ELSEVIER

Chemistry and Physics of Lipids
108 (2000) 37–52

CPL
CHEMISTRY AND PHYSICS OF LIPIDS

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Review

Molecular probes for the cannabinoid receptors

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Received 12 July 2000; received in revised form 18 July 2000; accepted 18 July 2000

Abstract

Cannabinoids produce most of their biochemical and pharmacological effects by interacting with CB1 and CB2 cannabinoid receptors, both of which are G-protein coupled membrane-bound functional proteins. CB1 is found in the central nervous system and in a variety of other organs including heart, vascular endothelium, uterus, vas deferens, testis and small intestine. Conversely, the CB2 receptor appears to be associated exclusively with the immune system and is found in the periphery of the spleen and other cells associated with immunochemical functions. Although both CB1 and CB2 have been cloned and the primary sequences are known, their three dimensional structures and the amino acid residues at the active site, critical for ligand recognition, binding and activation have not been characterized. In the absence of any X-ray crystallographic and NMR data, information on the structural requirements for ligand–receptor interactions is obtained with the help of suitably designed molecular probes. These ligands either interact with the receptor in a reversible fashion (reversible probes) or, alternatively, attach at or near the receptor active site with the formation of a covalent bond (irreversible probes). Subsequently, information related to ligand binding and receptor activation is further amplified with the help of receptor mutants and computer modeling. This review focuses on molecular probes related to the classical and non-classical cannabinoids that have been reported since the discovery of the first cannabinoid receptor over a decade ago.

Keywords: Classical cannabinoids; Cannabinoid receptors; Structure–activity relationships

Abbreviations: TMH, transmembrane helix; GPCR, G-protein coupled receptor; AEA, arachidonoylethanolamide; AG, arachidonoylglycerol; DMH, dimethylheptyl; HHC, hexahydrocannabinol; THC, tetrahydrocannabinol; SAR, structure–activity relationship.

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0009-3084/00/$ - see front matter
PII: S0009-3084(00)00186-9

1. Introduction

The discovery of the first cannabinoid receptor just over a decade ago has opened new horizons in cannabinoid research. To date, two such receptors, CB1 (Devane et al., 1988) and CB2 (Munro et al., 1993), have been identified and believed to be responsible for many of the biochemical and pharmacological effects produced by (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the active constituent of cannabis (Gaoni and Mechoulam, 1964) and its numerous synthetic congeners. CB1 and CB2 are membrane-bound G-protein coupled receptors (GPCR) with the characteristic seven transmembrane domains. CB1 is mainly found in the central nervous system with high density in the cerebellum, hippocampus and striatum (Herkenham et al., 1990; Herkenham, 1991; Mailleux et al., 1992; Matsuda et al., 1993). It is also found in the peripheral tissues such as testis (Gerard et al., 1991), small intestine (Pertwee et al., 1992, 1996a), urinary bladder (Pertwee and Fernando, 1996), vas deferens (Pertwee et al., 1992, 1996b), sperm (Chang et al., 1993) and uterus (Paria et al., 1998). On the other hand, the CB2 cannabinoid receptor is located outside the CNS primarily in the cells of immune system such as macrophages and B cells (Munro et al., 1993). The two receptors share an overall homology of ~44 and 68% in the transmembrane domains. The rat (Matsuda et al., 1990), mouse (Chakrabarti et al., 1995; Abood et al., 1997) and human (Gerard et al., 1990) CB1 receptors have been cloned and show 97–99% sequence identity across species while the mouse (Shire et al., 1996a,b) exhibit 82% sequence identity with the human CB2 (Munro et al., 1993).

A second area of cannabinoid research where significant progress has been made is the discovery of novel classes of cannabimimetic ligands which also include two families of endogenous ligands represented by anandamide (Devane et al., 1992a,b) and 2-arachidonoylglycerol (Mechoulam et al., 1995; Sugiura et al., 1995; Stella et al., 1997). Prior to the discovery of cannabinoid receptors, a number of independent research laboratories and pharmaceutical industries developed a large number of synthetic ligands as pharmacological and biochemical probes for studying cannabinoid biology and also as prototypes for developing new medications. All of the early analogs were classical cannabinoids and many of them were considerably more potent than the naturally occurring compounds (Razdan 1986;

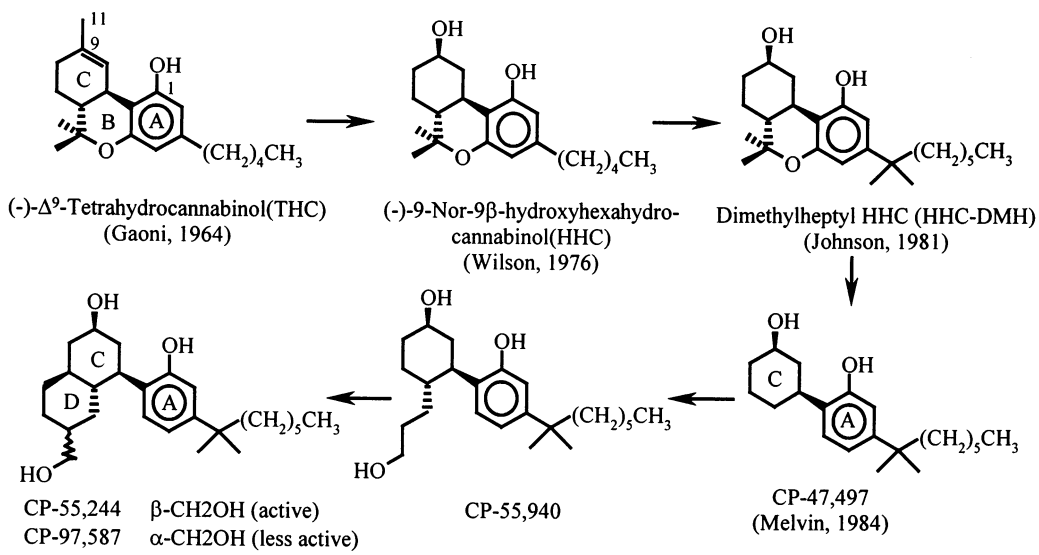


Fig. 1. Evolution in cannabinoid structures with progressively enhanced potencies.

