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One-pot heterogeneous synthesis of Δ^3-tetrahydrocannabinol analogues and xanthenes showing differential binding to CB_1 and CB_2 receptors

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ABSTRACT

Δ^9-tetrahydrocannabinol (Δ^9-THC) is the major psychoactive cannabinoid in hemp (*Cannabis sativa* L.) and responsible for many of the pharmacological effects mediated via cannabinoid receptors. Despite being the major cannabinoid scaffold in nature, Δ^9-THC double bond isomers remain poorly studied. The chemical scaffold of tetrahydrocannabinol can be assembled from the condensation of distinctly substituted phenols and monoterpenes. Here we explored a microwave-assisted one pot heterogeneous synthesis of Δ^3-THC from orcinol (**1a**) and pulegone (**2**). Four Δ^3-THC analogues and corresponding Δ^{4a}-tetrahydroxanthenes (Δ^{4a}-THXs) were synthesized regioselectively and showed differential binding affinities for CB_1 and CB_2 cannabinoid receptors. Here we report for the first time the CB_1 receptor binding of Δ^3-THC, revealing a more potent receptor binding affinity for the (*S*)-(−) isomer (hCB_1 K_i = 5 nM) compared to the (*R*)-(+) isomer (hCB_1 K_i = 29 nM). Like Δ^9-THC, also Δ^3-THC analogues are partial agonists at CB receptors as indicated by [^{35}S]GTPγS binding assays. Interestingly, the THC structural isomers Δ^{4a}-THXs showed selective binding and partial agonism at CB_2 receptors, revealing a simple non-natural natural product-derived scaffold for novel CB_2 ligands.

1. Introduction

Tethrahydrocannabinol (THC) is a naturally occurring tricyclic terpenophenolic natural product and the major psychoactive constituent of psychoactive hemp (*Cannabis sativa* L.). In nature it occurs in two isomeric forms, Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC) [1], both of which are psychoactive [2]. Overall, there are 7 double bond isomers and their 30 stereoisomers of the tetrahydrocannabinol scaffold. In the cannabis plant, THC occurs mainly as tetrahydrocannabinolic acid. In an enzymatically catalyzed reaction, geranyl pyrophosphate and olivetolic acid produce cannabigerolic acid which is cyclized by the enzyme THC acid synthase into Δ^9-THC acid. The carboxylate moiety in the aromatic ring stems from the olivetolic acid. Over time, or when heated, this natural product is decarboxylated to Δ^9-THC. Since the first isolation of Δ^9-THC and its seminal structure elucidation in 1964 [3], the interest in the total synthesis of THC-type cannabinoids [4,5] culminated with discovery of the cannabinoid receptors CB_1 and CB_2 [6,7] and the numerous findings about therapeutic and adverse effects mediated in particular via CB_1 receptors [8–14]. While numerous pharmacological studies have been performed on Δ^9-THC [1,2], little is known about the cannabinoid receptor interaction of Δ^9-THC double bond isomers and stereoisomers or the structurally similar non-natural analogues.

Among the various approaches to the synthesis of THC-like cannabinoids, the condensation between differently substituted phenols and monoterpenes was one of the first to be explored [15]. Here we employed this synthetic strategy to generate Δ^3-THC analogues regioselectively using the natural phenolic secondary metabolite orcinol (**1a**) and the widespread monoterpene pulegone (**2**) as starting materials, exploring yield and cannabinoid receptor binding affinity of the products.

The condensation between olivetol and **2** under acid catalysis for the preparation of Δ^3-THC, in its racemic form was investigated in the early 1940s [16]. Moreover, all the procedures indicated from the outset several drawbacks, such as the use of benzene, poor

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yields, long reaction times, harsh reaction conditions and homogeneous catalysis [15–19]. In the past, several attempts were made to obtain the enantiomers in a pure form. For instance, the synthesis and isolation of (*R*)-(+) Δ^3-THC and (*S*)-(−) Δ^3-THC was achieved in 1984 [20] but, even though the process of base-catalyzed isomerization gave good yields, it used Δ^9-THC as starting material which is legally restricted and difficult to obtain. The classic reaction of **1a** and **2** mediated by phosphorus oxychloride reported by Leaf et al. In 1942 [16] was revisited in 1968 by Chazan and Ourisson [18] who achieved the complete isolation and identification of all reaction products. The main products obtained were THC analogue (**3a**) and the tetrahydroxanthenes **4a** and **5a** (Suppl. Scheme 1), with isolated yields of 25%, 15% and 10% respectively, along with several side products.

The low regioselectivity and poor yields (up to 25%) showed by this reaction likely discouraged further exploration. Nevertheless, to date this approach represents the simplest way to synthesize THC analogues in a one-pot reaction. In addition, the availability of several substituted resorcinols (i.e. olivetol and orcinol) and (*R*)- or (*S*)-pulegone should make this approach more practical and easy. From the catalytic point of view, to our knowledge, Δ^3-THC syntheses by acidic heterogeneous catalysts have never been tested.

With the aim of evaluating the biological activities of Δ^3-THC and corresponding analogues, we explored the possibility to improve the yield and handleability of their syntheses by microwave-assisted heterogeneous catalysis.

2. Results and discussion

2.1. Chemistry

Two systems of number notation are commonly used for cannabinols, IUPAC dibenzopyran based numbering and monoterpene based numbering [21] (Suppl. Fig. 1). The two systems are often used simultaneously. For instance, Δ^9-THC and Δ^{6a}-tetrahydrocannabinol in the dibenzopyran numeration, are also called Δ^1-tetrahydrocannabinol and Δ^3-THC in monoterpene numeration. Here we refer to Δ^9-THC and Δ^{4a}-THX using the dibenzopyran (IUPAC) numeration and to Δ^3-THC using the monoterpenoid numeration (Scheme 1).

The presence of an acid catalyst is essential to obtain a successful condensation reaction of **2** and resorcinols for the preparation of THC–like compounds. Relying on our previous knowledge on organic synthesis mediated by heterogeneous acid catalysts, α-zirconium sulphophenyl phosphonate/methanphosphonate [22], ytterbium triflate ($Yb(OTf)_3$) [23] and sulfuric acid supported on silica gel [24] were selected as possible solid acid catalysts for the synthesis of Δ^3-THC analogues using resorcinol (**1b**) and **2** as educts.

In order to verify and compare the catalytic efficiency of the aforementioned solid acids, equimolar amounts of **1b** and **2** were allowed to react under conventional heating using either different organic solvents or in neat condition (Scheme 1).

Table 1
Reaction of resorcinol **1b** and pulegone **2** in the presence of optimized amount of catalyst under conventional heating.

Solvent[a]	T °C[b]	α-ZrP-SO_3H (12 mol %)[a]			H_2SO_4/SiO_2 (10 mol %)[a]			$Yb(OTf)_3$ (16 mol %)[a]		
		Time (day)	Yield[c]		Time (day)	Yield[c]		Time (day)	Yield[c]	
			3b	**4b**		**3b**	**4b**		**3b**	**4b**
DCM	40 R	2	25	3	2	35	–	2	45	5
DCE	84 R	2	35	5	2	32	–	2	51	6
D	101 R	7	–							
Neat	80	2	5							

[a] DCM: CH_2Cl_2, DCE: $(CH_2Cl)_2$, D: dioxane.
[b] R: reflux.
[c] Isolated yields (%).

