**19a**, PSB-SB-1201), a selective CB_1 antagonist (K_i CB_1 0.022 μM), 5-methoxy-3-(2-methoxybenzyl)-7-pentyl-2H-chromen-2-one (**21a**, PSB-SB-1202), a dual CB_1/CB_2 agonist (CB_1 K_i 0.032 μM, EC_{50} 0.056 μM; CB_2 K_i 0.049 μM, EC_{50} 0.014 μM), 5-hydroxy-3-(2-hydroxybenzyl)-7-(2-methyloct-2-yl)-2H-chromen-2-one (**25b**, PSB-SB-1203), a dual CB_1/CB_2 ligand that blocks CB_1 but activates CB_2 receptors (CB_1 K_i 0.244 μM; CB_2 K_i 0.210 μM, EC_{50} 0.054 μM), and 7-(1-butylcyclopentyl)-5-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (**27b**, PSB-SB-1204), a selective CB_2 receptor agonist (CB_1 K_i 1.59 μM; CB_2 K_i 0.068 μM, EC_{50} 0.048 μM).

■ INTRODUCTION

Cannabinoid (CB) receptors are rhodopsin-like G protein-coupled receptors (GPCRs) activated by lipid mediators, such as anandamide (**1**) and 2-arachidonoylglycerol (**2**) (Figure 1).[1] Even before the physiological agonists were known, CB receptors have been described to be activated by terpenoid plant constituents, e.g., by Δ^9-tetrahydrocannabinol (Δ^9-THC, **3**) from *Cannabis sativa*, and were therefore named accordingly.[2] Synthetic CB receptor agonists such as CP55,940 (**4**) and the indole derivative WIN55,212-2 (**5**) have been developed. Two distinct receptor subtypes have been described, CB_1 and CB_2, which are coupled to $G_{i/o}$ proteins mediating inhibition of adenylate cyclase.[3,4] The CB_1 receptor is highly expressed in the brain, e.g., cerebellum, hippocampus, cortex, and striatum, but also in peripheral organs, such as lung, liver, kidneys, and cardiomyocytes.[5−7] In contrast, the CB_2 receptor is mainly expressed in organs and cells associated with the immune system, including T-lymphocytes, macrophages, B-cells, hematopoietic cells, spleen, thymus, tonsils, and microglial cells, but also in bone cells and cardiomyocytes.[8,9] The CB_1 receptor subtype is known to play an important role in analgesia, memory impairment, regulation of appetite, spasmolysis, inhibition of nausea, and lipolysis.[2,10]

Synthetic Δ^9-THC (dronabinol, Marinol) is used for the therapy of AIDS-related anorexia and the treatment of cancer patients to enhance their appetite and to counteract vomiting.[11,12] Furthermore, it is used as an analgesic and spasmolytic agent, e.g., by patients suffering from multiple sclerosis.[13,14] The selective CB_1 inverse agonist rimonabant (**6**), which is structurally related to AM281 (**7**), another CB_1 selective inverse agonist, was marketed as an antiobesity drug
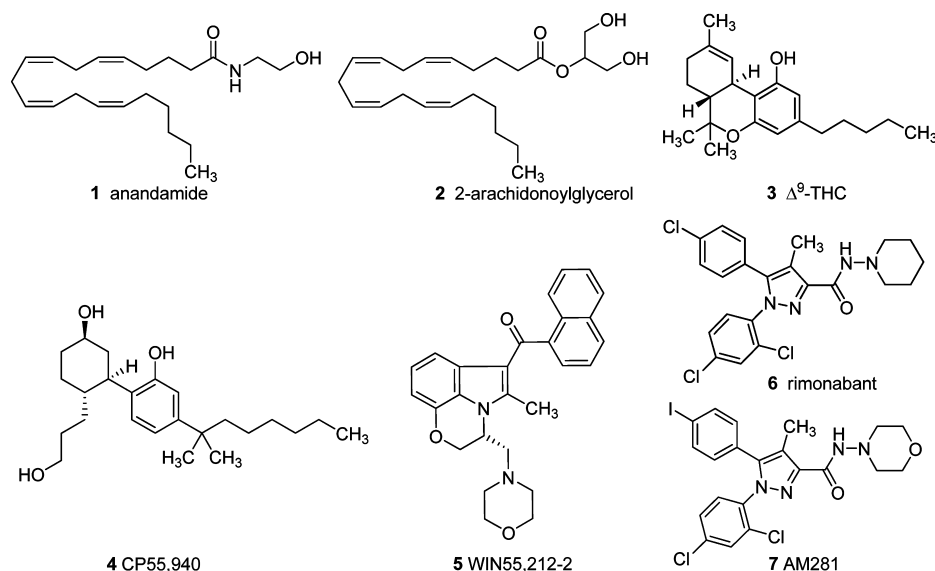
Figure 1. Structures of cannabinoid receptor agonists **1**–**5** and antagonists **6** and **7**.

but has been withdrawn due to side effects (depression, increased rate of suicide), which may have been due to its penetration into the central nervous system (CNS) and/or off-target effects.[2,12] Several studies have found that CB receptor ligands may exhibit neuroprotective properties and thus could be useful for the treatment of neurodegenerative diseases, including Alzheimer's and Parkinson's disease.[2,15] Selective agonists for CB$_2$ receptors exhibit antiinflammatory and analgesic properties in animal models; furthermore, they may have cardioprotective effects and be effective in the treatment of osteoporosis and arteriosclerosis and for the treatment of cancer, e.g., gliomas.[16−18]

Coumarins (chromen-2-ones, benzopyran-2-ones) are known to possess a variety of pharmacological activities, depending on their substitution pattern, and thus can be characterized as "privileged structures" in medicinal chemistry. For example, coumarin derivatives with anticoagulant, anti-tumor, anti-inflammatory, and antiviral effects have been described.[19,20] Coumarin derivatives, such as warfarin and phenprocoumon have been widely used therapeutically for many decades as vitamin K antagonists exhibiting anticoagulant properties. The lactone ring of coumarin derivatives has been shown to be highly stable under physiological conditions.[21,22]

Benzo[*c*]coumarin derivatives structurally related to canna-binoids and therefore termed "cannabilactones", e.g., com-pound **8** (Figure 2), were reported to possess CB$_2$-agonistic properties.[23] We have recently discovered that simple 3-benzyl-5-methoxycoumarin derivatives, such as **9**, interact with CB$_1$ as well as CB$_2$ receptors, showing antagonistic or inverse agonistic

activity at CB$_1$ receptors.[24,25] In the present study we present the results of systematic modifications of scaffold **9** leading to strongly increased potency, selectivity for either CB$_1$ or CB$_2$ receptors, and agonistic or antagonistic properties, depending on the substitution pattern.

RESULTS AND DISCUSSION

Compound Design. Comparison of the structural features of our new 3-benzylcoumarin scaffold[24] (lead structure **9**), a nonselective CB$_1$/CB$_2$ antagonist, with the potent, nonselective agonist Δ^9-THC (**3**) and the CB$_2$-selective agonistic benzo[*c*]-coumarin derivative **8** is depicted in Figure 3. The heterocyclic

Figure 3. Structural comparison of coumarin derivative **9** (CB$_1$/CB$_2$ antagonist) with the nonselective agonist Δ^9-THC (**3**) and the CB$_2$-selective agonist AM-1714 (**8**).
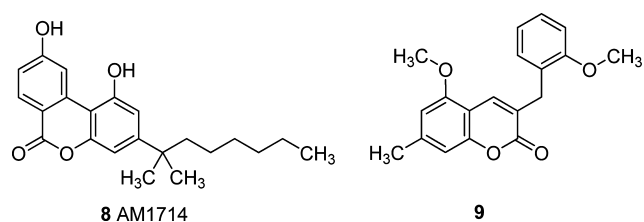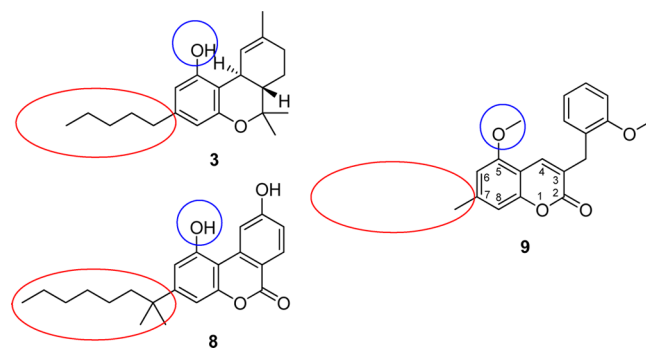