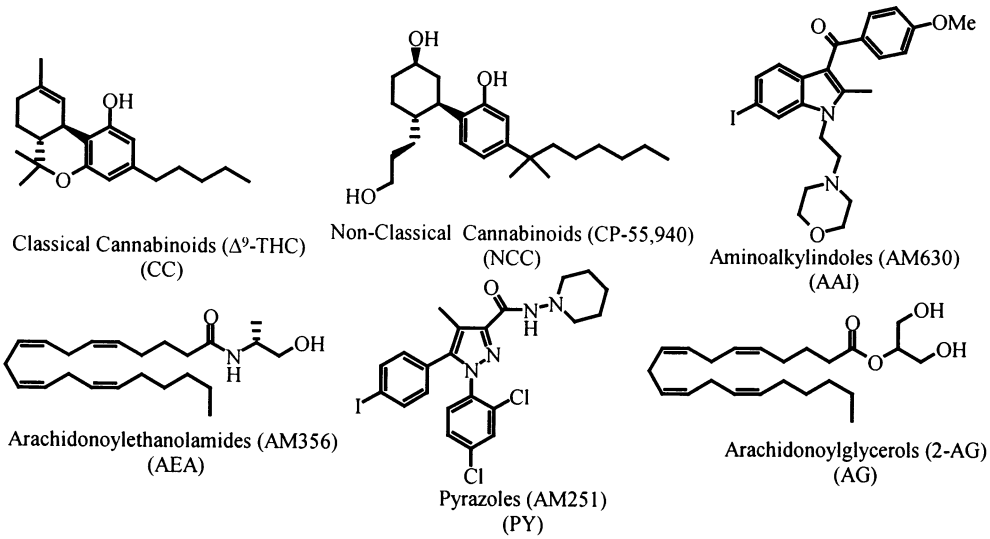


Fig. 2. Representative ligands from the six classes of cannabimimetic agents.

Makriyannis and Rapaka 1990). The evolution of such ligands from Δ^9-THC, the original prototypic cannabinoid, is depicted in Fig. 1. However, discovery of cannabinoid receptors has led to the development of structurally more diverse groups of analogs possessing similar pharmacological properties. Currently six major structurally diverse classes of cannabimimetics have been identified: (1) classical cannabinoids; (2) non-classical cannabinoids; (3) aminoalkylindoles; (4) arachidonoylethanolamides; (5) biarylpyrazoles; and (6) 2-arachidonoylglycerols (Fig. 2). Of these, the aminoalkylindoles (AAIs) were developed by Sterling Winthrop following leads from their program on non-steroidal anti-inflammatory agents and were the first non-cannabinoid ligands for CB1 and CB2 (D'Ambra et al., 1992). Biarylpyrazoles represented by SR141716A were the first group of cannabinoid receptor antagonists developed by Sanofi from their compound libraries using high-throughput screening (Rinaldi-Carmona et al., 1994).

Although there have been major advances in our understanding of cannabinoid biology and the availability of novel cannabimimetic ligands, the tertiary structure of cannabinoid receptors and the chemical nature of the amino acid residues involved in ligand binding are not yet known. Information regarding the structural features of a ligand that are associated with receptor recognition, activation, and deactivation is of great value in designing cannabinoid ligands with high affinities and selectivities. Reggio and coworkers have reported (Bramblett et al., 1995) a theoretical 3D model of the CB1 receptor using rhodopsin as the prototype GPCR. Based on this model, they have proposed a three-point agonist interaction between a cannabinoid ligand and the amino acid residues in TMH3, TMH5 and TMH6. Being membrane-bound proteins, cannabinoid receptors are difficult to isolate in pure form to obtain their crystal structures. For this reason, synthetic molecular probes incorporating specific pharmacophoric features have been used to explore the tertiary structure and ligand binding domain(s) of the cannabinoid receptors. These probes are either reversible or irreversible (covalent) probes. The former bind reversibly to the receptor while the latter form a covalent bond with the receptor, subsequent to the receptor binding. This review will focus on the molecular probes of the classical cannabinoid type compounds that have been reported since the discovery of the first cannabinoid receptor CB1 about a decade ago.

2. Reversible (non-covalent) cannabinoid receptor probes

The discovery of the new classes of cannabimimetic ligands has broadened the scope of cannabinoid medicinal chemistry. However, there is still a considerable amount of interest in developing more advanced classical cannabinoid prototypes. With the discovery of the CB2 receptor (Munro et al., 1993), research efforts have focused on developing receptor-selective cannabinoid analogs. Surprisingly, none of the existing cannabinoid ligands exhibit significant selectivities for CB1 and CB2. However, in the past few years several new classical highly potent cannabinoid analogs with very good receptor selectivity have been reported. Prior to the discovery of cannabinoids, structure–activity relationships (SAR) were studied using a variety of in vivo behavioral tests in mouse and dog models (Dewey 1986; Razdan 1986). At present, however, cannabinoid receptor binding assays using either native or cloned wild-type receptor preparations are routinely used in a high throughput mode to assess the affinities and selectivities of cannabinoid analogs.

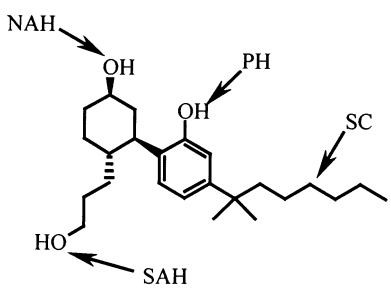
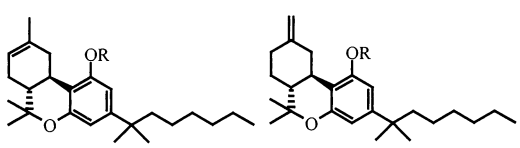


Fig. 3. The four cannabinoid pharmacophores.