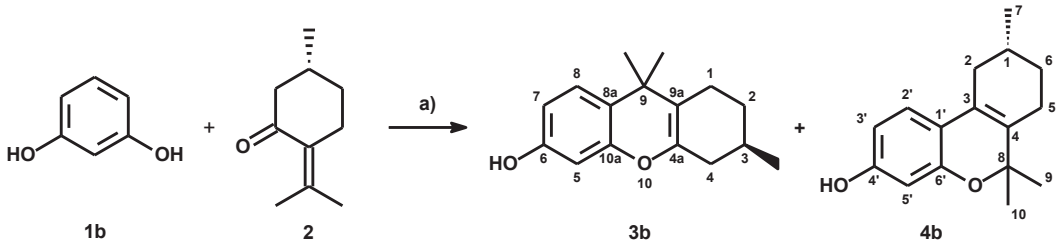
All experiments required long reaction times (up to 7 days). Surprisingly, under conventional heating the reaction of **1b** and **2** gave the corresponding Δ^{4a}-tetrahydro-1*H*-xanthene (Δ^{4a}-THX) **3b** as the main product (Table 1). The desired Δ^3-THC analogue **4b** was isolated in very poor yield (5%). Since halogenated solvents gave the best results in this preliminary test 1,2-dichloroethane was chosen for the reaction.

When the temperature plays a crucial role in a chemical process, more efficient heating system, such as microwave irradiation, can be exploited to increase yields and to reduce reaction time [25,26].

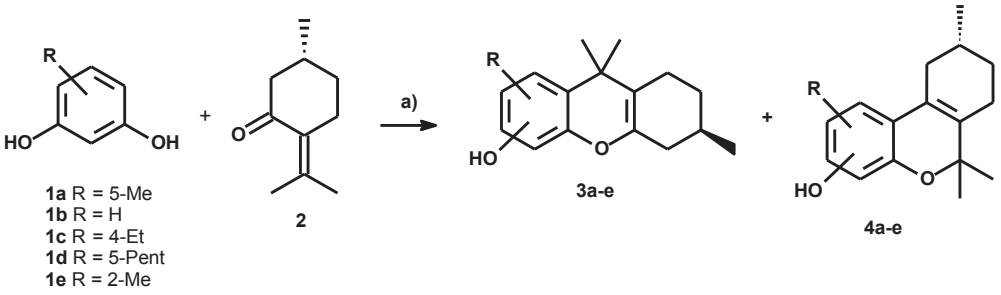
The role of the temperature and a possible improvement in the synthetic yield, the previous reaction setup was repeated under microwave irradiation in a sealed vial in 1,2-dichloroethane (Scheme 2, Table 2).

A general optimization of parameters was made to establish the best reaction conditions. The hermetically closed reaction environment allowed an evaluation of the temperature effect over the normal solvent boiling point. It was possible to explore a range of temperatures from 70 to 150 °C, reaching a maximum inner pressure of 13 atm. The reaction mixture was observed to turn into a very dark red coloration at the end of the reaction time. No internal cooling system was used in this instance. The optimal reaction time resulted 120 min. When the reactions were carried out for more than 120 h, an increase of by-products formation was observed along with a degradation of the desired products and a poor catalyst recovery. The results reported in Table 2 show an improvement in the yield of Δ^3-THC **4b** as a result of the increased temperature. Moreover, in this series, $Yb(OTf)_3$ showed a tendency to be the best performing catalyst and was readily available in our laboratory. In order to verify the effect of a substituent on the reactivity and regioselectivity of the reaction, the best reaction conditions were applied to different resorcinol derivatives (Scheme 2).

As shown in Table 3, the reaction yield of Δ^3-THC analogues was increased when C5-substituted resorcinols **1a** and olivetol (**1d**) were used as starting materials. Noteworthy, bioactive naturally



Scheme 1. Reagents and conditions: a) Catalyst, solvent, reflux (see Table 1 for detailed information).



Scheme 2. a) Catalyst, 1,2-dichloroehtane, Microwave Irradiation.

Table 2
Reaction of resorcinol **1b** and pulegone **2** in the presence of optimized amount of catalyst under microwave irradiation in 1,2-dichloroethane.[a]

T °C	α-ZrP-SO_3H (12 mol %)		H_2SO_4/SiO_2 (10 mol %)		$Yb(OTf)_3$ (16 mol %)	
	Yield (%)[b]		Yield (%)[b]		Yield (%)[b]	
	3b	**4b**	**3b**	**4b**	**3b**	**4b**
70	8	3	23	7	10	5
90	21	11	24	14	18	12
130	23	13	23	14	24	15
150	22	14	25	13	27	18

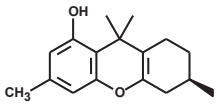
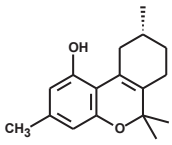
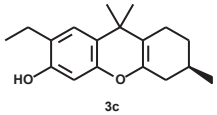
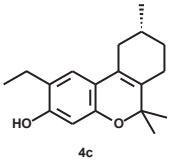
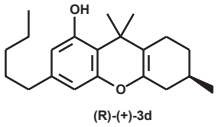
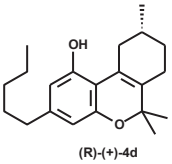
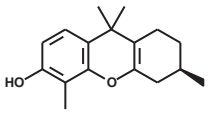
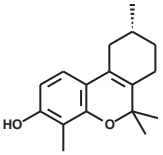
[a] Reaction time: 120 min.
[b] Isolated yields. Cooling OFF.

occurring THC analogues have an alkyl side chain at this position [27–31].

Intriguingly, 5-pentylresorcinol (olivetol, **1d**) was completely unreactive under conventional heating conditions, keeping constant all the other parameters, while under microwave irradiation it became the best starting material for the synthesis of the Δ^3-THC analogues. Although the use of **1a** and **1d** resulted in a more regioselective approach to the synthesis of Δ^3-THCs, the reaction yields (up to 23%) were not satisfactory. Under the above mentioned reaction conditions the Δ^{4a}-THX derivatives remained the main products in the case of using 5-unsubstituted resorcinols (Table 3, entries 2 and 4).

In view of the obtained results the attention was focused on the use of $Yb(OTf)_3$ with the aim to improve the yields of the Δ^3-THC analogues. Among several studies performed, the most promising

Table 3
Synthesis of tetrahydro-1*H*-xanthene and Δ^3-THC analogues from resorcinols and pulegone **2** in the presence of optimized amount of catalyst under microwave irradiation in 1,2-dichloroethane.

#	Main products		α-ZrP-SO_3H (12 mol %)		H_2SO_4/SiO_2 (10 mol %)		$Yb(OTf)_3$ (16 mol %)	
			(**3**) (%)[a]	(**4**) (%)[a]	(**3**) (%)[a]	(**4**) (%)[a]	(**3**) (%)[a]	(**4**) (%)[a]
1	 3a	 4a	18	10	20	12	12	17
2	 3c	 4c	15	9	18	7	27	8
3	 (R)-(+)-3d	 (R)-(+)-4d	18	19	11	11	15	23
4	 3e	 4e	15	3	19	5	19	6

Reaction temperature: 150 °C. Reaction time: 120 min.
[a] Isolated yields. Cooling OFF.