Figure 2. Structures of benzo[*c*]coumarin (**8**) and 3-benzylcoumarin (**9**) derivatives described to interact with cannabinoid receptors.[23,24]

core structures align well with the superimposed (modified) benzopyran structures. It becomes evident that the high affinity ligands **3** and **8** possess an alkyl side chain, which may be branched (in **8**). In contrast, coumarin derivative **9** does not occupy this lipophilic pocket. Because it is located in an area which could be filled by a large substituent in the 7-position of the 3-benzylcoumarins, we decided to synthesize suitably 7-substituted 3-benzylcoumarin derivatives in order to improve the affinity of lead structure **9**. A second goal was to remove the methyl ether in position 5 to obtain phenolic compounds in order to investigate the hypothesis that a free phenolic group

Scheme 1. Synthesis of 3-Benzylcoumarin Derivatives[a,b]
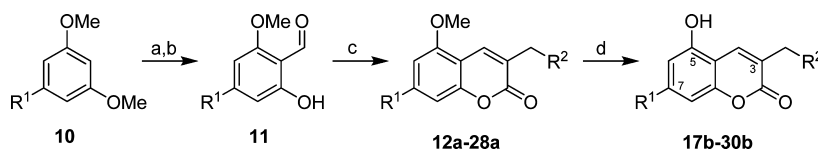
[a]Reagents and conditions: (a) DMF, *n*-BuLi, TMEDA, Et$_2$O, 0 °C to rt, 6 h; (b) NaI, AlCl$_3$, MeCN/CH$_2$Cl$_2$, 0 °C to rt, 1 h; (c) α,β-unsaturated aldehyde, 1,3-dimethylimidazolium dimethylphosphate, K$_2$CO$_3$, toluene, MW, 110 °C, 50 min; (d) BBr$_3$, CH$_2$Cl$_2$, −78 °C to rt, 24 h. [b]For R[1] and R[2], see Table 1.

might be responsible for agonistic activity like that in agonists **3**, **4**, and **8**.

Syntheses. The 5-methoxy-substituted 3-benzylcoumarin derivatives **12a**−**28a** were synthesized in a one-pot synthesis from appropriately substituted salicylaldehyde obtained from **10** and α,β-unsaturated aldehyde in the presence of potassium carbonate and 1,3-dimethylimidazolium dimethylphosphate in toluene under microwave irradiation (110 °C, 50 min) as previously described (see Scheme 1).[24,26] 3-(2-Hydroxyben-zyl)-5-isopropyl-8-methylcoumarin (**31**) was synthesized anal-ogously. Cleavage of the phenyl methyl ether by boron tribromide in dichloromethane at −78 °C yielded the phenols **17b**−**30b**. The products were purified by flash chromatog-raphy. The structures were confirmed by ^{1}H and ^{13}C NMR spectra, IR spectra, and EI-MS and EI-HRMS spectra; purity was confirmed by elemental analysis (for details, see Experimental Section and Supporting Information). For the two final products, **29b** and **31**, crystal structures were obtained (see Supporting Information).

Biological Evaluation. The affinities of the 7-alkyl-3-benzylcoumarin derivatives **12**−**31** were determined in radio-ligand binding studies at human CB$_1$ and CB$_2$ receptors using [^{3}H](−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940, **4**) as CB re-ceptor radioligand. As a source for human CB$_1$ and CB$_2$ receptors, membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were utilized (50 μg of protein/vial). Initially the compounds were screened at a concentration of 10 μM. In cases where inhibition of radioligand binding was at least about 50%, full concentration−inhibition curves were determined in order to calculate K_i values. Functional properties were investigated in cAMP assays using CHO cells stably expressing the human CB$_1$ or CB$_2$ receptor subtype, respectively. A radioactive filtration assay determining competition of [^{3}H]cAMP by formed cAMP to a binding protein isolated from bovine adrenal glands was applied.[27] Effects of test compounds (1 μM) on forskolin-stimulated cAMP levels were determined relative to the maximal effect observed with the full agonist CP55,940.

Structure−Activity Relationships (SARs). Biological data are collected in Table 1. A large variety of substituents was introduced in position 7, ranging from small (H, Br, methyl, bromomethyl, hydroxymethyl) to large substituents (*tert*-butyldiphenylsilyloxymethyl, 3-(2-methoxyphenyl)propionic acid methyl ester, pentyl, 1,1-dimethylheptyl, 1,1-dimethyloctyl, 1-butylcyclopentyl, and 1-butylcyclohexyl). In the 5-position, methoxy or hydroxy was present. One compound, **31**, contained an isopropyl residue at C5. Further modifications were conducted at the 3-benzyl residue in the ortho- and/or meta-position, whose substitution had been shown in our previous study to be favorable for CB receptor affinity, while para-substitution had led to inactive derivatives and was

therefore not considered.[24] The following small substituents were introduced: H, methyl, hydroxy, methoxy, and Cl.

As suggested by pharmacophore modeling, a lipophilic moiety in position 7 of the coumarin scaffold proved to be essential for high affinity of the compounds for CB receptors. If this position was not substituted as in **12a** (containing a methoxy group in the 5-position, combined with a *m*-methoxy group on the 3-benzyl residue), the compound showed only low affinity (K_i CB$_1$: 21.1, CB$_2$: ≫10 μM). After substitution with a bromomethyl residue, a small increase in affinity could be achieved (**14a**; K_i CB$_1$: 4.74 μM, CB$_2$: 13.3 μM), while replacement by a pentyl moiety (in compound **17a**) enhanced affinity dramatically, yielding a K_i value of 0.045 μM at CB$_1$ receptors (470-fold increase) and of 0.143 μM (>70-fold increase) at CB$_2$ receptors. Polar or other bulky substituents (compounds **15a**, **16a**) were not tolerated by the CB receptors.

In the next series of compounds we retained the 7-pentyl residue and modified the 5-substituent (OMe or OH), and the substituents on the benzyl ring (ortho- or meta-substitution). In almost all cases a hydroxy instead of a methoxy group in the 5-position led to a drastic reduction in CB$_1$ as well as CB$_2$ affinity (compare e.g., **19a/19b**, **20a/20b**, **22a/22b**, **23a/23b**). The 5-isopropylcoumarin **31** was virtually inactive. All 5-hydroxy-7-pentyl-3-benzylcoumarins showed only moderate affinities in the micromolar range.

Substitution of the benzyl ring in the ortho- or meta-position strongly contributed the affinity of the 7-pentyl-5-methoxy-3-benzylcoumarins for the CB$_1$ receptor (compare e.g., **17a** (*m*-OMe) and **21a** (*o*-OMe) with **18a** (unsubstituted benzyl)). The rank order of potency at the CB$_1$ receptor with regard to benzyl substitution was as follows: *o*-Me (K_i 0.022 μM) > *o*-OMe (0.032 μM) = *o*-Cl (0.033 μM) > *m*-OCH$_3$ (0.045 μM) ≫ *m*-Cl (0.637 μM) = *m*-Me (0.713 μM) > H (1.92 μM), indicating that ortho- was superior to meta-substitution. At the CB$_2$ receptor, the unsubstituted benzyl derivative **18a** was 7-fold more potent than at the CB$_1$ receptor (K_i CB$_2$ 0.267 μM). Ortho- or meta-substitution had only moderate effects on the CB$_2$ affinity of the compounds (**19a**, **20a**, **21a**). The best substituent was the *o*-OCH$_3$ residue, leading to a 5-fold increase in CB$_2$ affinity (K_i 0.049 μM, **21a**). In this series, the unsubstituted benzyl derivative (**18a**) showed the highest CB$_2$ selectivity (7-fold), while the *m*-methylbenzyl derivative (**19a**) exhibited not only the highest CB$_1$ affinity but also highest selectivity (18-fold) for that receptor subtype.

