

TEIJA PARKKARI

Synthesis of Novel Cannabinoid CBI Receptor Ligands

Doctoral dissertation

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ABSTRACT

The past fifteen years have been an exciting time for the cannabinoid research. Our understanding of the endogenous cannabinoid system (ECS) has continued to reveal the significant physiological role of this system in both the central nervous system (CNS) and peripheral tissues. The ECS has proved to be an important neuronal modulator with a novel mechanism of action, and therefore, a novel target for the drug discovery. Although cannabinoids still suffer from a negative reputation mainly due to the recreational use of marijuana, the exponential growth in a number of cannabinoid related patent applications is evidence that even in the near future, synthetic cannabinoids will achieve general acceptance as therapeutically significant agents.

The present study was focused on the design of novel metabolically stable endocannabinoid type CB1 receptor ligands, with the basis of the design based on the previously reported structure activity relationships of endocannabinoids. The synthesized compounds were divided into four different groups according to their chemical structures; (i) derivatives of arachidonyl alcohol, (ii) reversed amides of *N*-arachidonoyl ethanolamide (AEA), (iii) dimethylheptyl (DMH) derivatives of 2-arachidonoyl glycerol (2-AG) and 2-arachidonoyl glyceryl ether (2-AGE), and (iv) α -methylated derivatives of 2-AG. The study consisted of three main parts. Firstly, effective synthesis and purification methods for the novel CB1 ligands were developed. Secondly, the cannabinergic activity of the synthesized compounds was determined *in vitro* by a [³⁵S]GTP γ S binding assay (efficacy E_{max} and potency $-\log EC_{50}$). Thirdly, the chemical and enzymatic stabilities of the novel CB1 ligands were studied in rat brain homogenate and membrane-free buffer.

The series of the ester, carbamate and carbonate derivatives of arachidonyl alcohol were evaluated, and it was observed that these kinds of compounds are not potent ligands for the CB1 receptor. In addition, the compounds were difficult to synthesize and handle due to their degradation, polymerization, and high lipophilicity. The important finding emerging from the second series of compounds was that, in contrast to the previous belief, the reversed amide derivatives of endogenous AEA are able to activate both CB1 and CB2 receptors. The study also revealed that the reversed amides exhibit significant metabolic stability under conditions where AEA is almost completely degraded. The study of DMH derivatives of 2-AG and 2-AGE showed that unlike the case of the AEA-type compounds and classical cannabinoids, the activity properties of 2-AG and 2-AGE cannot be improved by the replacement of the end pentyl chain with the DMH structure. Finally, a study with the α -methylated derivatives of 2-AG indicated that even though the stereochemistry of the α -position of 2-AG does not play any role in its affinity for the CB1 receptor, it has a significant role in G-protein activation. The study also indicated that the α -methylation can provide protection against the enzymatic degradation, and therefore, a prolonged duration of action for these compounds is to be expected.

National Library of Medicine Classification: QV 744, QV 38, QV 77.7, QV 126

Medical Subject Headings: chemistry, pharmaceutical; receptors, drug; neurotransmitter agents; cannabinoids; endocannabinoids; receptor, cannabinoid, CB1; ligands / chemical synthesis; structure-activity relationship; drug design

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Boston, November 2006

Teija Parkkari

ABBREVIATIONS

| | |
|-----------------|--|
| A ₁ | adenosine A ₁ receptor |
| AA | arachidonic acid |
| AAI | aminoalkylindole |
| ACEA | arachidonoyl 2-chloroethylamide |
| AcOH | acetic acid |
| ACPA | arachidonoyl cyclopropylamide |
| AD | Alzheimer's disease |
| AEA | <i>N</i> -arachidonoyl ethanolamide, anandamide |
| 2-AG | 2-arachidonoyl glycerol |
| 2-AGE | 2-arachidonoyl glyceryl ether |
| AM251 | <i>N</i> -(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide |
| AM281 | <i>N</i> -(morpholin-4-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1 <i>H</i> -pyrazole-3-carboxamide |
| AIDS | Acquired Immune Deficiency Syndrome |
| aq | aqueous solution |
| AT | anandamide (AEA) transporter |
| Bn | benzyl |
| BSA | bovine serum albumin |
| CC | classical cannabinoid |
| cAMP | cyclic adenosine monophosphate |
| CB1 | cannabinoid receptor 1 (neuronal) |
| CB2 | cannabinoid receptor 2 (peripheral) |
| CB3 | cannabinoid receptor 3 |
| CBD | cannabidiol |
| CBN | cannabinol |
| Cbz | benzyloxycarbonyl |
| CHO | Chinese hamster ovary |
| CNS | central nervous system |
| COX-2 | cyclooxygenase-2 |
| CP55,940 | (-)- <i>cis</i> -3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]- <i>trans</i> -4-(3-hydroxypropyl) cyclohexanol |
| CT-3 | 1',1'-dimethylheptyl- Δ^8 -tetrahydrocannabinol-11-oic acid |
| Δ^9 -THC | Δ^9 -tetrahydrocannabinol |
| 3D | three dimensional |
| DCC | <i>N,N'</i> -dicyclohexylcarbodiimide |
| DFP | diisopropyl fluorophosphate |
| DIPEA | <i>N,N'</i> -diisopropylethylamine |
| DMAP | 4-(dimethylamino)pyridine |
| DMF | <i>N,N</i> -dimethylformamide |
| DMH | dimethylheptyl |
| DMSO | dimethyl sulfoxide |
| DPPA | diphenyl phosphoryl azide |
| DSE | depolarization-induced suppression of excitation |