1 **5**

		K_i (nM)[a]	
		CB_1	CB_2
1	R=H	0.83	0.49
	R=CH_3	15,850	20
5	R=H	1.82	0.58
	R=CH_3	>20,000	19.4

[a]hCB_1, hCB_2 receptors; vs. [^{3}H]CP-55,940

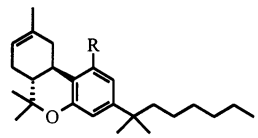
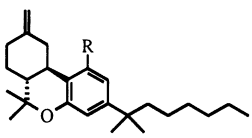
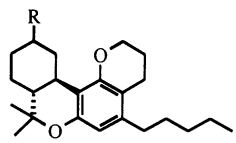
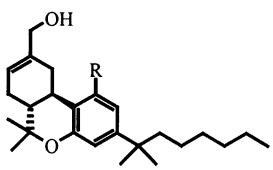
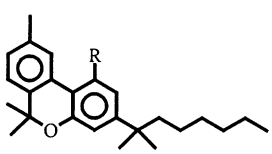
Fig. 4. Methyl ethers of classical cannabinoids with dimethylheptyl side chain possessing CB2-selectivity.

There are four pharmacophores (Fig. 3) in classical cannabinoids that are important for cannabimimetic activity: (1) a phenolic hydroxyl (PH); (2) a lipophilic alkyl side chain (SC); (3) a northern aliphatic hydroxyl (NAH); and (4) a southern aliphatic hydroxyl (SAH). The first two pharmacophores, PH and SC, are found in the plant-derived cannabinoids such as (−)-Δ^9-THC, the active principle of marijuana, while all four pharmacophores are found in the synthetic nonclassical cannabinoids, e.g. CP-55,940 (Fig. 2).

2.1. The phenolic hydroxyl group (PH)

Until recently, it was thought that a phenolic hydroxyl group (PH) at the C-1 position is essential for cannabimimetic activity. Analogs lacking this group, alkyl ethers and esters were either devoid of or had weak cannabimimetic activity (Razdan 1986). It is believed that the phenolic OH is involved in hydrogen bonding interaction with the CB1 cannabinoid receptor. Indeed, Song and Bonner have reported that mutation of lysine 192 to alanine in the wild-type CB1 receptor diminished affinity for classical, non-classical cannabinoids and anandamide (Song and Bonner 1996). However, the requirement of a phenolic hydroxyl group for cannabimimetic activity has recently come under scrutiny after the discovery of the CB2 cannabinoid receptor (Fig. 4). It was thus reported that cannabinoid analogs in which the phenolic hydroxyl was either absent or had a methyl substituent showed significant affinity and high selectivity for the peripheral CB2 receptor (Gareau et al., 1996). Thus, Gareau et al. found that 1-deoxy-DMH-Δ^8-THC (**2**) has ~12-fold selectivity for human CB2 which increases to ~800-fold in its methyl ether (**3**). Similarly for $\Delta^{9,11}$-DMH-THC (**5**), the corresponding methyl ether (**6**) exhibits more than 1000-fold CB2-selectivity. Similarly, Khanolkar et al. observed increased CB2 selectivity in the case of

Table 1
Modifications of the phenolic hydroxyl (PH)

Analog	R	K_i nM		CB1/CB2	Reference
		CB1	CB2		
	OH (**1**) H (**2**) OCH_3 (**3**) $OP(O)(OEt)_2$ (**4**)	0.83 249.7 15,850 >20,000	0.49 20.8 20.0 441.7	1.7 12 793 >45	Gareau et al 1996[a]
	OH (**5**) OCH_3 (**6**)	1.82 >20,000	0.58 19.4	3.1 >1000	Gareau et al 1996[a]
	Δ^9-CH_3 (**7**) $=CH_2$ (**8**) =O (**9**) β-OH (**10**)	41 884 90 26	36 200 23 5.8	1.1 4.4 3.9 4.5	Reggio et al. 1997[b]
	OH (**11**) H (**12**)	0.73 1.2	0.53 0.032	1.4 38	Huffman et al. 1996[b]
	OH (**13**) OCH_3 (**14**) OCH_2OCH_3 (**15**)	0.95 4188 1141	1.52 155 5718	0.63 27 5	Khanolkar and Makriyannis; unpublished results[c]

[a]hCB1, hCB2; [b]rat CB1, hCB2; [c]rat CB1, mouse CB2. [^{3}H] CP-55,940 was used as a radioligand in all receptor binding assays

dimethylheptylcannabinol analogs (**13**–**15**) (Khanolkar and Makriyannis, unpublished results). More recently, Reggio et al have shown that *O*,2-propano-Δ^8-THC analogs (**7**–**10**) possess modest CB_2-selectivity (Reggio et al., 1997). The authors speculate that this could be due to differences in solvation/desolvation when the ligand enters the receptor binding site or due to a change

Table 2
Modifications of the alkyl side chain (SC)

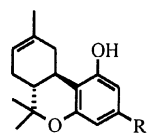
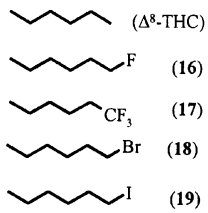
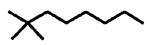
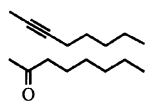
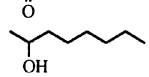
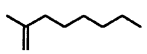
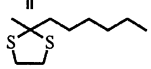
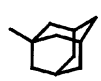
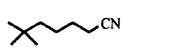
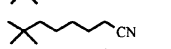
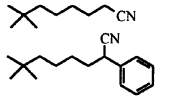
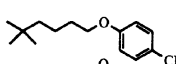
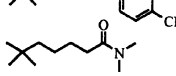
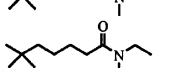
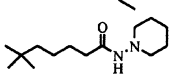
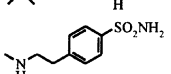
Analog	R	K_i, nM[a]		CB1/CB2	Reference
		CB1	CB2		
	 (Δ^8-THC)	122	ND	-	Charalambous et al. 1991
	(16)	45	ND		
	(17)	19.4	ND	-	
	(18)	6.0	ND	-	
	(19)	6.1	ND	-	
	 (20)	0.89	1.41	0.79	Martin et al. 1993
	 (21)	0.65	3.1	0.21	Papahatjis et al. 1998
	(22)	21.7	83.7	0.26	
	 (23)	86.4	65.6	1.3	
	 (24)	2.17	3.3	0.66	
	 (25)	0.32	0.52	0.62	
	 (26)	6.86	52	0.13	Makriyannis et al. Unpublished results
	 (27)	0.36	ND	-	Singer et al. 1999
	 (28)	0.60	ND	-	
	 (29)	9.2	ND	-	
	 (30)	3.0	ND	-	
	 (31)	0.86	ND	-	
	 (32)	13	ND	-	
	 (33)	1.2	ND	-	
	 (34)	29	ND	-	