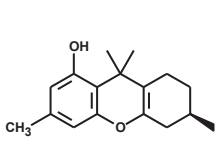
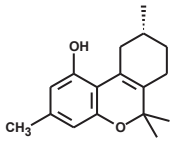
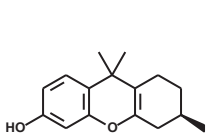
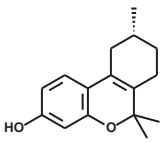
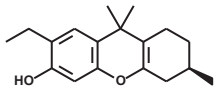
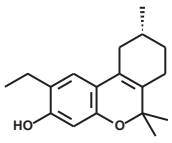
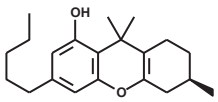
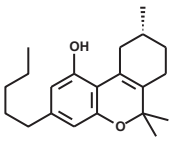
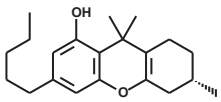
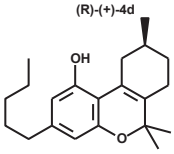
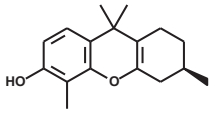
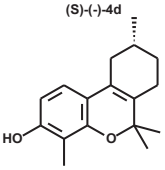
was the possibility to increase the catalytic capacity of $Yb(OTf)_3$ by the use of a co-catalyst. The combination of two components as catalytic system is known in organic synthesis and can produce several advantages [32,33].

The association of two acidic species as catalytic system has been studied and reported in the literature [34]. The concept of combined acids can be classified into Brønsted acid-assisted Lewis acid (BLA), Lewis acid-assisted Lewis acid (LLA), Lewis acid-assisted Brønsted acid (LBA), and Brønsted acid-assisted Brønsted acid (BBA) [35]. Under these premises, different organic and inorganic acids were tested in association with $Yb(OTf)_3$ and ascorbic acid was found to be the best performing. With reference to the above mentioned combined acids theory, $Yb(OTf)_3$ and ascorbic acid (named YTACA for short) can be defined as Lewis acid-assisted Brønsted acid (LBA) [36]. As shown in Table 4, the use of YTACA produced an overall improvement in the catalytic process, leading to a relevant increase of the reaction yield from 23% to 49% (see Table 4 for compound **4d**).

Moreover, the use of ascorbic acid as co-catalyst allowed to reduce the amount of $Yb(OTf)_3$ from 16 mol % to 2 mol %, with a great improvement in terms of the process economy. In addition, 1 mol % of the catalyst was still sufficient to catalyze the reaction when doubling the reaction time.

Unfortunately, despite the improvement of the reaction yields, YTACA did not allow a quantitative process. This is not addressable to the behavior of the catalyst but to the formation of unavoidable side products that lead to the consumption of the starting materials. In the presence of ascorbic acid alone the reactions between resorcinol derivatives and **2** did not take place. Also the addition of ascorbic acid to $ZrP{-}SO_3H$ and $H_2SO_4{-}SiO_2$ did not lead to any improvement in the reaction yield.

Table 4
Synthesis of tetrahydro-1*H*-xanthene and tetrahydrocannabinol analogues from resorcinols and pulegone **2** in the presence of $Yb(OTf)_3$ (2 mol %) and ascorbic acid (20 mol %) under microwave irradiation in 1,2-dichloroethane.

#		Main products		$Yb(OTf)_3$ (2 mol %)/ascorbic acid 1:10	
				Δ^{4a}-THX (3) (%)[a]	Δ^{3}-THC (4) (%)[a]
1		 3a	 4a	13 (12)[b]	43 (17)[b]
2		 3b	 4b	34 (27)[b]	12 (18)[b]
3		 3c	 4c	50 (27)[b]	11 (8)[b]
4	*i*	 (R)-(+)-3d	 (R)-(+)-4d	22 (15)[b]	49 (23)[b]
	ii	 (S)-(-)-3d	 (S)-(-)-4d	21	47
5		 3e	 4e	38 (19)[b]	8 (6)[b]

Reaction temperature: 150 °C. Reaction time: 120 min.
[a] Isolated yields. Cooling OFF.
[b] In brackets are reported the yields of the reactions carried out in the presence of $Yb(OTf)_3$ without the addition of ascorbic acid (See Table 3).

Table 5
K_i values for the binding of Δ^{4a}-THXs to CB receptors.

	Side chain (R)	CB_1 (K_i mean ± SE)	CB_2 (K_i mean ± SE)	[^{35}S]GTPγS EC_{50} on CB_2 (K_i mean ± SE)
3a	Me	>10 μM	>10 μM	n.d.
3b	H	>10 μM	4.2 ± 1.1 μM	n.d.
3c	Et	>10 μM	0.51 ± 0.21 μM	n.d.
3d (*S*)-(−)	Pent	2.1 ± 0.4 μM	0.18 ± 0.12 μM	0.82 ± 0.11 μM
3d (*R*)-(+)	Pent	1.1 ± 0.3 μM	0.47 ± 0.15 μM	0.93 ± 0.10 μM
3e	Me	>10 μM	1.0 ± 0.6 μM	n.d.
WIN55,212		2.5 ± 1.3 nM	0.45 ± 0.9 nM	n.d.
CP55,940		0.52 ± 0.08 nM	0.89 ± 0.04 nM	9.3 ± 8.1 nM

n.d. not determined.

The use of 5-substituted resorcinols (**1a** and **1d**) and **2** in the presence of YTACA and under microwave irradiation resulted in higher yields and regioselectivity for the Δ^3-THC analogues, keeping almost unchanged the yield of the Δ^{4a}-THX analogues (Table 4).

YTACA also improved the reactivity of 5-unsustituted resorcinols (Table 4, entries 2, 3, 5) yielding Δ^{4a}-THX derivatives as the main products.

This approach was successfully used also with (*S*)-(−) **2** and **1d** to generate the corresponding Δ^3-THC analogue (*S*)-(−) **4d**.

In microwave (MW) reactors equipped with an internal air-cooling system, the reaction can be carried out with the cooling mode ON at the lower temperature of 120 °C. The reaction afforded the final products with comparable isolated yields as with the cooling mode OFF at 150 °C. In MW-assisted syntheses there are two primary hypotheses about thermal and non-thermal MW effects ([37] and see Suppl. Methods for more details). We speculate that the two sets of condition may be equivalent for the final result. Given the comparability of the resulting yields, the lower temperature is more suitable and compliant with the presence of ascorbic acid in the catalytic association and resulted to be particularly important for the recyclability of the catalytic system. The recovered catalyst could be reused with a minimal refresh of ascorbic acid (20% of the initial amount of ascorbic acid), obtaining comparable reaction yields.

2.2. Characterization of Δ^3-THC analogues and corresponding Δ^{4a}-THX on cannabinoid receptor binding and function

Next we characterized the properties of the purified Δ^3-THC analogues (≥95% purity) and corresponding Δ^{4a}-THXs on cannabinoid receptor binding and function. As shown in Table 5, Δ^{4a}-THXs showed moderate binding interactions at CB_1 and CB_2 receptors. Despite the limited series of compounds, it is noteworthy that there is a 10-fold difference for CB_1 receptor affinity and 60-fold for CB_2 between the most active (**3d** isomers) and least active compounds in the series.