| | |
|--|--|
| DSI | depolarization-induced suppression of inhibition |
| E_{\max} | maximal efficacy |
| EC_{50} | concentration which evokes half maximal efficacy |
| ECS | endogenous cannabinoid system |
| EDCI | 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride |
| EDTA | ethylenediaminetetraacetic acid |
| eq | equivalent |
| ERK | extracellular signal-regulated kinase |
| FAAH | fatty acid amide hydrolase |
| FDA | Food and Drug Administration |
| FID | free induction decay |
| FSH | follicle-stimulating hormone |
| GABA | gamma-aminobutyric acid |
| GC-MS(EI) | gas-liquid chromatography with electron-ionization mass detector |
| GnRH | gonadotropin-releasing hormone |
| G protein | guanine nucleotide-binding protein |
| GPCR | G protein-coupled receptor |
| $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ | guanosine-5'- <i>O</i> -(3- $[^{35}\text{S}]$ thio)-triphosphate |
| hCB2 | human CB2 receptor |
| HD | Huntington's disease |
| HPLC | high performance liquid chromatography |
| HU-210 | (-)-(6 <i>aR</i> ,10 <i>aR</i>)-11-hydroxy-3-(1',1'-dimethylheptyl)- Δ^8 -tetrahydrocannabinol |
| HU-211 | (+)-(6 <i>aS</i> ,10 <i>aS</i>)-11-hydroxy-3-(1',1'-dimethylheptyl)- Δ^8 -tetrahydrocannabinol |
| $[^3\text{H}]\text{HU-243}$ | tritiated (-)-11-hydroxy-3-(1',1'-dimethylheptyl)-hexahydrocannabinol |
| HU-310 | 2-arachidonoyl glyceryl ether |
| IC_{50} | concentration causing half-maximal inhibitory effect |
| IOP | intraocular pressure |
| J | coupling constant (spin-spin) |
| JWH-133 | 3-(1',1'-dimethylbutyl)-1-deoxy- Δ^8 -tetrahydrocannabinol |
| K_D | dissociation constant |
| K_i | inhibition constant |
| LAH | lithium aluminium hydride |
| L-DOPA | levodopa (3,4-dihydroxy-L-phenylalanine) |
| LH | luteinizing hormone |
| MAFP | methyl arachidonoyl fluorophosphonate |
| MGL | monoacylglycerol lipase |
| mRNA | messenger ribonucleic acid |
| MS | multiple sclerosis |
| MsCl | methanesulfonyl chloride |
| NAAA | <i>N</i> -acylethanolamine-hydrolyzing acid amidase |
| NADA | <i>N</i> -arachidonoyl dopamine |
| NCC | non-classical cannabinoid |

| | |
|----------------|---|
| NESS 0327 | <i>N</i> -piperidinyl-[8-chloro-1-(2,4-dichlorophenyl)-1,4,5,6-tetrahydrobenzo [6,7]cyclohepta[1,2- <i>c</i>]pyrazole-3-carboxamide] |
| NHMDS | sodium bis(trimethylsilyl) amide |
| NMDA | <i>N</i> -methyl-D-aspartic acid |
| NMR | nuclear magnetic resonance |
| <i>p</i> | para |
| <i>p</i> -TSA | <i>para</i> -toluenesulfonic acid |
| PAG | periaqueductal grey |
| PE | petroleum ether 60/95 |
| PEA | <i>N</i> -palmitoyl ethanolamine |
| PET | positron emission tomography |
| PD | Parkinson's disease |
| PKA | protein kinase A |
| PMSF | phenylmethylsulfonyl fluoride |
| R _f | retention unit of TLC |
| RIO | Rimonabant In Obesity |
| RT | room temperature |
| R _t | retention time |
| SAR | structure-activity relationship |
| SEM | standard error of means |
| SR141716A | <i>N</i> -piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methylpyrazole-3-carboxamide |
| SR147778 | 5-(4-bromophenyl)-1-(2,4-dichlorophenyl)-4-ethyl- <i>N</i> -(1-piperidinyl)-1H-pyrazole-3-carboxamide |
| <i>t</i> | tertiary |
| TBDMS | <i>t</i> -butyldimethylsilyl |
| TBAF | tetrabutylammonium fluoride |
| TFAA | trifluoroacetic acid anhydride |
| THF | tetrahydrofuran |
| TIPS | triisopropylsilyl |
| TLC | thin-layer chromatography |
| TMS | tetramethylsilane |
| TS | Tourette's syndrome |
| URB597 | cyclohexylcarbamic acid 3'-carbamoylebiphenyl-3-yl ester |
| UV | ultraviolet |
| WIN-55,212 | [2,3-dihydro-5-methyl-3-[(morpholinyl)methyl]pyrrolo[1,2,3- <i>de</i>]-1,4-benzoxazin-yl)-(1-naphthalenyl)methanone |

LIST OF ORIGINAL PUBLICATIONS

The present doctoral dissertation is based on the following original publications I-IV.