As a next step, we further increased the size and bulk of the alkyl substituent in the 7-position and combined it with an ortho-substituted 3-benzyl residue (OCH$_3$- or OH-substituted) and a methoxy or hydroxy group in position 5. Due to synthetic reasons, both residues (R^5 and R^{ortho}) were always identical in this series, being either OCH$_3$ or OH. These were combined with four different residues in the 7-position: 1,1-dimethylhep-tyl, 1,1-dimethyloctyl, 1-butylcyclopentyl, and 1-butylcyclohex-

Table 1. Potencies and Efficacies of Coumarin Derivatives at Cannabinoid Receptor Subtypes[a]

9, 12-29   30b   31

compd	R[7]	R[5]	R[ortho]	R[meta]	radioligand binding assays vs [³H]CP55,940		cAMP assays, % inhibition of forskolin-stimulated cAMP accumulation at a concentration of 1 μM[b] (relative to maximal effect of full agonist CP55,940 (1 μM) = 100%) (EC$_{50}$ ± SEM (μM))	
					human CB$_1$ K_i ± SEM (μM)	human CB$_2$ K_i ± SEM (μM)	human CB$_1$	human CB$_2$
4		see Figure 1			0.00128 ± 0.00044	0.00142 ± 0.00075	100% (EC$_{50}$: 0.00228 ± 0.00137)	100% (EC$_{50}$: 0.00100 ± 0.00019)
6		see Figure 1			0.0126 ± 0.0039	0.900 ± 0.320	0%	0%
8		see Figure 2			0.400 (rCB$_1$)[21]	0.82 (mCB$_2$)[21]	nd[c]	nd
9	methyl	methoxy	methoxy	H	0.738 ± 0.414[22]	1.03 ± 0.22[22]	nd	nd
12a	H	methoxy	H	methoxy	21.1 ± 9.6	≫10 (27%)[a]	0%	nd
13a	Br	methoxy	H	methoxy	>10 (38%)[d]	2.45 ± 0.65	0%	0%
14a	bromomethyl	methoxy	H	methoxy	4.74 ± 1.35	13.3 ± 6.3	23%	0%
15a	hydroxymethyl	methoxy	H	methoxy	≫10 (8%)[d]	≫10 (16%)[d]	nd	nd
16a	CH$_2$O-TBDPS[e]	methoxy	H	methoxy	≫10 (12%)[d]	≫10 (4%)[d]	nd	nd
17a	pentyl	methoxy	H	methoxy	0.045 ± 0.020	0.143 ± 0.022	58% (EC$_{50}$: 0.430 ± 0.283)	93% (EC$_{50}$: 0.092 ± 0.028)
17b	pentyl	hydroxy	H	hydroxy	16.2 ± 4.1	5.15 ± 0.02	0%	0%
18a	pentyl	methoxy	H	H	1.92 ± 1.38	0.267 ± 0.060	58%	47%
18b	pentyl	hydroxy	H	H	1.27 ± 0.31	1.68 ± 0.15	0%	0%
19a (PSB-SB-1201)	pentyl	methoxy	methyl	H	0.022 ± 0.009	0.405 ± 0.086	0%	58% (EC$_{50}$: 0.213 ± 0.055)
19b	pentyl	hydroxy	methyl	H	1.64 ± 0.50	3.57 ± 0.47	9%	12%
20a	pentyl	methoxy	H	methyl	0.713 ± 0.393	0.392 ± 0.025	0%	50%
20b	pentyl	hydroxy	H	methyl	2.17 ± 0.91	1.76 ± 0.35	42 ± 3%	10 ± 6%
21a (PSB-SB-1202)	pentyl	methoxy	methoxy	H	0.032 ± 0.011	0.049 ± 0.006	93% (EC$_{50}$: 0.056 ± 0.028)	106% (EC$_{50}$: 0.014 ± 0.001)
21b	pentyl	hydroxy	hydroxy	H	9.82 ± 5.57	4.80 ± 0.43	109%	0%
22a	pentyl	methoxy	Cl	H	0.033 ± 0.012	0.185 ± 0.026	6%	47%
22b	pentyl	hydroxy	Cl	H	19.1 ± 17.6	1.94 ± 0.61	60%	2 ± 8%
23a	pentyl	methoxy	H	Cl	0.637 ± 0.344	0.350 ± 0.069	0%	40%
23b	pentyl	hydroxy	H	Cl	6.39 ± 3.33	2.20 ± 0.33	70%	0%
24a	1,1-dimethylheptyl	methoxy	H	H	1.43 ± 0.49	4.12 ± 0.31	27%	0%
24b	1,1-dimethylheptyl	hydroxy	H	H	2.63 ± 1.23	0.465 ± 0.024	84%	32%
25a	1,1-dimethylheptyl	methoxy	methoxy	H	1.02 ± 0.38	3.01 ± 4.81	0%	30%
25b (PSB-SB-1203)	1,1-dimethylheptyl	hydroxy	hydroxy	H	0.244 ± 0.051	0.210 ± 0.025	0%	76% (EC$_{50}$: 0.054 ± 0.026)
26a	1,1-dimethyloctyl	methoxy	methoxy	H	≈ 10 (51%)[d]	≈ 10 (47%)[d]	nd	nd
26b	1,1-dimethyloctyl	hydroxy	hydroxy	H	1.17 ± 0.37	0.292 ± 0.040	0%	52%
27a	1-butylcyclopentyl	methoxy	methoxy	H	0.598 ± 0.055	1.14 ± 0.11	110%	3%
27b (PSB-SB-1204)	1-butylcyclopentyl	hydroxy	hydroxy	H	1.58 ± 0.21	0.068 ± 0.005	18%	106% (EC$_{50}$: 0.048 ± 0.029)
28a	1-butylcylcohexyl	methoxy	methoxy	H	≤10 (42%)[d]	≤10 (45%)[d]	nd	nd
28b	1-butylcylcohexyl	hydroxy	hydroxy	H	4.89 ± 3.34	0.049 ± 0.002	0%	76% (EC$_{50}$: 0.179 ± 0.082)
29b	methyl	hydroxy	methyl	H	>10 (34%)[d]	>10 (38%)[d]	nd	nd
30b		see above for structure			>10 (30%)[d]	≫10 (23%)[d]	nd	nd
31		see above for structure			>10 (31%)[d]	≫10 (24%)[d]	nd	nd

[a]All data result from three independent experiments, performed in duplicates. [b]SEM was in most cases below 10%, and it never exceeded 14%. [c]nd = not determined. [d]% inhibition of radioligand binding at 10 μM. [e]TBDPS = *tert*-butyldiphenylsilyl.