The major structural differences among those compounds occur in the positioning and the hydrophobicity of the substituents present on the tetrahydroxanthene scaffold. In terms of CB_1 binding, most of the molecules were inactive and only the derivatives bearing a pentyl chain at C-3 exhibited a certain affinity to the receptor. Interestingly, most of the Δ^{4a}-THXs showed a better interaction with CB_2 receptors, with the (*S*)-(−) **3d** and (*R*)-(+) **3d** stereoisomers being the best ligands with K_i values in the submicromolar range (0.47 ± 0.15 μM and 0.18 ± 0.12 μM for (*R*)-(+) **3d** and (*S*)-(−) **3d**, respectively). Interestingly, the replacement of the hydrophobic pentyl chain with the hydrophilic hydroxyl group at C-3 did not impair the CB_2 receptor binding, and the compounds bearing a short hydrophobic substituent (methyl or ethyl) in the neighbor positions showed a similar K_i value as the pentyl derivatives (0.51 ± 0.21 μM and 1.0 ± 0.6 μM for **3c** and **3e**, respectively) (Fig. 1A and B). (*R*)-(+) and (*S*)-(−) **3d** isomers were further investigated for their functional activity at CB_2 receptors by using [^{35}S]GTPγS binding assays. As shown in Fig. 1C the two isomers behaved as agonists, by inducing a concentration-dependent increase of [^{35}S]GTPγS binding. The EC_{50} values for (*S*)-(−) **3d** and (*R*)-(+) **3d** do not significantly differ between each other and are slightly (2–4 times) higher than the calculated K_i values for the receptor binding (Table 5). When compared with the full agonist CP55,940, the **3d** isomers show partial agonism at CB_2 receptors (Emax value (% of vehicle) of 152 ± 11, 167 ± 14 and 230 ± 22 for (*R*)-(+) **3d**, (*S*)-(−) **3d** and CP55,940, respectively) (Fig. 1C and Table 5).

We also characterized the effects of Δ^3-THC and analogues on CB_1 and CB_2 receptors. As shown in Table 6, compound **4b**, **4c** and

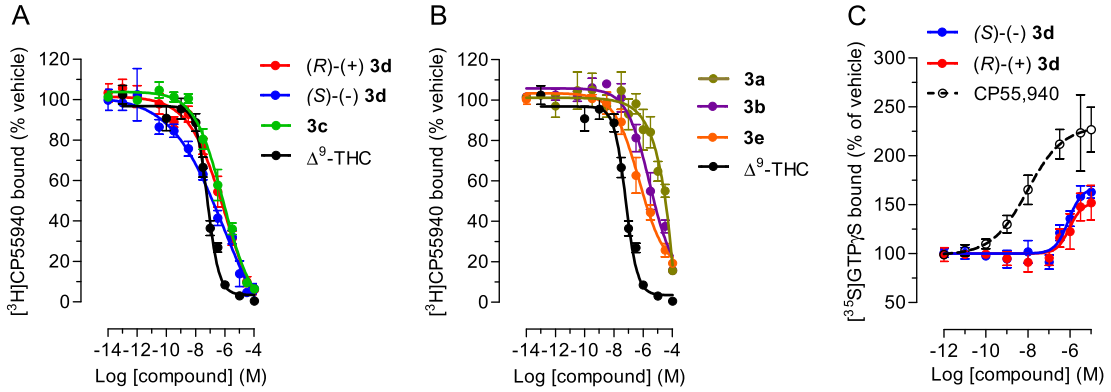


Fig. 1. Δ^{4a}-THXs binding and functional curves at CB_2 receptor. Concentration-dependent binding curve at CB_2 receptor for (A) the more potent (**3c**, (*S*)-(−) and (*R*)-(+) **3d**) and (B) less potent (**3a**, **3b** and **3e**) Δ^{4a}-THXs. Δ^9-THC was used as positive control. (C) [^{35}S]GTPγS binding induced by (*S*)-(−) **3d**, (*R*)-(+) **3d** and CP55,940 upon CB_2 activation. For A) and B) data were collected from 5 to 8 independent experiments, for C) data were collected from 3 independent experiments. All the experiments were carried out in triplicate and presented as mean ± S.E.

Table 6
K_i values for the binding of Δ^3-THCs to CB receptors.

	Side chain (R)	CB_1 (K_i mean ± SE)	[^{35}S]GTPγS on CB_1 (EC_{50} mean ± SE)	CB_2 (K_i mean ± SE)	[^{35}S]GTPγS on CB_2 (EC_{50} mean ± SE)
4a	Me	1.0 ± 0.2 μM	n.d.	0.66 ± 0.23 μM	n.d.
4b	H	>10 μM	n.d.	>10 μM	n.d.
4c	Et	>10 μM	n.d.	>10 μM	n.d.
4d (*S*)-(−)	Pent	5 ± 2 nM	87.5 ± 28.1 nM	17 ± 9 nM	10.9 ± 7.5 nM
4d (*R*)-(+)	Pent	29 ± 12 nM	126.5 ± 31.8 nM	18 ± 12 nM	17.4 ± 11.1 nM
4e	Me	>10 μM	n.d.	>10 μM	n.d.
Δ^9-THC	Pent	22 ± 13 nM	77.5 ± 30.4 nM	46 ± 10 nM	12.3 ± 6.8 nM
WIN55,212		2.5 ± 1.3 nM	n.d.	0.45 ± 0.9 nM	n.d.
CP55,940		0.52 ± 0.08 nM	3.9 ± 6.7 nM	0.89 ± 0.04 nM	9.3 ± 8.1 nM

n.d. not determined.

4e which lack the pentyl chain at C-3 and the phenolic hydroxyl group at C-1 did not show any significant binding, neither at CB_1 nor at CB_2.

The 3-methyl-1-hydroxy Δ^3-THC (**4a**) had a weak binding activity, at submicromolar concentrations, to both CB_1 and CB_2 receptors. Interestingly, compound (*R*)-(+) **4d** and (*S*)-(−) **4d** which bear the same substituents and in the same positions as Δ^9-THC, showed a potent binding to CB_1 and CB_2 receptors with the (*S*)-(−) isomer being more potent at CB_1 compared to the (*R*)-(+) isomer (K_i values: 5 ± 2 nM and 17 ± 6 nM for (*S*)-(−) **4d** and (*R*)-(+) **4d**, respectively). Both isomers also showed potent binding activities at CB_2 receptors, without significant difference between each other. Although we have investigated a limited set of compounds, we could observe an interesting preliminary structure–activity relationship in regard to CB_1 and CB_2 receptor binding. Compounds (*R*)-(+) **4d** and (*S*)-(−) **4d** resulted the best ligands for both receptors with an affinity that is comparable to Δ^9-THC (Fig. 2).