- I Teija Parkkari, Juha R. Savinainen, Anu L. Rauhala, Tiina L. Tolonen, Tapio Nevalainen, Jarmo T. Laitinen, Jukka Gynther, and Tomi Järvinen. Synthesis and CB1 Receptor Activities of Novel Arachidonyl Alcohol Derivatives. *Bioorg Med Chem Lett* 14: 3231-3234, 2004
- II Teija Parkkari, Juha R. Savinainen, Katri H. Raitio, Susanna M. Saario, Laura Matilainen, Tuomas Sirviö, Jarmo T. Laitinen, Tapio Nevalainen, Riku Niemi, and Tomi Järvinen. Synthesis, Cannabinoid Receptor Activity, and Enzymatic Stability of Reversed Amide Derivatives of Arachidonoyl Ethanolamide. *Bioorg Med Chem* 14: 5252-5258, 2006
- III Teija Parkkari, Outi M.H. Salo, Kristiina M. Huttunen, Juha R. Savinainen, Jarmo T. Laitinen, Antti Poso, Tapio Nevalainen, and Tomi Järvinen. Synthesis and CB1 Receptor Activities of Dimethylheptyl Derivatives of 2-Arachidonoyl glycerol (2-AG) and Arachidonoyl glyceryl ether (2-AGE). *Bioorg Med Chem* 14: 2850-2858, 2006
- IV Teija Parkkari, Mikko Myllymäki, Juha R. Savinainen, Susanna M. Saario, Joel A. Castillo-Meléndez, Jarmo T. Laitinen, Tapio Nevalainen, Ari M.P. Koskinen, and Tomi Järvinen. α -Methylated Derivatives of 2-Arachidonoyl glycerol: Synthesis, CB1 Receptor Activity, and Enzymatic Stability. *Bioorg Med Chem Lett* 16: 2437-2440, 2006

CONTENTS

| | |
|---|-----------|
| 1 INTRODUCTION TO THE CANNABINOIDS..... | 15 |
| 1.1 History of cannabinoids | 15 |
| 1.2 The endogenous cannabinoid system | 16 |
| 1.2.1 The CB1 receptor | 17 |
| 1.2.2 The endocannabinoids - biosynthesis and inactivation..... | 19 |
| 1.2.3 Physiological significance..... | 26 |
| 1.2.4 The endogenous cannabinoid system as the target for a drug discovery | 27 |
| 1.3 CB1 receptor ligands | 28 |
| 1.3.1 Agonists | 29 |
| 1.3.2 Antagonist/inverse agonists | 38 |
| 1.4 Therapeutic potential of the CB1 receptor ligands | 41 |
| 1.5 Nervous system..... | 42 |
| 1.5.1 Appetite and emesis | 43 |
| 1.5.2 Movement disorders..... | 44 |
| 1.5.3 MS-disease and spinal cord injury | 47 |
| 1.5.4 Neuroprotection | 48 |
| 1.5.5 Pain..... | 50 |
| 1.5.6 Cognition and memory..... | 51 |
| 1.6 Cancer | 51 |
| 1.7 Gastrointestinal system..... | 52 |
| 1.8 Cardiovascular system | 53 |
| 1.9 Respiratory system..... | 54 |
| 1.10 Reproductive system..... | 54 |
| 1.11 Eye | 55 |
| 1.12 The CB1 receptor agonists as therapeutics –useful or harmful? | 56 |
| 1.13 References..... | 57 |
| 2 AIMS OF THE STUDY | 80 |
| 3 GENERAL EXPERIMENTAL PROCEDURES | 82 |
| 3.1 Synthesis | 82 |
| 3.2 Analysis | 82 |
| 3.3 <i>In vitro</i> assay for the CB1 receptor activity..... | 83 |
| 3.4 References..... | 85 |
| 4 SYNTHESIS AND CB1 RECEPTOR ACTIVITIES OF NOVEL ARACHIDONYL ALCOHOL DERIVATIVES | 86 |
| 4.1 Introduction..... | 86 |
| 4.2 Synthesis | 87 |
| 4.3 Results and discussion | 89 |
| 4.4 Conclusions..... | 90 |
| 4.5 Experimental..... | 90 |
| 4.5.1. Synthesis | 90 |
| 4.6 References..... | 102 |