yl. The increase in chain length and bulk of the 7-pentyl substituent in **21a** (5-methoxy-3-*o*-methoxybenzyl-7-pentylcoumarin) led to a large drop in CB$_1$ as well as CB$_2$ affinity (compare **21a** with **25a**, **26a**, **27a**, and **28a**). The larger the substituent, the lower its affinity for the receptors. The best tolerated substituent was the 1-butylcyclopentyl residue (**27a**),
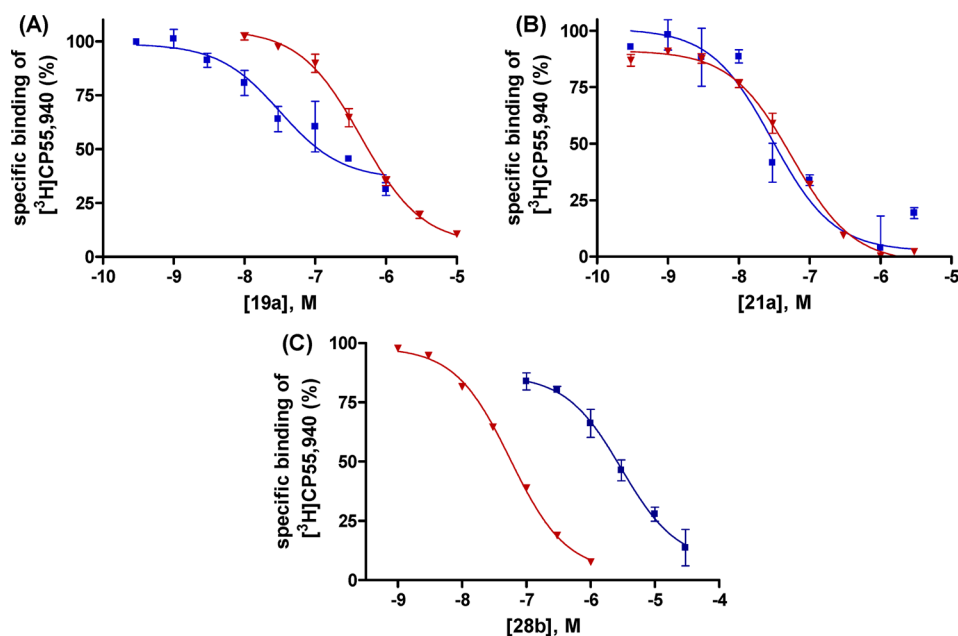
Figure 4. Concentration-dependent inhibition of specific [3H]CP55,940 binding by (A) **19a**, (B) **21a**, and (C) **28b** at membrane preparations of CHO cells expressing human CB$_1$ (■), or human CB$_2$ (▼) receptors, respectively. Data points represent means ± SEM of three independent experiments, performed in duplicates.

showing 19-fold lower affinity at CB$_1$ and 23-fold reduced affinity for CB$_2$ in comparison with the pentyl-substituted analogue **21a**.

In contrast to the findings with the 5-methoxy-3-(o-methoxybenzyl)coumarin derivatives, the corresponding 5-hydroxy-3-(o-hydroxybenzyl)coumarin derivatives showed very different structure−activity relationships. In this series, larger 7-substituents were tolerated at both receptor subtypes. At the CB$_1$ receptor, the 1,1-dimethylheptyl residue (compound **25b**, K_i CB$_1$ 0.244 μM) was best tolerated, leading to a 40-fold increase in CB$_1$ affinity compared with the corresponding pentyl derivative **21b** (K_i CB$_1$ 9.82 μM). However, further increase in the size of the 7-substituent reduced affinity. At the CB$_2$ receptor, the situation was different: large and especially bulky substituents led to a dramatic increase in CB$_2$ affinity. Because the CB$_1$ receptor did not tolerate such substituents, the resulting compounds showed high CB$_2$ selectivity. The best compound of this series was **28b** with a K_i value at CB$_2$ of 0.049 μM and 100-fold selectivity versus the CB$_1$ receptor.

Investigations of the SARs of THC derivatives have shown that a methylation of the phenolic hydroxyl group (in the C-1 position) resulted in a marked decrease of affinity for the CB$_1$ receptor and a high selectivity for the CB$_2$ receptor subtype.[8,28] Reported SARs of Δ^8-THC also indicated that large, bulky alkyl residues are beneficial for high CB$_2$ affinity, suggesting a similar binding mode of Δ^9-THC and 5-hydroxycoumarin derivatives.[8]

However, in our studies, we observed very different effects of methylation of the phenolic hydroxy group at the benzopyrane ring system (C5) in 7-alkyl-substituted 3-benzylcoumarin derivatives, depending on the size of the 7-substituent. The different SARs of the dimethoxy- versus the dihydroxy-substituted benzylcoumarin derivatives (and those of THC derivatives) might be explained by different binding modes and/or different conformations of these compounds.[28,29] Thus, 7-alkyl-3-benzylcoumarins are highly versatile scaffolds for obtaining a potent CB receptor ligand with high potency at

either CB$_1$ or CB$_2$ or both receptor subtypes by small modifications of the substitution pattern.

Figure 4 shows the radioligand competition binding curves of selected coumarin derivatives, including the potent CB$_1$-selective ligand **19a**, the nonselective potent CB$_1$/CB$_2$-ligand **21a**, and the potent and selective CB$_2$-ligand **28b**.

Functional Properties. Receptor ligands may exhibit agonistic, partial agonistic, antagonistic, or inverse agonistic activity. In order to study the intrinsic activity of the new coumarin derivatives at the G$_i$-coupled CB$_1$ and CB$_2$ recepor subtypes, their inhibitory effects on forskolin-stimulated adenylate cyclase was determined in cAMP accumulation assays at a concentration of 1 μM and compared to the maximal effect (set at 100%) achieved with the full CB$_1$ and CB$_2$ agonist CB55,940 (1 μM). For the most potent compounds, full concentration−response curves were measured and EC$_{50}$ values were determined. The obtained results are presented in Table 1. pK_i values determined in radioligand bindings studies using the agonist radioligand [3H]CP55,940 (**4**) were correlated with pEC$_{50}$ values for selected potent agonists determined in cAMP accumulation studies (see Supporting Information). A high correlation ($r^2 = 0.781$) between the determined CB$_2$ pK_i and pEC$_{50}$ values was observed.

In a previous series of moderately potent 3-benzyl-5-methoxycoumarin derivatives, neutral antagonists and those with inverse agonistic activity had been identified by [35S]GTPγS binding studies. The present series comprises compounds with full agonistic, partial agonistic, and antagonistic/inverse agonistic properties. Selected concentration−response curves of potent CB receptor agonists are depicted in Figure 5.