The two stereoisomers were also tested on [^{35}S]GTPγS binding assays to check their functional behavior at CB_1 and CB_2 receptors. The results showed that (*R*)-(+) and (*S*)-(−) **4d** activate CB_1 receptors with similar potencies and with a tendency of the (*S*)-(−) isomer to be more potent and efficient than the (*R*)-(+) isomer (Fig. 3A and Table 6). When compared with the full agonist CP55,940, both **4d** isomers behaved as partial agonists like Δ^9-THC

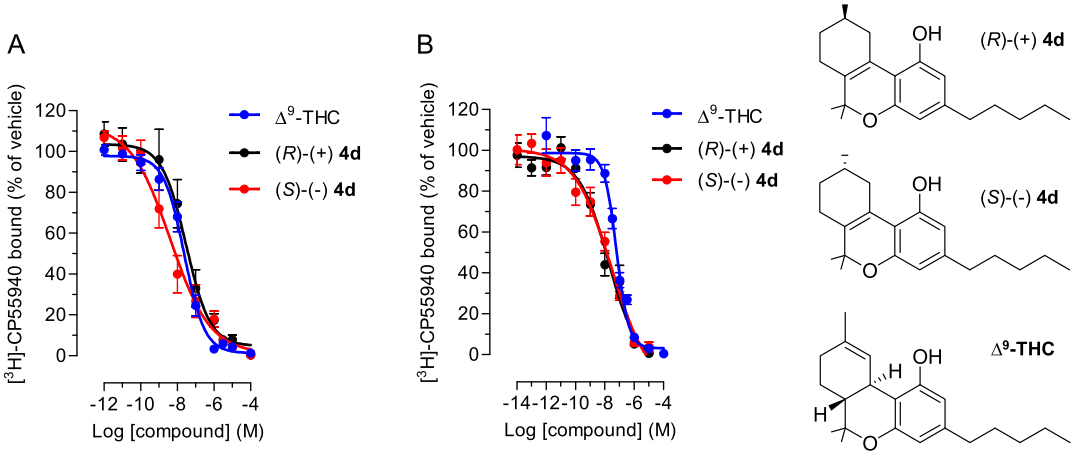


Fig. 2. (*R*)-(+) and (*S*)-(−) 4d binding curve at CB_1 and CB_2 receptor. Concentration-dependent binding curve at (A) CB_1 and (B) CB_2 receptor for (*R*)-(+) **4d**, (*S*)-(−) **4d** and Δ^9-THC. Data were collected from 5 to 6 independent experiments carried out in triplicate and presented as mean ± S.E.

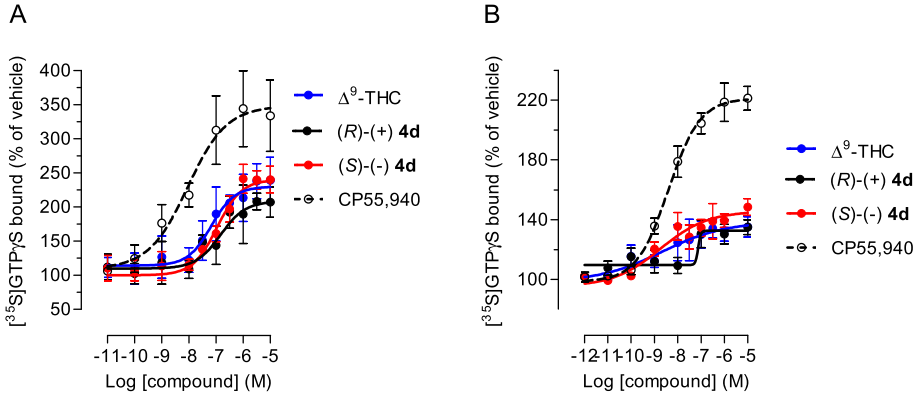


Fig. 3. [^{35}S]GTPγS binding induced by (*R*)-(+) **4d**, (*S*)-(−) **4d** and Δ^9-THC. Concentration-dependent [^{35}S]GTPγS binding curves at (A) CB_1 and (B) CB_2 receptor for (*R*)-(+) **4d**, (*S*)-(−) **4d** and Δ^9-THC. Data were collected from 4 to 5 independent experiments carried out in triplicate and presented as mean ± S.E.

(Emax value of 209 ± 16, 240 ± 10, 236 ± 12 and 348 ± 39 for (*R*)-(+) **4d**, (*S*)-(−) **4d**, Δ^9-THC and CP55,940, respectively) (Fig. 3A). (*R*)-(+) **4d**, (*S*)-(−) **4d** and Δ^9-THC also exerted partial agonistic effect at CB_2 receptors (Emax value of 133 ± 6, 146 ± 9, 139 ± 13 and 222 ± 9 for (*R*)-(+) **4d**, (*S*)-(−) **4d**, **Δ^9**-THC and CP55,940, respectively) (Fig. 3B and Table 6).

Despite the **4d** isomers have already been described more than twenty years ago, no biological and pharmacological investigation has been carried out. Interestingly, the only report on the functional activity of those molecules was performed in human volunteers [38]. In that study, both 1S and 1R Δ^3-THC were injected i.v. in six individuals and the "cannabis-like" effects became evident at doses of 4, 8 and 16 mg for the 1S isomer, while the 1R isomer elicited such effects only at 16 mg [38]. As in 1987, the cannabinoid receptors were not identified yet, this at least suggested that Δ^3-THCs behave similarly to Δ^9-THC, which was also included in the test, by interacting with a specific common cellular target. Unlike other THCs (i.e. Δ^8-THC [39,40]) no further investigation of Δ^3-THC activity at CB receptors was performed. We now describe for the first time the potent CB_1 binding of both (*R*)-(+) and (*S*)-(−) Δ^3-THC (**4d**), with the (*S*)-(−) isomer showing a 6-fold lower K_i value. Although in the [^{35}S]GTPγS assay we could not detect any significance difference, (*S*)-(−) **4d** and Δ^9-THC showed a tendency to be more potent and efficacious than (*R*)-(+) **4d**. In addition, other signal transduction pathways (e.g. β-arrestin and Ca^{2+}) could be differentially involved in the CB_1-mediated biological effects of **4d** isomers. This may account for the different potency in triggering the "cannabis-like" effects in human subjects [38]. In terms of the SAR for cannabinoid receptor binding, several studies have investigated the main structural requirements that make classical cannabinoids good or bad ligands for both CB_1 and CB_2 receptors. Among the main chemical features for CB_1 binding, the presence of a phenolic hydroxyl group at C-1 is usually essential as well as a suitable substituent and geometry at C-9 [41]. In general, an 11- or 11-nor-9-hydroxyl group enhances the affinity at CB_1 receptors [42–44]. The alkyl chain present at C-3 clearly plays a relevant role in the interaction with CB_1 receptors by eliciting an optimal binding when is composed by five to eight carbon atoms [45]. SAR data for classical cannabinoids on CB_2 receptors have been reported to minor degree, but some important features which make the compounds more selective for CB_2 have been suggested, based upon the observation that some ligands (i.e. JWH-051, JWH-057, 1-deoxy-Δ^8-THC analogues and L59633, see Suppl. Fig. 2 for chemical structures) are more selective for this cannabinoid receptor subtype [46]. In particular, the phenolic hydroxyl group at C-1 hinders CB_2 selectivity which is increased when it is replaced by hydrogen. Unlike for CB_1 receptors, the length of the alkyl chain at C-3 plays a marginal role for CB_2 binding [40,46]. The limited Δ^3-THCs SAR described in our study strictly follows these structural requirements. In fact, compound **4b**, **4c** and **4e**, which bear a hydroxyl group instead of an alkyl chain at C-3 and lack the hydroxyl group at C-1 resulted totally inactive at CB_1 and CB_2 receptors while only (*R*)-(+) **4d** and (*S*)-(−) **4d**, which show the same chemical features as Δ^9-THC possess a high affinity binding at both CB receptors. In keeping with this similarity in the binding potency, the two Δ^3-THC stereoisomers also behave as partial agonists at CB_1 and CB_2 like Δ^9-THC ([47,48] and our data).