| | |
|---|------------|
| 5 SYNTHESIS, CANNABINOID RECEPTOR ACTIVITIES AND ENZYMATIC STABILITY FOR REVERSED AMIDE DERIVATIVES OF ARACHIDONOYL ETHANOLAMIDE | 104 |
| 5.1 Introduction..... | 104 |
| 5.2 Synthesis | 105 |
| 5.3 Results and discussion | 106 |
| 5.4 Conclusions..... | 108 |
| 5.5 Experimental..... | 109 |
| 5.5.1 Synthesis | 109 |
| 5.5.2 Enzymatic stability..... | 115 |
| 5.5.3 <i>In vitro</i> assay for the CB2 activity | 116 |
| 5.6 References..... | 116 |
| 6 SYNTHESIS AND CB1 RECEPTOR ACTIVITIES OF DIMETHYLHEPTYL DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL (2-AG) AND ARACHIDONYL GLYCERYL ETHER (2-AGE) | 118 |
| 6.1 Introduction..... | 118 |
| 6.2 Synthesis | 119 |
| 6.3 Factor analysis | 121 |
| 6.4 Results and discussion | 121 |
| 6.5 Conclusions..... | 123 |
| 6.6 Experimental..... | 123 |
| 6.6.1 Synthesis | 123 |
| 6.6.2 Factor analysis..... | 129 |
| 6.7 References..... | 130 |
| 7 α-METHYLATED DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL : SYNTHESIS, CB1 RECEPTOR ACTIVITY AND ENZYMATIC STABILITY..... | 132 |
| 7.1 Introduction..... | 132 |
| 7.2 Synthesis | 133 |
| 7.3 Results and discussion | 134 |
| 7.4 Conclusions..... | 135 |
| 7.5 Experimental..... | 136 |
| 7.5.1 Synthesis | 136 |
| 7.5.2 Enzymatic stability..... | 142 |
| 7.6 References..... | 143 |
| 8 SUMMARY AND CONCLUSIONS..... | 144 |
| 8.1. Summary for novel CB1 receptor ligands | 144 |
| 8.1.1 Arachidonyl alcohol derivatives | 144 |
| 8.1.2 Reversed amides of AEA | 144 |
| 8.1.3 DMH derivatives of 2-AG and 2-AGE | 145 |
| 8.1.4 α -Methylated derivatives of 2-AG | 146 |
| 8.2 Conclusions..... | 147 |

1 INTRODUCTION TO THE CANNABINOIDS

1.1 History of cannabinoids

The history of cannabinoids can be traced back to ancient China where *Cannabis sativa*, the plant producing cannabinoids, was cultivated for its fiber and oil for over 5000 years ago. Long before cannabis reached Europe, it was widely recognized not only as a medical but also as a recreational plant in Asia and Africa. Cannabis did not reach Europe until the 19th century when cannabis extract was used in the treatment of epilepsy, rheumatism, menstrual cramps, convulsions, chorea, hysteria, depression, tetanus, gout, and neuralgia (Hart 1999). The mystery compounds responsible for these curing effects of cannabis remained unclear for decades due to difficulties with the isolation and identification techniques. However, in the 1930's the first two cannabinoids, cannabinol (Table 1.5, **26**) and cannabidiol (Table 1.5, **27**) were finally isolated (Mechoulam 2000). But soon after, researchers realized that the biological activity and UV spectra of these compounds did not match to the active compound of cannabis, and it took another two decades and the development of chromatographic and NMR techniques to finally reveal the main active constituent of *Cannabis sativa*, (-)-*trans*- Δ^9 -tetrahydrocannabinol (Δ^9 -THC, Table 1.5, **22**) (Gaoni and Mechoulam 1964). The discovery of Δ^9 -THC was the spark for the development of novel synthetic cannabinoids, and their pharmacological profile was intensively studied, although cannabis had by then been banned from medical use and the mechanism of action of cannabinoids was a mystery (Pertwee 2006). It was a general assumption that hydrophobic cannabinoids mediate their actions via cell membranes since Δ^9 -THC did not show any stereoselectivity, which is one characteristic of compounds acting through biological proteins, such as receptors. However, in the mid-1980's, purification methods were further improved, and it was observed that the biological activity of cannabinoids was indeed highly stereoselective. Finally, when it was reported that cannabinoids could inhibit adenylate cyclase, an intense search for a specific cannabinoid receptor was launched.

1.2 The endogenous cannabinoid system

Although cannabinoids had been used for medicinal purposes for centuries, their mechanism of action remained unclear for a surprisingly long time. However, when the crucial key was finally discovered, this opened a new door and entirely new opportunities for cannabinoid scientists, especially for those involved in the drug discovery. The key, the first cannabinoid receptor, was discovered in 1988 as a result from the persistent work of Allyn Howlett and co-workers (Devane et al. 1988). The receptor, which we now call the CB1 receptor (see chapter 1.2.1.), was determined from rat brain by means of a tritium labeled, selective cannabinoid receptor agonist, CP55,940 (Table 1.5, **28**). This significant finding has been followed by a series of interesting discoveries deepening our understanding of the endogenous cannabinoid system (ECS) as it is currently recognized (Figure 1.4).