We observed that minor structural modifications in ligands with a 7-alkyl-substituted 3-benzylcoumarin scaffold could have dramatic effects on the functionality of the compounds. At CB$_1$ receptors, the full range of efficacies was observed from antagonists (e.g., **19a**), and partial agonists (e.g., **17a**), to full agonists (e.g., **21a**). The three examples are all 3-benzyl-5-
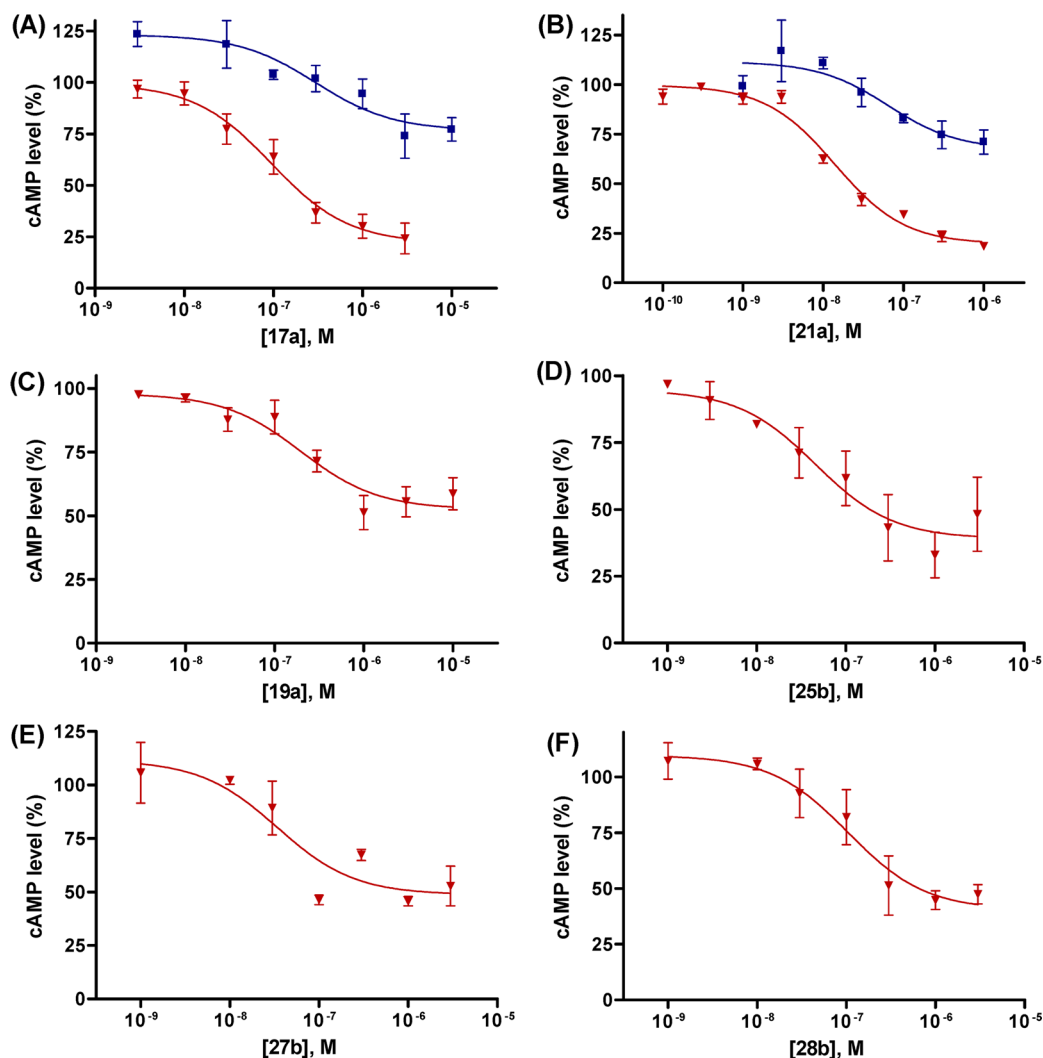
Figure 5. Concentration-dependent inhibition of forskolin-induced cAMP-level by (A) **17a**, (B) **21a**, (C) **19a**, (D) **25b**, (E) **27b**, and (F) **28b** in CHO cells stably expressing human CB_1 (■) and human CB_2 (▼) receptors. Data points represent means ± SEM of three experiments, performed in duplicates.

methoxy-7-pentylcoumarin derivatives differing merely in the substituent on the benzyl residue (**19a**: *o*-methyl, **17a**: *m*-methoxy, **21a**: *o*-methoxy). This shows that small modifications are sufficient to change the conformation of the receptor and consequently lead to its activation.

At the CB_2 receptor, all of the most potent compounds were agonists, with varying degrees of efficacy. For example, **21a** was found to be a full agonist, and **17a** and **25b** were almost full agonists, while **19a** was a partial agonist in our test system. Again, only minor modifications accounted for the differences in efficacy; for example, the partial CB_2 agonist **19a** and the full agonist **21a**, both of which are 3-benzyl-5-methoxy-7-pentylcoumarin derivatives, only differ in the substitution of the benzyl residue, the partial agonist bearing an *o*-methyl group, and the full agonist bearing an *o*-methoxy group.

The efficacy of the benzylcoumarin derivatives at CB_1 and CB_2 receptors did not always correlate. While **21a** was a full agonist at both receptor subtypes, other compounds showed different behavior; for example, **19a** and **22a** were antgonists at CB_1 but partial agonists at CB_2. Compound **25b**, for example, which was equipotent at both receptor subtypes in binding studies, was found to be an antagonist at CB_1 while it was

highly efficacious (76% compared to the full agonist **4**) at CB_2 receptors. The opposite was observed, for example, for **24b**, which showed a higher efficacy at CB_1 than at CB_2. Thus, SARs are not only different at CB_1 and CB_2 receptors with regard to affinity but also in terms of efficacy.

Cannabinoids that have been approved as drugs include the synthetic Δ^9-THC derivate nabilone (Cesamet) and Δ^9-THC, in combination with cannabidiol (Sativex). Nabilone is used for the treatment of chemotherapy-induced nausea and vomiting as well as anorexia and weight-loss of AIDS patients, while the combination of Δ^9-THC and cannabidiol are approved for the treatment of spasticity and neuropathic pain in multiple sclerosis patients.[1,10,13,14] These drugs are about equipotent at CB_1 and CB_2 receptors, and both Δ^9-THC and nabilone have been reported to be partial agonists when compared to the efficiency of CP55,940. A similar profile can also be found among the new compounds described in the present study; for example, **17a** binds with an affinity to CB receptors comparable to that of Δ^9-THC and is also a partial agonist at CB_1 receptors.

Receptor Docking Studies. In addition to a ligand-based structural comparison (see above), we performed docking studies of the compounds using homology models of the
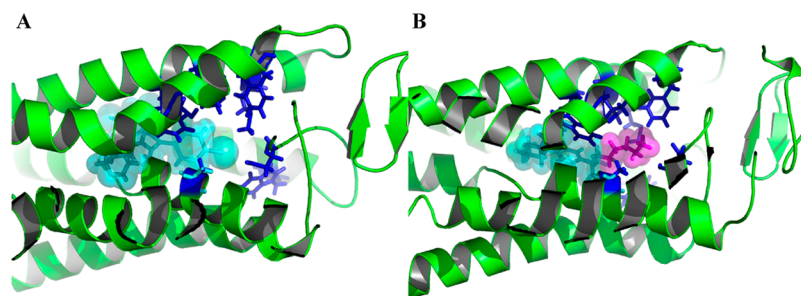
Figure 6. Hydrophobic pocket formed by ALA198, CYS264, TRP279, TRP356, LEU359, MET363, PHE379, CYS386 (in blue) of the CB_1 receptor interacting with the reference ligands (A) inverse agonist **7** and (B) agonist **4**. We note that the aliphatic side chain in **4** (shown in magenta) occupies the hydrophobic pocket.

human CB_1 to try to understand how modifications of the R7 substituent would affect ligand binding. We constructed a model for the CB_1 receptor based on homology using as a common template the crystal structure of bovine rhodopsin (pdb-code 1U19). Site-specific mutation studies[30−32] on the rhodopsin subfamily of receptors, including CB_1/CB_2, suggest that many ligands bind to CB_1/CB_2 within the transmembrane (TM) core region in the crevice formed by TM3, TM4, TM5, and TM6. Initial docking studies using the CB_1-selective inverse agonist AM281 (**7**), an analogue of rimonabant (**6**), and the agonists CP55,940 (**4**) and WIN5,212−2 (**5**) demonstrated that these reference ligands are well accommodated within the transmembrane region (see Figure S3 in Supporting Information). This is in agreement with studies that reported alanine substitution of LYS192 to result in a significant loss in affinity for the agonist ligand **4**.[30] On the other hand, mutation of PHE191, TRP279, and TRP356 to ALA showed a reduction in affinity for the agonist **5**. In addition, it was suggested that TRP356 might be important for binding of **5** to the CB_1 receptor.

When docking the inverse agonist **7** as a reference ligand, we found a large hydrophobic pocket in the vicinity of the docking pose (Figure 6A) which is not occupied by the ligand. Modifications of the ligand that would fill this hydrophobic pocket with apolar substituents should dramatically improve the binding energy by exploitation of the hydrophobic effect. This observation is supported by that fact that in the binding pose of the agonist **4** we find its aliphatic side chain to fill exactly this pocket (Figure 6B).