Δ^{4a}-THXs and classical cannabinoids (THCs) are structural isomers and show a certain degree of similarity in the chemical structure. Based on this we assume that similar structural requirements described for classical cannabinoids would also be essential for Δ^{4a}-THXs binding at CB receptors. All Δ^{4a}-THXs resulted inactive or poorly active at CB_1. On the other side, we noticed an increasing affinity to CB_2 receptor depending on the type and position of the substituents on the tetrahydroxanthene scaffold. The most potent compounds (*S*)-(−) **3d** and (*R*)-(+) **3d** bear the pentyl chain at C-3 and the hydroxyl group at C-1 in agreement with the SAR described for classical cannabinoids. Interestingly, the presence of a methyl or an ethyl group in the *orto*-position of the hydroxyl group in C-3 increases the affinity at CB_2 (**3c** and **3e**), while the type of alkyl chain C-3 is not a strict requirement for the binding at CB_2 receptors.

Despite the landscape of CB_2 ligands has exploded over the last years, leading to the identification of numerous potent and selective receptor agonists, antagonists and inverse agonists [see [46,49] for review], our findings with the THX analogues open the possibility to investigate an alternative natural product-like scaffold as selective CB_2 ligands. We also describe that (*R*)-(+) **3d** and (*S*)-(−) **3d** behave as partial agonists, by increasing the CB_2-mediated activation of G-protein. A deeper investigation of the SAR for Δ^{4a}-THXs binding at cannabinoid receptors needs to be performed together with the characterization of the functional behavior for the main CB_2-mediated transduction pathways (i.e. cAMP, β-arrestin and Ca^{2+}). In the past decade it has become clear that the concept of agonist at a certain receptor does not exhaustively describe the pharmacological properties of a ligand. Different agonists at the same metabotropic receptor can transduce the signal by recruiting different pathways leading to a diverse pharmacology and/or toxicology [50]. For example, Δ^9-THC has been recently reported to trigger some of its CB_1-mediated behavioral effects by increasing COX-2 expression through the preferential activation of the G-protein βγ-subunit signaling, while 2-AG does the opposite (i.e. reducing the COX-2 expression) by recruiting the $G\alpha_i$-mediated signaling pathway [51]. Thus, new scaffolds with particular CB_2 receptor binding and functional properties may lead to the identification of molecules which exhibit new pharmacological features.

3. Conclusions

Ytterbium triflate-ascorbic acid (named YTACA) was used as association catalyst in the MW-assisted one pot synthesis of Δ^3-THC and analogues. The use of YTACA led to an improvement in the Δ^3-THC yield (up to 49%) and in the Δ^3-THC/Δ^{4a}-THX ratio (2:1) compared with previously reported data reported [16,18]. The heterogonous catalysis described in our article allowed a fast and regioselective preparation of the desired products in sufficient yields for full biological characterizations.

We show for the first time that Δ^3-THC binds to CB_1 and CB_2 receptors with a similar potency and exert partial agonism at both receptor subtypes like Δ^9-THC. In addition, we report that the (*S*)-(−) isomer shows a 6-fold higher affinity at CB_1 than the (*R*)-(+) isomer in agreement with previous functional results obtained in human volunteers. We also show that any changes in the type and position of the substituents on the THC scaffold leads to a loss of receptor binding. Importantly, we report for the first time the moderate but selective CB2 receptor binding properties for Δ^{4a}-THXs, which are structural isomers of Δ^3-THCs.

In future studies, a more detailed investigation of the SAR for Δ^{4a}-THXs binding at cannabinoid receptors needs to be performed as well as an *in vivo* pharmacological characterization of the two (*S*)-(−) and (*R*)-(+) Δ^3-THC isomers.

4. Experimental section

4.1. Materials and methods

All chemicals were purchased from the major chemical suppliers as highest purity grade and used without any further purification. Solvents were dried over standard drying agent and freshly

distilled prior to use. Column chromatography was performed by Merck silica gel 60 (70–230 mesh ASTM) with Hexane/Et_2O 98:2 as eluent, and monitored by TLC on silica gel 60 F254 with detection by charring with sulfuric acid/p-anisaldehyde 0.5%/0.5% in EtOH followed by heating at 110 °C. ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ with a Bruker Avance DPX 400 spectrometer at a frequency of 400 and 100 MHz, respectively. GC–MS analysis were obtained by HP-6890 gas chromatograph (dimethyl silicone column, 12.5 m) equipped with an HP-5973 mass-selective detector at an ionizing voltage of 70 eV.

GC–MS analysis were obtained with HP-6890 gas chromatograph (dimethyl silicone column, 12.5 m) equipped with an HP-5973 mass-selective detector at an ionizing voltage of 70 eV. Optical rotations ($[\alpha]_D$) were measured with a JASCO DIP-1000 digital polarimeter.

For HRESI-MS all samples were diluted with methanol in order to obtain stock solutions (5 ng/mL), to be analyzed by a Thermo Scientific LTQ-Orbitrap XL mass spectrometer equipped with an electrospray ion source (Thermo Scientific, Germany) and operated under Xcalibur 2.1 version software. Negative ionization mode for the MS analysis was used with data-dependent automatic switching between MS and MS/MS acquisition modes. The instrument was calibrated using the manufacturer's calibration standards. The scan was collected in the Orbitrap at a resolution of 30,000 in a m/z range of 150–800 amu. The source voltage was 4.5 kV and capillary voltage −35 kV was, the tube lens offset 126 V and the capillary temperature was set at 280 °C, auxiliary gas was set at 5 and no sheath gas was used.

HPLC analysis was performed using a HP 1100 system equipped with a UV/Vis detector with chiral column Lux Amilose-2 (chlorinated amylose phenylcarbamate) (250 × 4.6 mm ID). Eluent Hexane/Isopropanol 98:2. Flow 1 mL/min. Detection at wavelength 254 nm. All chromatograms were run at 25 °C.

Microwave-assisted reactions were carried out using a single-mode cavity dedicated reactor (Biotage Initiator™). Reactions were performed either with temperature or power-controlled programs in glass vials (0.5–2 mL, 2–5 mL or 5–25 mL, depending on the scale) sealed with a Teflon septum. Power-controlled experiments were performed with simultaneous cooling of the vials by means of pressurized air (maximum 15 bar). All temperatures were measured externally by an IR sensor. The reaction time was counted when the reaction mixture reached the stated temperature for temperature-controlled experiments. In the case of power controlled experiments the specified reaction time corresponds to the total irradiation time. Pressure was measured by a non-invasive sensor integrated into the cavity lid.

4.2. General procedure for THXs and Δ^3-THCs synthesis

In a microwave hermetically sealable vial an equimolar quantity of R-(+)-pulegone or S-(−)-pulegone and resorcinol derivatives **1a**–**e** are placed. 1,2-dichloroethane (1–4 mL) was used as solvent. The catalyst is added (either α-zyrconium sulphenylphosphonate [(α-$Zr(O_3PCH_3)_{1.2}(O_3PC6H_4SO_3H)_{0.8}$], sulfuric acid supported on silica gel [H_2SO_4–SiO_2], ytterbium triflate [$Yb(OTf)_3$], ytterbium triflate-ascorbic acid [YTACA 1:10]. The vial was sealed with its cap and a microwave irradiation (20–240 min, cooling OFF/ON, 90 °C–150 °C, 150–350 W mean irradiation depending on reaction temperatures) was applied. At the end of the programmed reaction time, the resulting mixture was filtered on a Buchner funnel in order to recover the catalyst and evaporated under vacuum. Efficient and complete separation of the major reaction product is achieved by column chromatography on silica gel, eluting with Hexane/Et_2O (98:2).