Five years after the discovery of the CB1 receptor, another receptor, named the CB2 receptor, was cloned (Munro et al. 1993). The CB2 receptor is expressed widely in tissues involved in immune responses, such as spleen, tonsils, and the immune cells (Sugiura and Waku 2002). Until recently, this receptor type was believed to be only a peripheral receptor, however, the CB2-like immunoreactivity was detected in rat brain raising the possibility of a neglected role of the CB2 receptor in the central nervous system (CNS) (Gong et al. 2006). In addition to these two main receptor types, studies with CB1 knockout mice have shown that all the effects of cannabinoids are not mediated through currently known cannabinoid receptors, pointing to the existence of a “CB3” receptor (Breivogel et al. 2001; Monory et al. 2002; Begg et al. 2005; Demuth and Molleman 2006).

In 1992, the first compound that deserved to be called as an endogenous cannabinoid was identified in porcine brain (Devane et al. 1992). The compound, *N*-arachidonoyl ethanolamide (AEA, anandamide, Figure 1.2), was found to inhibit the specific binding of a tritiated cannabinoid receptor ligand, [³H]HU-243 (Table 1.5, **24**), to synaptosomal membranes, and it produced effects typical of psychotropic cannabinoids, such as inhibition of the electrically evoked twitch response of the mouse vas deferens (Devane et al. 1992). A few years later, another significant endocannabinoid finding was reported (Mechoulam et al. 1995; Sugiura et al. 1995). The compound, 2-

arachidonoyl glycerol (2-AG, Figure 1.2), was shown to be present in the brain at much higher concentrations than AEA, pointing to an important role in the ECS. Indeed, according to present knowledge, 2-AG is the main endogenous ligand for both cannabinoid receptors (Mechoulam et al. 1995; Sugiura et al. 1995; Sugiura et al. 1999; Sugiura et al. 2000; Savinainen et al. 2001). Subsequently, a variety of other arachidonoyl derivatives, such as *O*-arachidonoyl ethanolamine (virodhamine), *N*-arachidonoyl dopamine (NADA), and 2-arachidonoyl glyceryl ether (2-AGE), have been suggested to act as endocannabinoids (Figure 1.2). However, it is unclear whether these have any significant role in the ECS.

In addition to the cannabinoid receptors, the proteins responsible for the biosynthesis and inactivation of the endocannabinoids are viewed as the members of the ECS. At present, the most extensively studied targets have been the metabolizing enzymes, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL) as well as the transporter protein, anandamide transporter (AT). The endocannabinoids as well as their metabolic pathways are further discussed in chapter 1.2.2.

1.2.1 The CB1 receptor

The first evidence for the existence of a specific cannabinoid receptor was provided by Howlett et al. (1984, 1985) in the mid-80's. They demonstrated that cannabinoids decrease cyclic adenosine monophosphate (cAMP) accumulation, which pointed to the involvement of a G protein-coupled receptor (GPCR). Two years later the same group reported that the receptor-mediated inhibition of cAMP requires the presence of the G_i-protein (Howlett et al. 1986). In 1988 they finally swept away the old theories about how cannabinoids mediate their actions by characterizing the CB1 receptor from rat brain (Devane et al. 1988). Two years later, the CB1 receptor gene was cloned from cerebellar cortex, and the CB1 receptor distribution in the brain was determined (Herkenham et al. 1990; Matsuda et al. 1990). The study of Herkenham et al. showed that the CB1 receptors are highly abundant in the CNS. In fact, they were reported to be as abundant as glutamate and GABA receptors, i.e. they were more abundant than many other GPCRs in the brain. The CB1 receptor localization in the CNS is clearly associated with their pharmacological actions. They are most abundantly localized in

areas that control movement (basal ganglia and cerebellum), and cognition and memory (hippocampus and cerebral cortex) (Demuth et al. 2006). However, it is noteworthy from the safety point of view that the CB1 receptors are not abundant in those brain areas controlling heart rate and respiration (medulla) (Herkenham et al. 1990). In addition to the CNS, the CB1 receptors are also expressed in some peripheral tissues, such as spleen, tonsils, small intestine, urinary bladder, vas deferens, smooth muscle cells, vascular smooth muscle cells, adrenal gland, heart, prostate, uterus and ovary (Demuth et al. 2006). Two splice variants of the CB1 receptor have also been reported (Shire et al. 1995; Ryberg et al. 2005).

The CB1 receptor has a typical G-protein-coupled receptor structure with seven, membrane-penetrating α -helical structures, and an extracellular amino and an intracellular carboxyl terminus. The human CB1 receptor consists of 472 amino acids, and it shares 44% homology to the CB2 receptor (Sugiura and Waku 2002). The actual three-dimensional structure of the CB1 receptor has still not been clarified, as a membrane-bound protein it is difficult to crystallize. However, models based on the crystal structure of the rhodopsin have been described (Barnett-Norris et al. 2002; Shim et al. 2003; Salo et al. 2004). As mentioned earlier, the activation of the CB1 receptor leads to the inhibition of the cAMP accumulation via G_i -type receptors. In addition, some data exist that the CB1 receptors are able to activate G_s -type-linked receptors (Glass and Felder 1997; Abadji et al. 1999). They also inhibit Ca^{2+} influx and activate K^+ ion channels via G_o -type receptors, and activate MAP kinase pathway (Figure 1.1) (Mackie and Hille 1992; Bouaboula et al. 1995; Felder et al. 1995).