Table 2 (*Continued*)

	(35)	5.8	61.6	0.09	Busch-Petersen,1996
	(36)	0.8	9.5	0.08	
	(37)	1.2	5.3	0.23	
	(38)	8.7	14.3	0.61	
	R_1= (39)	703	ND	-	Huffman et al 1998
	R_1= (40)	402	162	2.48	Khanolkar et al 1999
	R_2= (41)	22.3	58.6	0.38	

[a] rat brain CB1, mouse spleen CB2; vs. [^{3}H] CP-55,940

in amino acid from hydrogen bond-accepting in CB1 to hydrogen-bond donating in the CB2 receptor. However, results reported by Huffman et al. have confounded the situation. They reported that 1-deoxy-11-hydroxy-DMH-Δ^8-THC (**11**) and 1-deoxy-DMH-Δ^8-THC (**12**) have significant affinities for both CB1 and CB2 with modest selectivity for the CB2 suggesting that phenolic hydroxyl is not essential for cannabinoid activity (Huffman et al., 1996). Computer molecular modeling studies postulate that with the PH forming H-bond with Lys192, the C-3 alkyl side chain interacts with a hydrophobic pocket formed by Val 351 and Ile 354 while Tyr 275 at the top of Helix 5 forms a hydrogen bond with the northern aliphatic hydroxyl (Table 1). Based on this postulate, cannabinoid analogs in which the phenolic hydroxyl is absent, Lys192 forms a hydrogen bond with the northern aliphatic hydroxyl (Huffman et al., 1996). According to this hypothesis, the minimal structural features required for CB1 activity are the presence of an O atom provided by either a phenolic or northern aliphatic hydroxyl to which Lys 192 can hydrogen bond and an alkyl side chain which can interact simultaneously with the lipophilic pocket on TMH 6 of CB1.

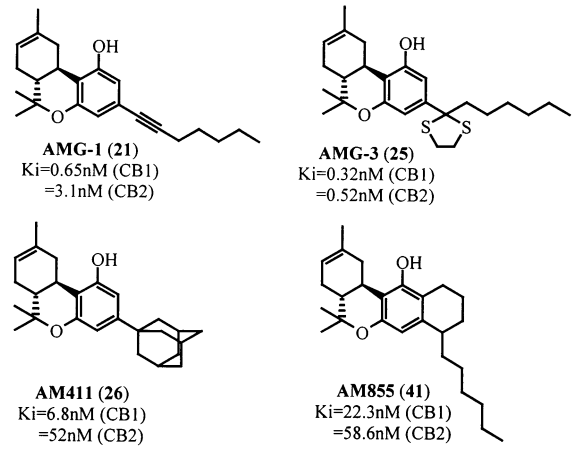


Fig. 5. Novel side chain analogs of (–)-Δ^8-tetrahydrocannabinol.

2.2. The side chain (SC)

The hydrophobic alkyl side chain is a key pharmacophore in classical cannabinoids. The plant-derived cannabinoids e.g. (–)-Δ^9-THC, possess an *n*-pentyl side chain. The early SAR work by Roger Adams in 1940s showed that a 1,1-dimethylheptyl (DMH) or 1,2-dimethylheptyl side chain is optimal for activity (Adams et al., 1949). Several side chain analogs have since been reported with methyl groups at various positions on the side chain with no significant enhancement in receptor affinity and potency. Stereochemically defined 1,2-dimethylheptyl analogs of Δ^8-THC have also been synthesized (Huffman et al., 1998).

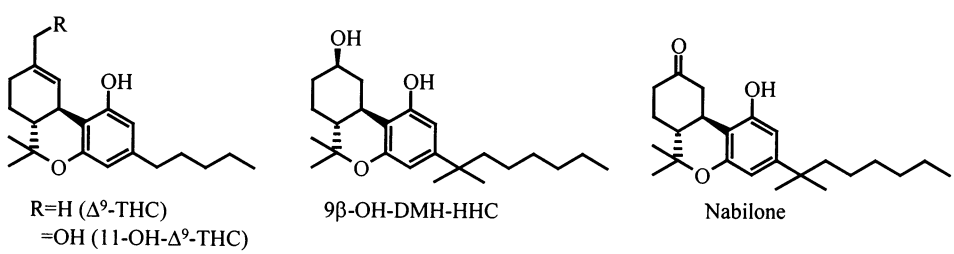


Fig. 6. Cannabinoid analogs possessing northern aliphatic hydroxyl (NAH) group.

Again no noteworthy increase in potency or stereoselectivity in cannabinoid receptor binding was observed. Therefore, the 1,1-dimethylheptyl is still the alkyl side chain of choice for classical cannabinoid analogs.