4.2.1. (9R)-3,9,9-trimethyl-2,3,4,9-tetrahydro-1H-xanthen-6-ol (**3b**)

Yield 34%. Yellow oil, $[\alpha]_D^{22.2}$ + 64.0° (c 0.17 in $CHCl_3$) ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 1.05 (d, J = 6.0 Hz, 3H, CH_3), 1.29 (m, 1H), 1.34 (s, 3H, CH_3), 1.36 (s, 3H, CH_3), 1.81 (m, 3H), 2.18 (m, 3H), 5.08 (bs, 1H, OH), 6.37 (d, J = 2.6 Hz, 1H, ArH–X), 6.53 (dd, J = 8.5, 2.6 Hz, 1H, ArH–Y), 7.16 (d, J = 8.5 Hz, 1H, ArH–Z). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 21.7, 22.6, 29.2, 29.4, 30.2, 31.6, 33.3, 35.5, 102.6, 110.4, 111.7, 123.3, 127.6, 142.1, 151.4, 154.5. **GC–MS *m/z***: 242, 217, 172, 145, 118, 102, 78. $C_{16}H_{20}O_2$ HRESI-MS: $[M-H]^-$, m/z 243.13853 calctd = 244.14633, purity = 98%.

4.2.2. (3R)-7ethyl-3,9,9-trimethyl-2,3,4,9-tetrahydro-1H-xanthen-6-ol (**3c**)

Yield 50%. Dark Yellow oil, $[\alpha]_D^{20.6}$ + 43.6° (c 0.16 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 1.04 (d, J = 6.0 Hz, 3H, CH_3), 1.24 (t, J = 7.5 Hz, 3H, CH_3) 1.28 (m, 1H), 1.35 (s, 3H, CH_3), 1.37 (s, 3H, CH_3), 1.81 (m, 3H), 2.21 (m, 3H), 2.61 (q, J = 7.5 Hz, 2H, CH_2), 4.89 (bs, 1H, OH), 6.31 (s, 1H, ArH), 7.02 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 14.7, 21.7, 22.6, 23.1, 29.2, 29.4, 30.2, 31.6, 33.3, 35.5, 102.3, 110.5, 122.8, 124.7, 126.9, 142.1, 149.4, 152.1. **GC–MS *m/z***: 272, 257, 241, 227, 215, 200, 128, 115. $C_{18}H_{24}O_2$ HRESI-MS: $[M-H]^-$, m/z 271.16923 calctd = 272.17763, purity = 98%.

4.2.2.1. (3R)-6-penthyl-3,9,9-trimethyl-2,3,4,9-tetrahydro-1H-xanthen-8-ol ((R)-(+)**3d**).

Yield 22%. Pale-brown oil, $[\alpha]_D^{24.0}$ + 36.0° (c 0.19 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 0.91 (t, J = 7 Hz, 3H, CH_3), 1.04 (d, J = 6.2 Hz, 3H, CH_3), 1.28 (m 7H, 3× CH_2, 1× CH) 1.52 (s, 3H, CH_3), 1.55 (s, 3H, CH_3), 1.61 (m, 2H, CH_2), 1.84 (m, 2H, CH_2), 2.22 (m, 2H, CH_2), 2.46 (t, J = 7.6 Hz, 2H, CH_2), 4.77 (bs, 1H, OH), 6.19 (d, J = 1.7 Hz, 1H, ArH) 6.31 (d, J = 1.7 Hz, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 14.4, 21.9, 22.2, 23.0, 26.6, 27.0, 29.4, 31.0, 31.90, 31.92, 33.6, 35.5, 35.6, 109.0, 111.1, 112.4, 113.9, 140.3, 142.7, 151.7, 154.6. **GC–MS *m/z***: 314, 299, 285, 271, 257, 243, 228, 218, 205, 189, 125, 96. $C_{21}H_{30}O_2$ HRESI-MS: $[M-H]^-$, m/z 313.21632 calctd = 314.22445, purity = 98%.

4.2.2.2. (3S)-6-penthyl-3,9,9-trimethyl-2,3,4,9-tetrahydro-1H-xanthen-8-ol ((S)-(−)**3d**).

Yield 22%. Pale-brown oil, $[\alpha]_D^{24.0}$−35.6° (c 0.78 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 0.91 (t, J = 7 Hz, 3H, CH_3), 1.04 (d, J = 6.2 Hz, 3H, CH_3), 1.28 (m 7H, 3× CH_2, 1× CH) 1.52 (s, 3H, CH_3), 1.55 (s, 3H, CH_3), 1.61 (m, 2H, CH_2), 1.84 (m, 2H, CH_2), 2.22 (m, 2H, CH_2), 2.46 (t, J = 7.6 Hz, 2H, CH_2), 4.77 (bs, 1H, OH), 6.19 (d, J = 1.7 Hz, 1H, ArH) 6.31 (d, J = 1.7 Hz, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 14.4, 21.9, 22.2, 23.0, 26.6, 27.0, 29.4, 31.0, 31.90, 31.92, 33.6, 35.5, 35.6, 109.0, 111.1, 112.4, 113.9, 140.3, 142.7, 151.7, 154.6. **GC–MS *m/z***: 314, 299, 285, 271, 257, 243, 228, 218, 205, 189, 125, 96. $C_{21}H_{30}O_2$ HRESI-MS: $[M-H]^-$, m/z 313.21553 calctd = 314.22458, purity = 98%.

4.2.3. (3R)-3,5,9,9-tetramethyl-2,3,4,9-tetrahydro-1H-xanthen-6-ol (**3e**)

Yield 38%. Yellow oil, $[\alpha]_D^{23.1}$ + 53.6° (c 0.35 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 0.99 (d, J = 6.0 Hz, 3H, CH_3), 1.23 (m, 1H), 1.28 (s, 3H, CH_3), 1.30 (s, 3H, CH_3), 1.78 (m, 3H), 2.10 (s, 3H, CH_3), 2.15 (m, 3H), 4.72 (bs, 1H, OH), 6.47 (d, 1H, J = 8.5 Hz, ArH), 6.97 (d, 1H, J = 8.5, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 8.1, 21.4, 22.3, 28.9, 29.0, 29.9, 31.4, 33.3, 35.2, 109.3, 110.1, 111.2, 122.6, 123.5, 142.0, 149.1, 152.1. **GC–MS *m/z***: 258, 243, 227, 216, 201, 189, 175, 115. $C_{17}H_{22}O_2$ HRESI-MS: $[M-H]^-$, m/z 257.15363 calctd = 258.16198, purity = 98%.

*4.2.4. (9R)-6,6,9-trimethyl-7,8,9,10-tetrahydro-6H-benzo[c] chromen-3-ol (**4b**)*

Yield 12%. Pale yellow oil, $[\alpha]_D^{22.2}$ + 98.6° (c 0.18 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.28 (m, 2H, CH_2), 1.32 (s, 3H, CH_3), 1.43 (s, 3H, CH_3), 1.79 (m, 2H, CH_2), 1.93, (m, 1H, CH), 2.11 (m, 1H, CH), 2.43 (m, 1H, CH), 4.97 (bs, 1H, OH), 6.35 (d, J = 2.8 HZ, 1H, ArH), 6.38 (dd, J = 2.8, 8.3 Hz, 1H, ArH), 6.99 (d, J = 8.3 Hz, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 21.9, 24.5, 25.5, 25.7, 28.3, 30.9, 32.9, 78.6, 103.6, 107.5, 117.9, 122.7, 122.8, 131.3, 153.2, 155.5. **GC–MS *m/z***: 244, 229, 214, 202, 174, 136, 118, 102, 78. $C_{16}H_{20}O_2$ HRESI-MS: $[M-H]^-$, m/z 243.13829 calctd = 224.14633, purity = 98%.