Since even the endogenous ligands can produce very different responses depending on the tissue and method applied, the traditional two-state receptor model has been replaced by new, more complicated theories where receptors are able to couple to several different signaling pathways. It has been proposed that a ligand can promote ligand-selective receptor conformations which have different abilities to regulate these pathways (Clarke 2005). Many outcome results are thus possible from ligands promoting different receptor conformations, differences in expression of the G proteins capable of activating the signaling pathways, and naturally the response being measured.

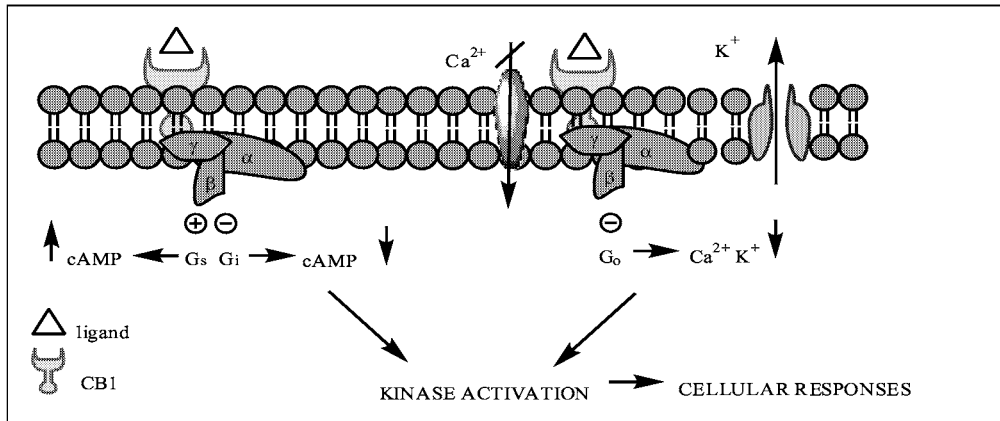


Figure 1.1. Signal transduction mechanism of the CB1 receptors.

In addition to the novel receptor models, a phenomenon that has gained considerable attention recently is that the cannabinoid receptors seem to be active also without the presence of their endogenous and exogenous ligands, i.e. the cannabinoid receptors are constitutively active. The constitutive activity of the CB1 receptors has been observed while searching for compounds with antagonist properties. Various drug development processes have revealed that compounds believed to act as neutral antagonists actually produce effects that are opposite to those of agonists, i.e. these compounds act as so called inverse agonists. The mechanism(s) behind this phenomenon is not fully understood; however, some hypotheses have been proposed. First, there is the possibility that inverse agonists compete in a surmountable manner for the CB1 receptors with the endocannabinoids. The second possibility is negative modulation, possible allosteric, of the constitutive activity of the CB1 receptors. Finally, inverse agonism may occur by some CB1 receptor-independent manner, such as by antagonism of endogenously released adenosine at A₁ receptors (Pertwee 2005a).

1.2.2 The endocannabinoids - biosynthesis and inactivation

Endocannabinoids. Depending on how strict definitions are used, from two to five endogenous cannabinoids have been reported so far. Although AEA had the privilege to be discovered first, and it fulfills all criteria so that it deserved to be classified as an

endocannabinoid, 2-AG is currently considered as the main endocannabinoid for the both cannabinoid receptors (Figure 1.2) (Sugiura et al. 1999; Sugiura et al. 2000; Savinainen et al. 2001). Other naturally occurring fatty acid derivatives possessing activity for the cannabinoid receptors are *O*-arachidonoyl ethanolamine (virodhamine), 2-arachidonoyl glyceryl ether (2-AGE, HU-310, noladin ether), and *N*-arachidonoyl dopamine (NADA) but only 2-AGE will be discussed in more detail in this chapter (Figure 1.2).

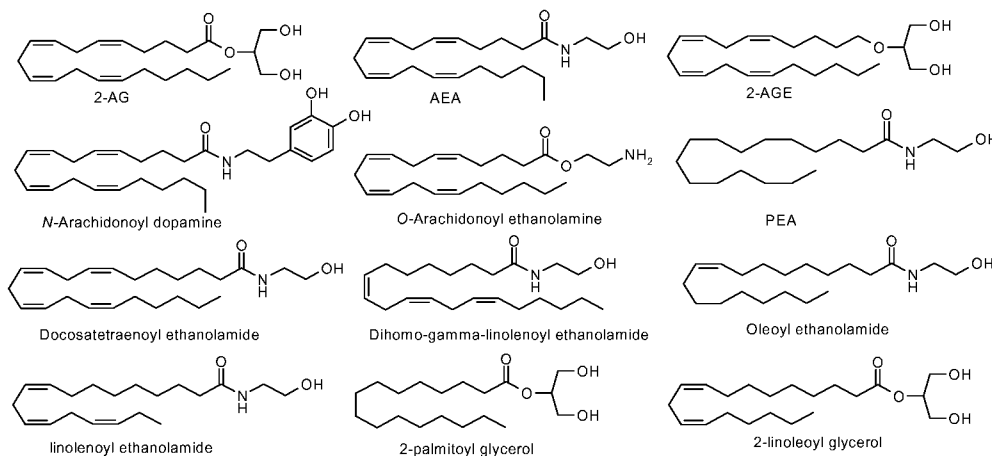


Figure 1.2. Chemical structures of endocannabinoids and related lipid mediators.