A number of other (-)-Δ^8-THC analogs with novel side chains have been reported in the past few years (Table 2). Introduction of a triple bond in the benzylic position of a heptyl-Δ^8-THC analog leads to classical cannabinoid analog **21** with high CB1 affinity (Papahatjis et al., 1998) while a similar substitution in the 11-hydroxyhexahydrocannabinol (11-OH-HHC) series affords analog **35** with considerably lower affinity (Busch-Petersen et al., 1996). This perhaps indicates that the presence of a C1′–C2′ triple bond in the 11-OH-HHC series does not allow for the simultaneous optimal alignment of both the OH and the side chain pharmacophore at the CB1 binding site. The dithiolane analog **25** (AMG-3) with subnanomolar affinities for both cannabinoid receptors corroborates the requirement for hydrophobic groups at C1' for optimal interaction with cannabinoid receptors suggested by the high affinity of 1′,1′-dimethylheptyl side chain analogs. The fact that a bulky adamantyl-Δ^8-THC **26** (AM411) exhibits considerable affinity and selectivity for CB1 points to a greater tolerance for steric bulk in that receptor subsite especially with regard to CB1 (Fig. 5). A polar group, e.g. carbonyl (**22**) or a hydroxyl (**23**) at the benzylic position significantly reduces cannabinoid receptor affinity indicating a requirement for a lipophilic group which interacts with this hydrophobic subsite at the receptor (Papahatjis et al., 1998).

The spatial orientation of the side chain plays an important role in cannabinoid activity. Khanolkar et al. have reported Δ^8-THC analogs **40** and **41** in which the *n*-heptyl side chain is restricted by a C2–C3 cyclohexyl ring (Khanolkar et al., 1999) and showed that analog **41** (AM855) in which the side chain is fixed at a 1–1.5 Å from the phenol ring and has its side chain pointing downwards has an 18-fold higher affinity for CB1 than analog **40** in which the side chain is fixed at a distance of at least 3–3.5 Å with a lateral orientation. Analog **41** also exhibits about three-fold higher affinity for CB2 than **40**. The decrease in CB1 affinity upon introduction of the fourth ring could be due to the additional carbon atoms of the ring that may prevent a favorable interaction with the receptor (Khanolkar et al., 1999). Huffman and Yu have also reported a tetracyclic analog of Δ^8-THC **39** which was found to have weak CB1 receptor affinity (Huffman and Yu 1998).

Introduction of a halogen group does not substantially alter the receptor affinity of the parent ligand and, in some cases, increases receptor affinity significantly, e.g. analogs **18** and **19** (Charalambous et al., 1991). We have used this finding to introduce ^{125}I-radiolabel at the terminal carbon of the alkyl side chain in the novel cannabinoid photoaffinity labels AM869 and AM1708 (Fig. 13) (Ramamurthy et al., 2000).

Singer et al. have recently reported Δ^8-THC analogs possessing cyano, *N*-substituted carboxamido and sulfonamido groups at the terminal carbon of the alkyl side chain (Singer et al., 1998). The cyano analogs (**27**–**30**) showed high CB1 binding affinity (K_i 0.36–13 nM) and in vivo potency as cannabinoid agonists. The dimethylcarboxamido analog **31** also showed high CB1 affinity (K_i 0.86 nM) and pharmacological profile similar to that of the cyano analogs **27** and **28**. Interestingly, the sulfonamide analog (**34**) exhibited greater affinity than Δ^8-THC but had no agonist effects (Singer et al., 1998).

Finally, Makriyannis and co-workers have reported that CB2-selectivity can be accomplished by incorporating terpenoid bicyclic side chains and structurally more defined alkyl chains (Lu et al., 1997).

2.3. The northern aliphatic hydroxyl (NAH) group

Introduction of a hydroxyl group in this molecular region leads to significant enhancement in potency and affinity for CB1 and CB2. Thus, (–)-11-hydroxy-Δ^9-THC (Fig. 6), a key product of THC metabolism, is significantly more potent than the parent natural product. Also, (–)-11-hydroxydimethylheptyl-Δ^8-THC (HU210), a ligand that has received a great deal of attention because of its high affinity for CB1 and CB2 receptors is

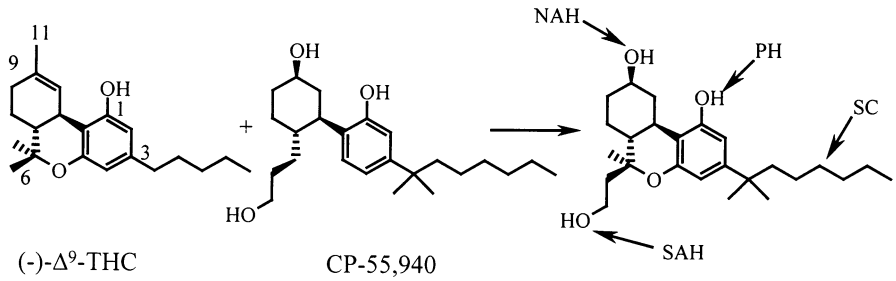
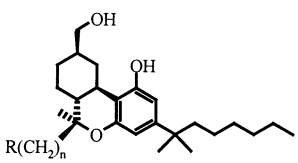
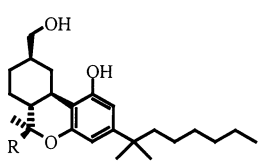
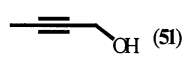


Fig. 7. Hybrid classical/non-classical (CC/NCC) cannabinoids.

more potent than the parent analog with no 11-hydroxy substitution (Mechoulam et al., 1987, 1988). This is also the case for the cannabinol series in which the C-ring is fully aromatized (Rhee et al., 1997). The hexahydrocannabinol (HHC) series encompasses two types of isomers based on the relative configuration at the C-9 position. Of the two, the β-epimer (9β-OH-DMH-HHC) in which the C-9 substituent is equatorial has been shown to be consistently more potent than the α-axial counterpart (Fig. 6). This holds true for both the 9-hydroxy (Wilson et al., 1976) and 11-hydroxymethyl (Devane et al., 1992a,b; Yan et al., 1994) analogs. Presence of a C-9 carbonyl group is also known to significantly enhance cannabimimetic activity (Archer et al., 1986). Thus, nabilone exhibits much higher potency than (−)-Δ^9-THC in behavioral tests. More recently, Makriyannis and collaborators have shown that this is also true in the case of 9-azido analogs in which the 9β-isomers (AM869, AM1708) have been shown to exhibit remarkably

Table 3
Structure–activity relationships of the Southern Aliphatic Group (SAH) group