We therefore docked a set of new ligands proposed by the rationale discussed above with various aliphatic substitutions into the same binding pocket and observed that the new ligands assumed binding poses in which the coumarin and aliphatic parts of the ligands changed their binding pose only very little. It should be noted that the CB_1 receptor exhibits 97−99% amino acid sequence identity across species comparing human, rat, and mouse sequences. In our own competition experiments at the rat and human cannabinoid CB_1 receptors only very minor species differences were observed for the CB_1 receptor; for example, the determined K_i values for CP55,940 at rat and human receptors were quite similar (1.24 nM at rat CB_1 and 0.71 nM at human CB_1 receptors).[24] Therefore, K_i values determined at rat CB_1 receptors can be expected to be highly predictive of K_i values at human CB_1 receptors.[1,24] In analyzing the effect of substitutions at the 7-position, we first concentrated on compounds related by a single substitution of a side group: the previously published ligands[24] 3-(2-methylbenzyl)-5-methoxy-7-methyl-2H-chromen-2-one, 3-(2-

chlorobenzyl)-5-methoxy-7-methyl-2H-chromen-2-one, 3-benzyl-5-methoxy-7-methyl-2H-chromen-2-one, and 3-(2-methoxybenzyl)-5-methoxy-7-methyl-2H-chromen-2-one each differ from **19a**, **22a**, **18a**, and **25a**, respectively, by the aliphatic substitution at the 7-position. In addition, **23a** and **20a** are similar to ligands 3-(2-chlorobenzyl)-5-methoxy-7-methyl-2H-chromen-2-one and 3-(2-methylbenzyl)-5-methoxy-7-methyl-2H-chromen-2-one, but here the positions of the chlorine and the methyl group are also changed. Comparison of the binding poses between the new compounds and the structurally related previously published derivatives[24] demonstrate that the substitution of the aliphatic side group at the 7-position has little effect on the binding pose of the scaffold (as shown for **22a** and 3-(2-chlorobenzyl)-5-methoxy-7-methyl-2H-chromen-2-one in Figure 7) but improves the binding energy (see Table
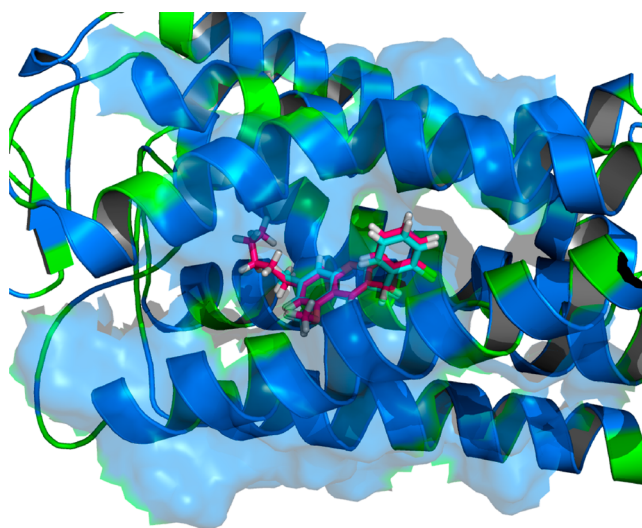

Figure 7. Binding poses between 3-(2-chlorobenzyl)-5-methoxy-7-methyl-2H-chromen-2-one (cyan) and **22a** (magenta) in the CB_1 receptor model. The substitution of the aliphatic side chain at the 7-position has little effect on the overall binding pose.

S2, Supporting Information). For **25a**, the replacement of methyl (in lead structure **9**) by 1,1-dimethylheptyl (**25a**) results in a large increase in the binding energy. Correlating the relative order of binding energies in the aliphatic substitutions at the 7-position, we find the relative order **21a** (pentyl), **25a** (1,1-dimethylheptyl), **27a** (1-butyl-cyclopentyl), **26a** (1,1-dimethyloctyl), and **28a** (1-butylcyclohexyl) in comparison with the experimental order **21a**, **27a**, **25a**, **26a**, and **28a**. There is a large jump in binding energy between **21a** and **25a**, **27a** and

another jump between **27a** and **26a**, **28a**, indicating that there is an optimal size for the substituent at the 7-position.

Physicochemical Properties. Molecular weights, log P values, and polar surface areas of selected coumarin derivatives (**19a**, **21a**, and **28b**), which possess high affinity, were calculated and compared to those of commercially available CB receptor ligands some of which are therapeutically used (see Supporting Information, Table S3). The physicochemical properties of the new coumarin derivatives are in the same range as those of commercially available CB ligands, indicating that they may show similar in vivo behavior.

CONCLUSIONS

A series of 7-alkyl-3-benzylcoumarin derivatives was designed and synthesized as high affinity CB_1 and/or CB_2 receptor ligands, tested in radioligand binding studies, and functionally characterized in cAMP accumulation assays. On the basis of the obtained structure−activity relationships, the synthesized compounds can be separated into two classes: 5-methoxy- and the 5-hydroxy-substituted coumarins. The two groups differ in their structure−activity relationships and their functional properties. In particular, the SARs of 5-hydroxy-coumarins correlate better with those of classical cannabinoids. The physicochemical properties of the synthesized compounds are comparable to those of commercially available and therapeutically applied CB receptor ligands. The developed series provides the advantage of high versatility, allowing us to obtain potent CB receptor ligands with high affinity and potency at either CB_1 or CB_2, or both receptor subtypes. Furthermore fine-tuning of compound properties with regard to affinity, selectivity, and efficacy could be achieved by small modifications of the substitution pattern. Thus, 7-alkyl-3-benzylcoumarins are highly versatile scaffolds for obtaining potent CB receptor ligands. The relatively simple structures, which do not possess any stereocenters, are easily accessible by a straightforward one-pot synthetic procedure. Derivatives which possess high potency at either CB_1, or CB_2, or both receptor subtypes and a broad spectrum of efficacies at each receptor subtype could be obtained. The most potent compounds of the present series include 5-methoxy-3-(2-methylbenzyl)-7-pentyl-2H-chromen-2-one (**19a**, PSB-SB-1201), a potent and selective CB_1 antagonist (K_i CB_1 0.022 μM, 18-fold selective), 5-methoxy-3-(2-methoxybenzyl)-7-pentyl-2H-chromen-2-one (**21a**, PSB-SB-1202), a potent dual CB_1/CB_2 agonist (CB_1 K_i 0.032 μM, EC_{50} 0.056 μM; CB_2 K_i 0.049 μM, EC_{50} 0.014 μM), 5-hydroxy-3-(2-hydroxybenzyl)-7-(2-methyloct-2-yl)-2H-chromen-2-one (**25b**, PSB-SB-1203), a dual CB_1/CB_2 ligand that blocks CB_1 but activates CB_2 receptors (CB_1 K_i 0.244 μM; CB_2 K_i 0.210 μM, EC_{50} 0.054 μM), and 7-(1-butylcyclopentyl)-5-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (**27b**, PSB-SB-1204), which is a selective CB_2 receptor agonist (CB_1 K_i 1.59 μM; CB_2 K_i 0.068 μM, EC_{50} 0.048 μM). Selected compounds of the present series will be further investigated in animal models to explore the pharmacological properties of these new cannabinoid receptor ligands.

EXPERIMENTAL SECTION

Syntheses. *General Procedures for the Preparation of Coumarin Derivatives.* Under an atmosphere of argon, 1.00 equiv of substituted salicylaldehyde, 1.20 equiv of potassium carbonate, 2.50 equiv of α,β-unsaturated aldehyde, and 1.20 equiv of 1,3-dimethylimidazolium dimethylphosphate were suspended in toluene (3.3 mL/mmol salicylaldehyde). The reaction vessel was subjected to microwave irradiation and kept at a constant temperature of 110 °C for 50 min (max. 200 W) while being stirred. After being cooled to rt, the reaction was quenched by addition of water. The aqueous layer was extracted with EtOAc, the combined organic phases were dried over sodium sulfate, and the solvent was removed under reduced pressure. The products were purified by flash column chromatography. Purity was determined by ^{1}H NMR, MS, and elemental analysis and was ≥95%.