AEA produces the effects characteristic of psychotropic cannabinoids, such as hypothermia, analgesia, decreased locomotion, and catalepsy (Fride and Mechoulam 1993). In addition, a variety of other biological effects have been reported, such as effects on cell proliferation and apoptosis, and modulation of reproduction and feeding (Gomez et al. 2002; Maccarrone and Finazzi-Agro 2003; Fride 2004). The levels of AEA in the tissues are generally very low indicating that it is synthesized on-demand and inactivated via reuptake into the cells followed by enzymatic hydrolysis. In the brain, the highest AEA concentrations are found in hippocampus, parahippocampal cortex, cerebellum, striatum, and thalamus (Felder et al. 1996). AEA has also been found in peripheral tissues, such as human and rat spleen, and human heart and rat skin (Felder et al. 1996). Although there is no doubt that AEA is an endocannabinoid, the

facts that AEA is only a partial agonist for the cannabinoid receptors, and its levels in the tissues are usually very low, indicate that its role in the ECS is not very significant. Indeed, all the effects of AEA are not mediated through the ECS, since AEA has been reported to interact also with other biological systems, of which the effects on the vanilloid receptors are the best known (Zygmunt et al. 1999; Smart et al. 2000; Di Marzo et al. 2002; Ross 2003).

In 1995, two independent research groups described a novel endogenous compound possessing cannabimimetic activity (Mechoulam et al. 1995; Sugiura et al. 1995). The compound had an ester linkage and a glycerol head, distinguishing it from AEA, and its concentration in the rat brain was almost 800 times higher than that of AEA. This novel endocannabinoid, 2-AG possessed typical cannabimimetic effects. It was active in the tetrad of mice behavioral assays, inhibited cAMP, and induced elevation of intracellular Ca^{2+} concentration (Mechoulam et al. 1995; Sugiura et al. 1996). 2-AG was neglected for some time due to its poor receptor binding affinity properties as compared to AEA (K_i (CB1) for 2-AG 2.4 μ M, K_i (CB1) for AEA 99 nM) (Sugiura et al. 1995). At that time, it was probably 2-AG's susceptibility to enzymatic degradation and acyl migration that made it difficult to determine and handle. After modifying the experimental conditions by inclusion of serine esterase inhibitors, first with diisopropyl fluorophosphate (DFP) and later with the more potent phenylmethylsulfonyl fluoride (PMSF) and methyl arachidonoyl fluorophosphonate (MAFP) (Savinainen et al. 2003), more reliable and promising results of 2-AG and its role in the endocannabinoid system have been achieved. Although the first CB1 receptor binding studies indicated that perhaps the role of 2-AG as an endocannabinoid was not very significant, it has been later shown that 2-AG is a potent, full efficacy agonist for both cannabinoid receptors (Sugiura et al. 1999; Gonsiorek et al. 2000; Sugiura et al. 2000; Savinainen et al. 2001). It is also most probably the main endocannabinoid and it exhibits interesting pharmacological properties, such as modulation of fasting and feeding, induction of hypotension, inhibition of cell proliferation, and neuroprotection after brain injury, to mention only a few (Varga et al. 1998; Sugiura et al. 1999; Jarai et al. 2000; Melck et al. 2000; Sugiura and Waku 2000; Kirkham et al. 2002; Panikashvili et al. 2005).

In the 1990's, 2-AGE was better known as a stable ether analogue of 2-AG also called as HU-310 (Mechoulam et al. 1998; Sugiura et al. 1999; Suhara et al. 2000). 2-AGE was then used as a pharmacological tool in various *in vitro* and *in vivo* studies due to its metabolic stability and appreciable agonistic activity. 2-AGE seemed to be more potent *in vivo* than 2-AG in reducing blood pressure (Mechoulam et al. 1998; Suhara et al. 2000). However, results comparing the 2-AG and 2-AGE biological activities *in vivo* need to be evaluated with caution since under normal physiological conditions 2-AG is rapidly metabolized into arachidonic acid (AA). In 2001, Hanus et al. reported that this compound was present in porcine brain, and they claimed that it represented the third endocannabinoid since it had good affinity for the CB1 receptor, and it caused typical cannabimimetic effects, such as sedation, hypothermia, and intestinal immobility. However, 2-AGE's (also known as noladin ether) status as the third endocannabinoid has been under debate since this initial finding because it has taken time for other research groups to repeat this finding. In addition, it is noteworthy, that if 2-AGE was naturally present in the brain, it would be the first 2-*O*-alkyl ether-linked lipid so far found in mammalian tissues. Di Marzo's group showed in 2002 that the compound possessing the same mass and similar chemical properties (i.e. chromatographical properties) as 2-AGE could be found in the brain (Fezza et al. 2002). Nevertheless, 2-AGE's occurrence in the brain did not fully overlap with the regional distribution of the CB1 receptors. Furthermore, opposite findings were reported by Oka et al. (2003) who were not able to detect appreciable amounts of 2-AGE from the distinct mammalian brain tissues using GC-MS and fluorometric HPLC. Due to these conflicting findings and the unclear biosynthetic route of 2-AGE, perhaps it is too early to consider 2-AGE as the third endocannabinoid.