*4.2.5. (9R)-2-ethyl-6,6,9-trimethyl-7,8,9,10-tetrahydro-6H-benzo [c]chromen-3-ol (**4c**)*

Yield 11%. Yellow oil, $[\alpha]_D^{22.7}$ + 19.9° (c 0.17 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 1.09 (d, J = 6.5 Hz, 3H, CH_3), 1.23 (t, J = 2.5 Hz, 3H, CH_3), 1.27 (m, 2H, CH_2), 1.31 (s, 3H, CH_3), 1.42 (s, 3H, CH_3), 1.80 (m, 2H, CH_2), 1.96, (m, 1H, CH), 2.13 (m, 1H, CH), 2.45 (dd, J = 4.4, 16.3 Hz, 1H, CH), 2.59 (q, J = 7.5, 2H, CH_2), 4.72 (bs, 1H, OH), 6.35 (d, J = 2.8 HZ, 1H, ArH), 6.29 (s, 1H, ArH), 6.88 (s, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 14.9, 22.3, 23.0, 25.2, 25.6, 26.1, 28.7, 31.4, 33.4, 78.6, 103.8, 118.1, 122.0, 122.9, 123.1, 131.6, 151.5, 153.2. **GC–MS *m/z***: 272, 257, 243, 230, 215, 202, 136, 115, 94. $C_{18}H_{24}O_2$ HRESI-MS: $[M-H]^-$, m/z 271.07982 calctd = 272.17763, purity = 98%.

*4.2.6. (9R)-4,6,6,9-tetramethyl-7,8,9,10-tetrahydro-6H-benzo[c] chromen-3-ol (**4e**)*

Yield 8%. Yellow oil, $[\alpha]_D^{24.5}$ + 49.3° (c 0.25 in $CHCl_3$), ^1H NMR (400 MHz, $CDCl_3$, 25 °C, TMS) δ = 1.07 (d, J = 6.5 Hz, 3H, CH_3), 1.27 (m, 2H, CH_2), 1.30 (s, 3H, CH_3), 1.44 (s, 3H, CH_3), 1.79 (m, 2H, CH_2), 1.95, (m, 1H, CH), 2.12 (s, 3H, CH_3), 2.13 (m, 1H, CH), 2.40 (m, 1H, CH), 4.77 (bs, 1H, OH), 6.38 (d, J = 8.3 HZ, 1H, ArH), 6.87 (d, J = 8.3 Hz, 1H, ArH). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C, TMS) δ = 7.9, 21.9, 24.9, 25.1, 25.7, 28.3, 30.9, 33.0, 78.2, 106.7, 111.4, 117.7, 119.4, 123.0, 131.0, 150.9, 153.5. **GC–MS *m/z***:258, 243, 232, 227, 216, 202, 189, 176, 112, 94, 70, 58. $C_{17}H_{22}O_2$ HRESI-MS: $[M-H]^-$, m/z 257.15311 calctd = 258.161980, purity = 98%.

*4.2.7. (3R)-3,6,9,9-tetramethyl-2,3,4,9-tetrahydro-1H-xanthen-8-ol (**3a**)*

Yield 13%, (9R)-3,6,6,9-tetramethyl-7,8,9,10-tetrahydro-6*H*-benzo[*c*]chromen-3-ol (**4a**) yield 43%, (R)-(+)-Δ^3-THC (**4d**) yield 49%, (S)-(−) Δ^3-THC (**4d**) yield 47%: data in accordance to the literature [18,20,38]. Purity of **3a**, **4a**, (R)-(+) and **4d** (S)-(−)**4d** resulted to be 98%.

4.3. Radioligand binding assays on cannabinoid receptors

Receptor-binding experiments were performed with membrane preparations as previously reported [52]. Briefly, 18 μg of crude membrane expressing hCB$_1$ or hCB$_2$ receptors (prepared as described earlier) were re-suspended in 300 μL of binding buffer (50 mM Tris–HCl, 2.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mg/mL fatty acid free BSA, pH 7.4) in silanized glass tubes and co-incubated different concentrations (10^{-13}–10^{-4} M) of Δ^3-THCs, Δ^{4a}-THXs or vehicle and 0.5 nM of [^{3}H]-CP-55940 (168 Ci/mmol) for 2 h at 30 °C. Non-specific binding of the radioligand was determined in presence of 10 μM of WIN55,512-2. Non-specific binding was around 5%. After the incubation time, membrane suspensions were rapidly filtered through a 0.5% polyethyleneimine pre-soaked 96-well microplate equipped with GF/B glass fiber filters (UniFilter-96 GF/B, PerkinElmer Life Sciences) under vacuum and washed 12 times with 150 μL of ice-cold washing buffer (50 mM Tris–HCl, 2.5 mM EGTA, 5 mM $MgCl_2$, 0.5% fatty acid free BSA, pH 7.4). Filters were added to 45 μL of MicroScint20 scintillation liquid and radioactivity measured with the 1450 MicroBeta Trilux top counter (PerkinElmer Life Sciences). Data were collected from at least three independent experiments performed in triplicate and the non-specific binding was subtracted. Results were expressed as [^{3}H]-CP55940 bound in % of binding to vehicle-treated samples. K_i values were calculated applying the Cheng–Prusoff equation. IC_{50} values were derived from the concentration-dependent curves.

4.4. [^{35}S]GTPγS binding assay

The assay was carried out using 5 μg of clean membrane prepared in-house from CHO-hCB$_2$ and CHO-hCB$_1$ cells as previously reported [52]. Membranes were diluted in silanized plastic tubes with 200 μL of GTP*γ*S binding buffer (50 mM Tris–HCl, 3 mM $MgCl_2$, 0.2 mM EGTA and 100 mM NaCl at pH 7.4 supplemented with 0.5% of BSA fatty acid free) in the presence of 10 μM of GDP and 0.1 nM of [^{35}S]-GTP*γ*S 1250 Ci/mmol). The mixture was kept on ice until the binding reaction was started by adding the tested compound, vehicle (negative control) or CP55940 (positive control). Non-specific binding was measured in presence of 10 μM GTP*γ*S (Sigma). The tubes were incubated at 30 °C for 90 min under shaking and then they were put on ice to stop the reaction. An aliquot (185 μL) of the reaction mixture was rapidly filtered through a 96-well microplate bonded with GF/B glass fiber filters (UniFilter-96 GF/B, PerkinElmer Life Sciences) previously pre-soaked with ice-cold washing buffer (50 mM of Tris–HCl pH 7.4 plus of 0.1% BSA fatty acid free). The filters were washed 6 times with 180 μL of washing buffer under vacuum and let them dry under the air drier flow. The radioactivity was measured with the 1450 Microbeta WallacTrilux Top counter after the addition of 45 μL of scintillation cocktail. Specific binding was calculated by subtracting the residual radioactivity signal obtained in presence of an excess of GTPγS and the results were expressed as % of vehicle control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.07.062.

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