5-Methoxy-3-(2-methylbenzyl)-7-pentyl-2H-chromen-2-one (19a). Yield 56.3 mg, 36% (scale: 450 μmol). R_f (c-Hex/EtOAc 40:1) = 0.06. mp: 173−175 °C. ^{1}H NMR (400 MHz, CDCl$_3$): δ/ppm = 0.92 (t, ^3J = 6.9 Hz, 3 H, CH$_2$-CH$_3$), 1.32−1.39 (m, 4 H, 2 × CH$_2$), 1.62−1.68 (m, 2 H, CH$_2$), 2.30 (s, 3 H, C$_{ar}$-CH$_3$), 2.66 (t, ^3J = 7.7 Hz, 2 H, C$_{ar}$-CH$_2$-CH$_2$), 3.84 (s, 3 H, OCH$_3$), 3.89 (s, 2 H, CH$_2$), 6.50 (s, 1 H, H_{ar}), 6.77 (s, 1 H, H_{ar}), 7.20−7.25 (m, 4 H, 4 × H_{ar}), 7.46 (s, 1 H, CH). ^{13}C NMR (100 MHz, CDCl$_3$): δ/ppm = 14.0 (p, CH$_2$-CH$_3$), 19.5 (p, C$_{ar}$-CH$_3$), 22.5 (s, CH$_2$), 30.8 (s, CH$_2$), 31.4 (s, CH$_2$), 34.0 (s, CH$_2$), 36.6 (s, C$_{ar}$-CH$_2$-CH$_2$), 55.8 (p, OCH$_3$), 105.5 (t, C$_{ar}$H), 108.0 (q, C$_{ar}$-CH), 108.4 (t, C$_{ar}$H), 125.1 (q, C$_{ar}$-CH$_2$), 126.2 (t, C$_{ar}$-H), 127.0 (t, C$_{ar}$-H), 130.2 (t, C$_{ar}$-H), 130.5 (t, C$_{ar}$-H), 134.1 (t, CH), 136.1 (q, C-CH$_2$), 136.9 (q, C$_{ar}$-CH$_3$), 147.6 (q, C$_{ar}$-CH$_2$CH$_2$), 154.1 (q, C$_{ar}$-O-CO), 155.4 (q, C$_{ar}$-OCH$_3$), 162.2 (q, C=O). IR (DRIFT): ν/cm^{-1} = 2924 (w), 2858 (w), 1725 (m), 1618 (m), 1573 (w), 1494 (w), 1455 (w), 1423 (w), 1352 (w), 1244 (w), 1167 (w), 1142 (w), 1117 (m), 1051 (w), 957 (w), 881 (w), 833 (w), 760 (w), 745 (w), 728 (w), 468 (w), 408 (w). EI-MS m/z (%): 351 (29) [M$^+$ + H], 350 (100) [M$^+$], 333 (21), 294 (19) [C$_{19}$H$_{18}$O$_3^+$], 259 (22) [C$_{16}$H$_{19}$O$_3^+$]. EI-HRMS (C$_{23}$H$_{26}$O$_3$): calcd 350.1882, found 350.1885.

General Procedures for the Deprotection of Coumarin Derivatives. Under an atmosphere of argon, 5.00 equiv BBr$_3$ (1 M, CH$_2$Cl$_2$) was added to a solution of 1.00 equiv of coumarin in dichloromethane (20 mL/mmol coumarin) at −78 °C, and the resulting mixture was stirred for 30 min at this temperature and for 24 h at rt. For work up, saturated NaHCO$_3$ solution was added at 0 °C, and the aqueous layer was extracted with CH$_2$Cl$_2$. The combined organic phases were washed with water and brine and were dried over sodium sulfate. The solvent was removed under reduced pressure, and the product was purified by flash column chromatography.

7-(1-Butylcyclopentyl)-5-hydroxy-3-(2-hydroxybenzyl)-2H-chromen-2-one (27b). Yield 317 mg, 99% (scale: 820 μmol). R_f (c-Hex/EtOAc 7:1) = 0.12. mp: 67−70 °C. ^{1}H NMR (400 MHz, CDCl$_3$): δ/ppm = 0.74 (t, ^3J = 7.3 Hz, 3 H, CH$_3$), 0.84−0.92 (m, 2 H, CH$_2$), 1.07−1.16 (m, 2 H, CH$_2$), 1.49−1.54 (m, 2 H, CH$_2$), 1.56−1.61 (m, 2 H, CH$_2$), 1.64−1.72 (m, 3 H, CH$_2$), 1.74−1.85 (m, 3 H, CH$_2$), 3.87 (s, 2 H, CH$_2$), 6.69 (d, ^4J = 1.4 Hz, 1 H, H_{ar}), 6.78 (d, ^4J = 1.4 Hz, 1 H, H_{ar}), 6.87 (ddd, ^3J = ^3J = 7.5 Hz, ^4J = 1.2 Hz, 1 H, H_{ar}), 6.95 (dd, ^3J = 8.1 Hz, ^4J = 1.2 Hz, 1 H, H_{ar}), 7.13 (ddd, ^3J = ^3J = 8.1 Hz, ^4J = 1.7 Hz, 1 H, H_{ar}), 7.23 (dd, ^3J = 7.5 Hz, ^4J = 1.7 Hz, 1 H, H_{ar}), 7.40 (bs, 1 H, OH), 8.16 (s, 1 H, OH), 8.36 (bs, 1 H, OH). ^{13}C NMR (100 MHz, CDCl$_3$): δ/ppm =13.9 (p, CH$_3$), 23.1 (s, 2 × CH$_2$), 23.2 (s, CH$_2$), 27.4 (s, CH$_2$), 32.1 (s, CH$_2$), 37.5 (s, 2 × CH$_2$), 41.4 (s, CH$_2$), 51.6 (q, C(CH$_2$)$_3$), 106.9 (t, C$_{ar}$-H), 107.2 (q, C$_{ar}$-CH), 109.4 (t, C$_{ar}$-H), 118.1 (t, C$_{ar}$-H), 121.1 (t, C$_{ar}$-H), 124.7 (q, C-CH$_2$), 125.4 (q, C$_{ar}$-CH$_2$), 128.6 (t, C$_{ar}$-H), 130.6 (t, C$_{ar}$-H), 136.6 (t, CH), 152.5 (q, C$_{ar}$-C), 153.7 (q, C$_{ar}$-O-CO), 154.2 (q, C$_{ar}$-OH), 165.6 (q, C=O), 171.9 (q, C$_{ar}$-OH). IR (ATR): ν/cm^{-1} = 3276 (w), 2927 (w), 2859 (w), 1674 (m), 1615 (m), 1489 (w), 1455 (m), 1422 (m), 1343 (w), 1234 (m), 1176 (m), 1054 (m), 933 (w), 845 (w), 751 (m), 732 (w), 671 (w), 523 (w). FAB-MS m/z (%): 393 (100) [M$^+$ + H], 299 (82) [C$_{19}$H$_{23}$O$_3^+$], 107 (37) [C$_7$H$_7$O$^+$]. FAB-HRMS (C$_{25}$H$_{28}$O$_4$ + H$^+$): calcd 393.2066, found 393.2064.

Retroviral Transfection. CHO K1 cells stably transfected with the human CB_1 and CB_2 receptor were generated with a retroviral transfection system. Packaging cells (1.5 × 10^6 GP+envAM12 cells) were plated into 25 cm^2 cell culture flasks 24 h before the transfection and grown in 5 mL of DMEM medium containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1% ultraglutamine, and 0.2 mg/mL hygromycin B. A few hours before the transfection, the medium was replaced with 6.25 mL of DMEM medium supplemented with 10% FCS and 1% ultraglutamine without antibiotics. Receptor

DNA (6.75 μg of pLXSN-CB$_1$ or pLXSN-CB$_2$, respectively) and 3.75 μg of a vesicular stomatitis virus G protein (VSV-G), which pseudotypes the generated viruses and therefore increase their infection efficiency, were cotransfected. The transfection reagent Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) was used in a ratio of 1:2.5 (DNA:Lipofectamine). After 12−15 h of incubation, the medium was removed and replaced with 3 mL of DMEM medium containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% ultraglutamine. Additionally, 30 μL of 500 mM sodium butyrate dissolved in water were added to the flasks, and the cells were incubated for 48 h at 32 °C, 5% CO$_2$. Then the supernatants (3 mL) that contained the virus were filtered and transferred into 25 cm^2 cell culture flasks of ∼60% confluent CHO-K1 cells. A Polybrene solution (6 μL, 4 mg/mL in water) was added. After an infection time of 2.5 h at 32 °C, 5% CO$_2$, the medium was removed and replaced with 6 mL of DMEM/F12 supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% ultraglutamin. After 48 h, cells were selected by adding 0.8 mg/mL of G418 to the cell culture medium. After one week, the G418 concentration was reduced to 0.2 mg/mL.