Analog	R	K_i nM[a] CB1	K_i nM[a] CB2	Reference
	OH (**42**)	1.9	1.4	Tius et al 1995
	I (**43**)	12.1	10.1	
	H (**44**) } n=1	2.3	2.3	
	OH (**45**)	2.8	2.3	
	I (**46**)	40.7	9.7	
	H (**47**) } n=2	11.1	21.5	
	OH (**48**)	2.2	3.4	
	I (**49**)	2.2	4.3	
	H (**50**) } n=3	14.4	38.9	
	 (**51**)	1.21	0.3	Harrington et al 2000

[a] rat brain CB1, mouse spleen CB2; vs. [^{3}H] CP-55,940

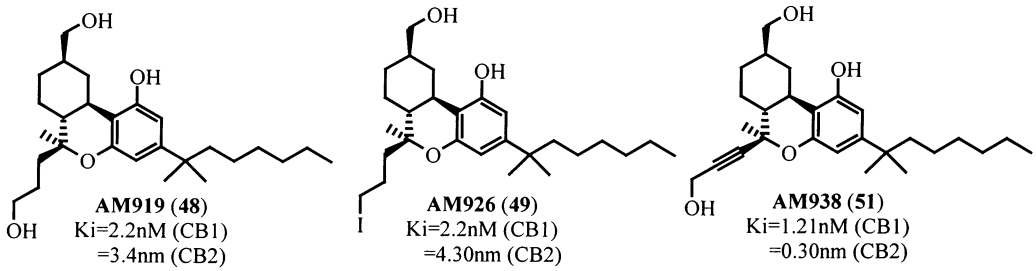


Fig. 8. Southern aliphatic hydroxyl (SAH) analogs.

high affinities for both CB1 and CB2 receptors (Khanolkar et al., 2000).

The requirement for a 9β-substitution in the HHC series can be linked to stereochemical consideration related to C-ring conformation. Early on, the conformational properties of the C-ring were probed by Makriyannis and co-workers who used 1D and 2D NMR to compare Δ^9-THC with its active isomer $\Delta^{9,11}$-THC (Kriwacki and Makriyannis, 1989). The studies showed that the two cannabinoids have similar conformations with regard to the dihydropyran B-ring and the side chain but different C-ring conformations. In Δ^9-THC, the C-ring assumes a somewhat flattened chain conformation causing the C-9 methyl group to project above the aromatic A-ring. Conversely, in $\Delta^{9,11}$-THC, the C-ring exists in a chair conformation with the exocyclic methylene group being coplanar with the aromatic phenolic ring (Kriwacki and Makriyannis, 1989).

The C-ring stereochemical requirements for cannabinoid activity were further elaborated by Reggio and co-workers using computational methods (Reggio et al., 1989). For this purpose, conformational preferences for seven classical cannabinoid analogs possessing a wide spectrum of potencies were compared while focusing on the C11–C9–C1–O dihedral angle which describes relative orientation of the C-ring with respect to that of the C1–OH phenolic bond. The study showed that in the biologically more active cannabinoids such Δ^9- and Δ^8-THC the angle in question has a large negative value with the C-9 methyl group projecting above the top face of the molecule. Conversely, in the less active cannabinoids such as $\Delta^{9,11}$-THC, the dihedral angle has a positive value and the C9-substituent is either coplanar with the aromatic ring or protruding towards the bottom face of the cannabinoid. Based on these results, the authors suggest that, within the CB1 active site, the C9-methyl group of the biologically active cannabinoid ligands is capable of occupying a subsite through which their interaction with the receptor is enhanced (Reggio et al., 1989). It should be pointed out that the presence of a C-9 substituent is not absolutely essential for activity because 9-nor-Δ^9-THC exhibits significant cannabinoid activity (Martin et al., 1975). However, an unfavorable orientation of the C-9 substituents, e.g. 9α-hydroxyl or 9α-hydroxymethyl, can seriously interfere with this ligand–receptor interaction.

2.4. The southern aliphatic hydroxyl (SAH) group

This pharmacophore is absent in the naturally occurring cannabinoids and was first introduced in the non-classical cannabinoids, a group of cannabinoid-like compounds lacking the pyranoid B-ring. The best known representative of this new class of synthetic cannabimimetics, first developed by Pfizer, is CP-55,940 (Fig. 2). To study more precisely the stereochemical requirements of this pharmacophore Makriyannis and co-workers designed a group of hybrid ligands that incorporated all of the structural features of both classical and non-classical cannabinoids (Tius et al., 1994, 1995) (Fig. 7).

This new class of analogs (CC/NCC hybrids) had the added advantage of being conformationally more defined three-dimensional probes for the CB1 and CB2 active sites than their non-classical counterparts. Initially, the two 6β-hydroxyethyl

and 6α-hydroxyethyl epimers were synthesized in order to determine the preferred orientation of the SAH group (Tius et al., 1994). Receptor binding data showed that the β-hydroxyethyl analog had 32-fold higher affinity than the α-axial epimer. Thus, favorable ligand–receptor interaction results when the SAH group is in the equatorial position.

To investigate whether the length of the southern hydroxyalkyl chain is important for activity, 6β-hydroxymethyl (**42**), 6β-hydroxyethyl (**45**) and 6β-hydroxypropyl (**48**) analogs with 1,1-dimethylheptyl side chain at the C-3 and a β-CH_2OH at the C-11 position were synthesized (Tius et al., 1995Drake et al., 1998). The three analogs **42**, **45** and **48** readily displaced CP-55,940 in a dose-dependent manner with K_is of 1.9, 2.8 and 2.2 nM, respectively, indicating little change in the CB1 receptor affinity with increasing chain length (Table 3). From these results, it can be concluded that while the relative configuration (6α or 6β) of the SAH appears to be critical, the length of the southern hydroxyl alkyl chain is of lesser importance in determining the cannabinoid activity. This could be due to conformational flexibility of the respective SAH chains which allows for optimal interaction between the OH group at a subsite on the receptor with which the tricyclic cannabinoid component interacts. More refined topological examination of this interaction required the synthesis of analog **51** in which the southern aliphatic chain is more confined (Harrington et al., 2000). From the receptor binding data, it can be concluded that, in addition to length, conformation of the side chain is not important for ligand–receptor interaction since the alkyne analog (**51**) exhibits equal receptor affinity to that of the hydroxyalkyl analogs (Fig. 8).

The southern cannabinoid subsite was further explored by extending the hydroxyalkyl series to the corresponding *n*-alkyl and ω-iodoalkyl analogs (Drake et al., 1998). For the alkyl series, increase in chain length from methyl to propyl (**44, 47, 50**)

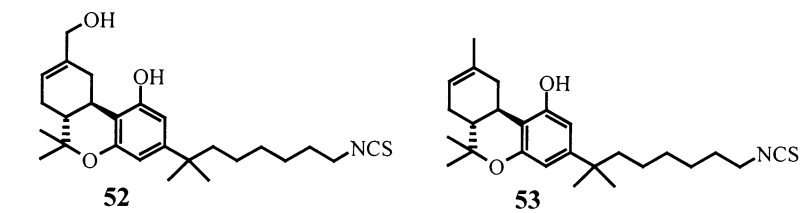


Fig. 9. Electrophilic affinity probes for cannabinoid receptors.

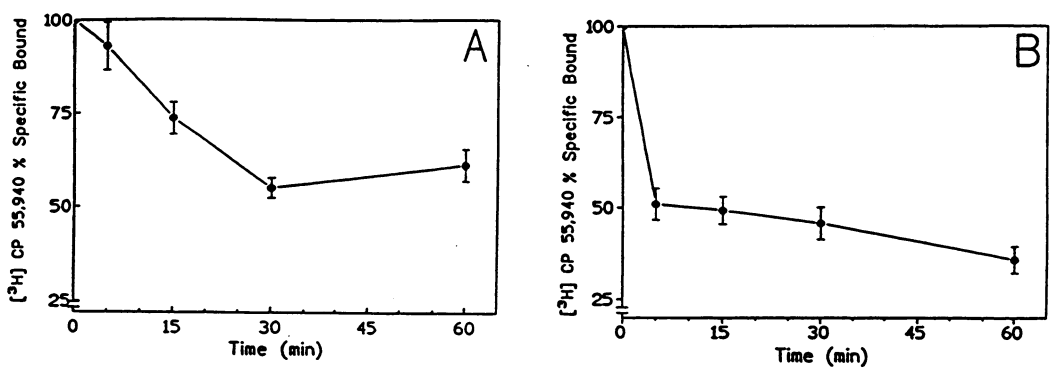


Fig. 10. Time course of rat brain CB1 labeling with AM708 (**52**): (A) 0.5 nM; (B) 2 nM.

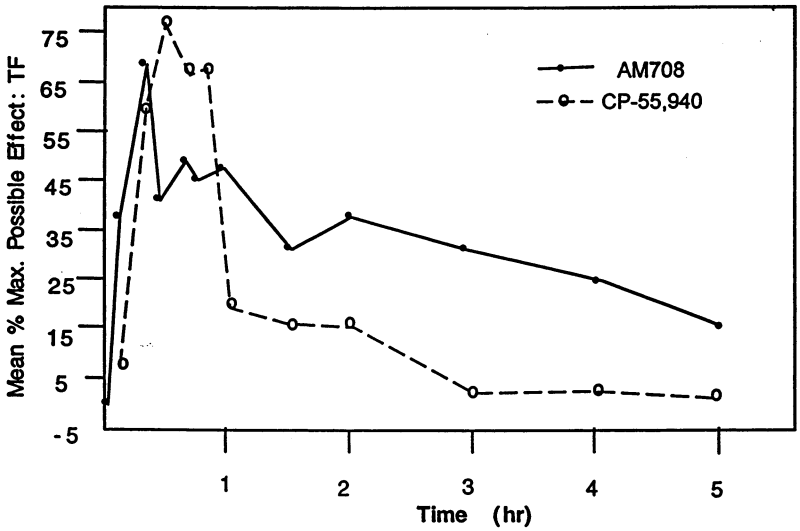


Fig. 11. Time course of CP-55,940 and AM708 (**52**) on rat tail flick latency.

resulted in progressively lower affinities toward both CB1 and CB2 (Table 3). These results define the limits of steric tolerance for hydrophobic groups at that subsite in both cannabinoid receptors and demonstrate that the 6β-alkyl pharmacophore exhibits different structure–activity relationship characteristics than the corresponding 6β-hydroxyalkyl group. The data also suggest that the 6-alkyl and the 6-hydroxyalkyl pharmacophores interact differently with the receptor subsite (different binding motifs). The 6β-(ω-iodoalkyl) series was expected to exhibit SAR profile similar to the corresponding 6β-alkyl series due to its hydrophobic character. Indeed, going from iodomethyl (**43**) to iodoethyl (**45**) substituent, receptor affinity decreases as expected. However, the most interesting SAR effect in this series was observed with the iodopropyl compound (**49**) which showed increased affinity for both CB1 and CB2 receptors compared to its lower homologs. The effect is most striking with the CB1 receptor for which the bulky iodopropyl analog exhibits 20-fold higher affinity than the iodoethyl analog. For the CB2 receptor, the corresponding increase in receptor affinity was moderate. The above results can be interpreted by postulating the presence of a hydrophobic cavity in the southern cannabinoid region. This subsite may resemble a 'baseball glove' with which the 'baseball'-like iodo group interacts and significantly enhances receptor affinity (Drake et al., 1998).