Cell Culture. GP+envAM12 packaging cells were cultured at 37 °C, 5% CO$_2$ in HXM medium which consisted of DMEM, 10% FCS, 100 U/mL penicillin G, 100 μg/mL streptomycin, 1% ultraglutamine, 0.2 mg/mL hygromycin B, 15 μg/mL hypoxanthine, 250 μg/mL xanthine, and 25 μg/mL mycophenolic acid. CHO K1 cells were maintained in DMEM/F12 medium with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1% ultraglutamine under the same conditions. CHO cells stably transfected with the human CB$_1$ and CB$_2$ receptors were maintained at 37 °C and 5% CO$_2$ in the same medium, however in the presence of 0.2 mg/mL G418.

Membrane Preparations for CB Receptor Assays. Membranes of CHO cells expressing the respective human CB receptor subtype were prepared by scratching the cells off the previously frozen cell culture dishes in ice-cold hypotonic buffer (5 mM Tris-HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice for 1 min using an Ultra-Turrax followed by further homogenization for 1 min with a Douncehomogenizer and subsequently spun down for 10 min at 4 °C and 1000g. The supernatant was subsequently centrifuged for 60 min at 48 000g. The obtained membrane pellets were resuspended and homogenized in the required amount of 50 mM Tris-HCl puffer, pH 7.4, to obtain a protein concentration of 5−7 mg/mL. Aliquots of the membrane preparation (1 mL each) were stored at −80 C° until used.

Radioligand Binding Assays at CB$_1$ and CB$_2$ Receptors. Competition binding assays were performed using the CB agonist radioligand [^{3}H](−)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940, **4**, final concentration 0.1 nM). As a source for human CB$_1$ and CB$_2$ receptors membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were used (50 μg of protein/vial). Stock solutions of the test compound were prepared in DMSO. The final DMSO concentration in the assay was 2.5%. After addition of 15 μL of test compound in DMSO, 60 μL of [^{3}H]CP55,940 solution in assay buffer, and 60 μL of membrane preparation to 465 μL of assay buffer (50 mM TRIS, 3 mM MgCl$_2$, 0.1% BSA, pH 7.4), the suspension was incubated for 2 h at rt. Total binding was determined by adding DMSO without test compound. Nonspecific binding was determined in the presence of 10 μM of unlabeled CP55,940 (**4**). Incubation was terminated by rapid filtration through GF/C glass fiber filters presoaked for 0.5 h with 0.3% aq polyethyleneimine solution, using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with ice-cold washing buffer (50 mM TRIS, 0.1% BSA, pH 7.4) and then dried for 1.5 h at 50 °C. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard/Perkin-Elmer) after 6 h of preincubation with 3 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates.

cAMP Accumulation Assays. Inhibition of adenylate cyclase activity was determined in CHO cells stably expressing the CB$_1$ or the CB$_2$ receptor subtype, respectively, using a competition binding assay

for cAMP. Cells were seeded into a 24-well plate at a density of 200 000 cells/well 24 h before performing the assay. After the incubation (see below), the cells were washed with Hank's buffered saline solution (HBSS) consisting of NaCl (13 mM), HEPES (20 mM), glucose (5.5 mM), KCl (5.4 mM), NaHCO$_3$ (4.2 mM), CaCl$_2$ × 2 H$_2$O (1.25 mM), MgSO$_4$ (0.8 mM), MgCl$_2$ (1 mM), KH$_2$PO$_4$ (0.44 mM), and Na$_2$HPO$_4$ (0.34 mM) dissolved in deionized, autoclaved water. After addition of 190 μL of HBSS per well, cells were incubated for 2 h at 37 °C. After this period of time, the phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)-2-imodazolidinone] (final concentration: 40 μM) dissolved in HBSS, test compound, and forskolin (final concentration: 10 μM), all dissolved in HBSS containing 10% DMSO, were added to each well. The final DMSO amount was 1.9%. The suspension was incubated for 10 min after the addition of Ro-20-1724, for 5 min after the addition of test compound, and for another 15 min after adding forskolin. cAMP accumulation was stopped by removing the supernatant from the cell suspension with a membrane pump and subsequently lysing the cells with 500 μL of hot lysis buffer (100 °C; 4 mM EDTA, 0.01% Triton X-100). Aliquots of 50 μL of cell suspension were transferred to 2.5 mL tubes, 30 μL of [^{3}H]cAMP and 40 μL of cAMP-binding protein were added, followed by 1 h of incubation at room temperature. The cAMP binding protein was obtained from bovine adrenal cortex as previously described.[25] Bound and free radioligand were separated by rapid filtration through GF/B glass fiber filter. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard/Perkin-Elmer) after 6 h of preincubation with 3 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer). Data were obtained from three independent experiments, performed in duplicates.

Receptor Models. All-atom models for the CB$_1$ and CB$_2$ receptors were constructed using the crystal structure of bovine rhodopsin (pdb-code 1U19)[31] as structural template, to which both CB receptors have a strong similarity in sequence. We constructed a model on the region from 80 to 439 for CB$_1$ and from 1 to 349 for CB$_2$. Template selection was performed using Phyre[32] using the default protocol, and the alignment between the receptors and the template was assessed using ClustalW.[33] ClustalW calculates the best match for the selected sequences and lines them up so that the identities, similarities, and differences can be seen. On the basis of the resulting alignment, 10 different models were built using MOE of which the model with the lowest energy profile was chosen for this investigation.

Docking Simulations. Docking simulations were performed using the FlexScreen[34,35] receptor−ligand docking software with a SASA-based implicit solvation model.[36] All simulations were performed using the homology models described above. FlexScreen performs fully automated in silico screening of a large 3D database of ligands against a structurally resolved protein receptor. In this study we used two different protocols: in the automatic docking protocol: each ligand was docked against the receptor with the stochastic tunneling method using an all-atom representation of both ligand and receptor using a cascadic docking protocol. Both ligand and receptor can change their conformation in the docking process. SASA, the accessible surface area (ASA), is the surface area of a protein that is accessible to a solvent. In the relaxation protocol, we started from the known binding mode of one ligand, superimposed, as closely as possible related to ligands synthetically derived by altering one or more substituents from the original ligand and performed only one long relaxation simulation. At the end of each simulation, a binding energy for the ligand was computed as the difference between the unbound and bound complex using the biophysical scoring function of FlexScreen. The scoring function of FlexScreen was scaled by a constant reflecting the proportionality constant between measured K_i and computed binding energies.

■ ASSOCIATED CONTENT

S Supporting Information

Synthetic procedure and analytical data for all compounds; X-ray structures of compounds **29b** (CCDC-861561) and **31** (CCDC-861562); receptor models and docking studies,

correlation between the computed and measured K_i values, and correlation between K_i and EC_{50} values at CB_2 receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CB, cannabinoid; CHO cells, Chinese hamster ovary cells; nd, not determined; DMF, dimethylformamide; PSB, Pharmaceutical Sciences Bonn; SARs, structure−activity relationships; THC, tetrahydrocannabinol; TMEDA, N,N,N',N'-tetramethylethylendiamine

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