3. Covalent probes for cannabinoid receptors

Both CB1 and CB2 receptors have been cloned and their primary amino acid sequence is known (Matsuda et al., 1990; Munro et al., 1993). Evidence to date indicates that the active site(s) for both receptors are located within the seven transmembrane helices (Xie et al., 1996). There is also evidence from receptor mutation experiments suggesting that classical and non-classical cannabinoids and arachidonoylethanolamides interact with the CB1 receptor differently compared with the aminoalkylindoles (Shire et al., 1996a,b;

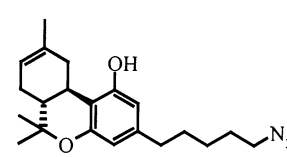
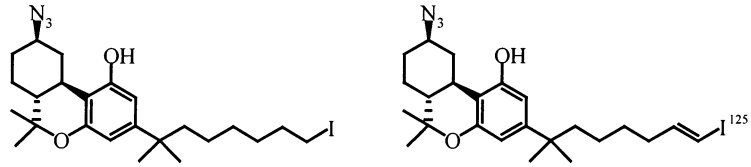


Fig. 12. (−)-5′-Azido-Δ^8-THC, the first photoactivatable cannabinoid receptor probe.



AM869
K_i=0.67nM (CB1)
=0.72nM (CB2)

AM1708
K_i=0.80nM (CB1)
=0.85nM (CB2)

Fig. 13. High affinity photoactivatable probe for cannabinoid receptors.

Song and Bonner, 1996). Thus, Lys192Ala mutation attenuated binding of CP-55,940 and anandamide but not that of WIN-55,212-2, an aminoalkylindole cannabimimetic prototype (Song and Bonner 1996). Notwithstanding the above work, we have only limited information on the nature of the amino acid residues involved in ligand recognition and binding. A more direct approach for obtaining such information is through receptor labeling experiments using high affinity covalent probes. To that end, Makriyannis and co-workers have developed several novel receptor affinity ligands that possess reactive groups at judiciously chosen positions within the classical cannabinoid structure. Two types of reactive groups were incorporated: (1) an electrophilic isothiocyanato group (NCS) which targets nucleophilic amino acid residues such as lysine, histidine and cysteine at or near the active site; and (2) a photoactivatable azido group (N_3) capable of labeling the amino acid residues at the active site via a highly reactive nitrene intermediate. Both types of probes were shown to successfully label the cannabinoid receptors.

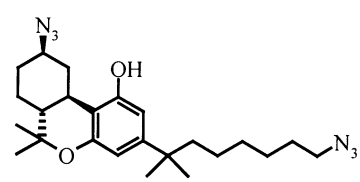
3.1. Electrophilic affinity probes

Two novel high affinity electrophilic covalent probes for cannabinoid receptors in which the isothiocyanate group is the reactive moiety have been reported (Guo et al., 1994; Morse et al., 1995), namely (−)-11-hydroxy-7′-isothiocyanato-Δ^8-THC (AM708; **52**) (IC_{50} for CB_1 1.6 nM) and (−)-7′-isothiocyanato-Δ^8-THC (**53**) (IC_{50} for CB_1 0.66 nM) (Fig. 9). Incubation with a 10 nM concentration of analog AM708 (**52**), led to an 80% reduction in the number of CB1 binding sites in a rat brain membrane preparation. Similar results were obtained with **53**. A 100 nM concentration of **52**, virtually depleted all CB1 binding sites. The covalent occupation of the cannabinoid receptor by **52** was shown to be time dependent and highly selective (Fig. 10) (Guo et al., 1994).

Interestingly, when tested on an adenylate cyclase membrane preparation **52** was shown to behave as an irreversible agonist (Howlett and Makriyannis, unpublished results). A similar result was recently obtained when analog **52** was tested in vivo by i.c.v. administration in rats where it behaved as a long acting analgesic (Fig. 11) (Martin and Walker, unpublished results).

3.2. Photoactivatable cannabinoid receptor probes

In 1992, the first successful photoactivatable probe for the CB1 receptor was reported (Charalambous et al., 1992) (Fig. 12). 5′-Azido-Δ^8-THC, with an aliphatic azido group attached to the terminal carbon of the alkyl side chain, had good affinity for CB1 with a K_i of 19 nM and was shown to selectively label the receptor active site



AM859
Ki=1.60nM (CB1)
=2.65nM (CB2)

Fig. 14. A novel bifunctional cannabinoid receptor covalent probes.

(Burstein et al., 1991; Charalambous et al., 1992). This unusual photoaffinity probe which incorporates an aliphatic azido group as the reactive moiety in lieu of the traditionally used aromatic azido group served as a prototype for later generation ligands two of which are shown in Fig. 13. Thus, the azido group was introduced at the C9-position of the tricyclic ring system in a series of photoactivatable classical cannabinoid probes (AM869, AM1708). The resulting novel compounds proved to be excellent ligands for both CB1 and CB2. A significant improvement in the design of these new probes was the introduction of ^{125}I-substituent in the ligand without compromising its high receptor affinity. These radioiodinated probes can serve as valuable tools for receptor purification and characterization. Furthermore, introduction of an azido group at different strategic positions within these prototypic probes and formation of covalent bonds with different amino acid residues allows for a more thorough exploration of the stereochemical features of the receptor active site(s).

^{125}I-Labeled AM869 was prepared via an S_N2 displacement of its tosylate with Na^{125}I (Khanolkar et al., unpublished results). However, a radiosynthetically more efficient approach was developed using an iododestannylation reaction in order to obtain the respective iodovinyl analog (Khanolkar et al., 2000). This procedure led to the synthesis of ^{125}I-AM1708 with a specific activity of $\sim$2500 Ci per mmol. Recently, this excellent photoactivatable radioligand was used to successfully label and purify the CB1 receptors either from rat brain membrane preparations or from transfected cell lines (Ramamurthy et al., 2000).

A novel concept in covalent labeling of cannabinoid receptors involves the developemnt of bifunctional covalent ligands which incorporate two reactive groups within the same molecule (Khanolkar et al., 1996). If successful, such probes will result in the formation of two covalent bonds at the receptor active site with the same ligand. Characterization of the double covalent attachment points will, in turn, provide more detailed three-dimensional information on the ligand–receptor binding motifs. An example of such a probe, AM859, is currently being explored (Fig. 14).

Acknowledgements

This work was supported by Grants DA-00355 (A.D.K.), DA-3801, DA-152, DA-7215 and DA-9158 (A.M.) from the National Institute on Drug Abuse.

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