Biosynthetic pathways. The endocannabinoids differ from traditional neurotransmitters since they are not stored but they are synthesized rapidly from membrane phospholipid precursors upon stimulation. Two synthesis mechanisms for AEA have been proposed. One is direct *N*-acylation of ethanolamine catalyzed by fatty acid amide hydrolase (FAAH) (Hillard 2000). Since this pathway requires reverse reaction of FAAH, its physiological relevance is dubious. Instead, AEA is more likely formed from phosphatidylethanolamine as presented in Figure 1.3 (Di Marzo 1998;

Hillard 2000). Figure 1.3 also shows that for 2-AG, several biosynthesis routes are available, which may partly explain why in the brain 2-AG levels are much higher than those of AEA. Finally, it is tempting to hypothesize that both 2-AG and AEA can also be formed simultaneously, since their biosynthesis routes may overlap via *sn*-1,2-diarachidonoyl phosphatidylcholine, phosphatidylethanolamine, and the enzyme *trans*-acylase (Figure 1.3) (Di Marzo 1998).

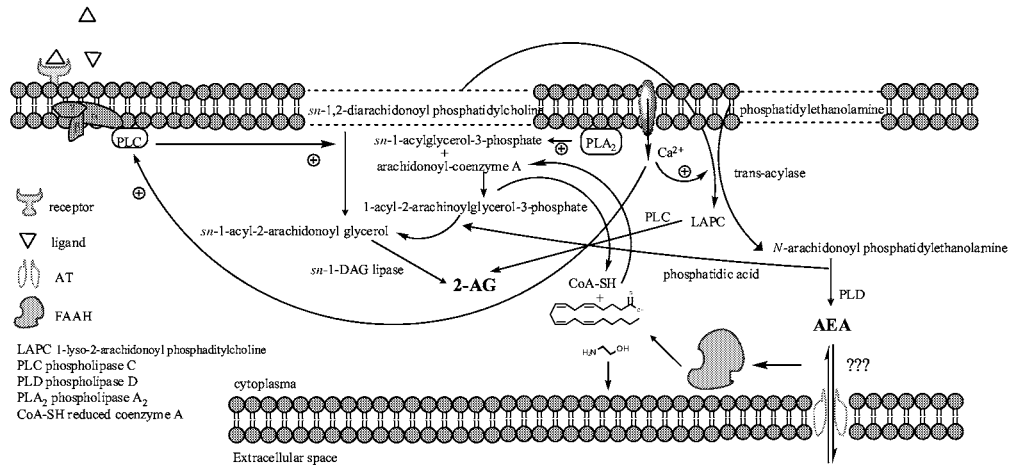


Figure 1.3. Possible biosynthesis and inactivation mechanisms for 2-AG and AEA.

Reuptake and Inactivation. Endocannabinoids have been proposed to reach their target proteins by rapid lateral diffusion within the membrane leaflet (Makriyannis et al. 2005). After interacting with the receptors, endocannabinoids are rapidly taken back into the cells and hydrolysed into arachidonic acid (AA) by an enzyme-catalyzed reaction. Since the enzymes responsible for the inactivation are located within the cell, the compounds need to be transported across the cell membrane. The transport mechanism is not fully understood, and the putative mechanism has been changed within last ten years from being ATP-independent, protein facilitated transport to simple concentration gradient driven diffusion across the membrane (Glaser et al. 2005). There are a few facts which support the existence of a specific transporter protein; AEA transport can be inhibited in a dose-dependent manner, it is rapid and temperature-

dependent, it is known to be linked to second messengers and signal pathways, and finally, it is saturable (McFarland and Barker 2004). However, no specific AEA transporter (AT) has been cloned, and therefore, the transport mechanism can only be hypothesized. Currently, there are three predominant hypotheses but these are not mutually exclusive, since different mechanisms may exist in different cell-types. In the first theory, introduced by Hillard and Jarrahian (2003), AEA is accumulated into cells by facilitated diffusion i.e. the transmembrane movement of AEA is regulated by the concentration gradient between the levels of extracellular and intracellular free AEA. In addition, they suggested that intracellular AEA may be partly sequestered, for example, by binding to some protein. The second theory is rather similar to the first but it proposes that the carrier molecule is not bound to the membrane but it is located intracellularly. One interesting point of view is that the carrier molecule may in fact be some already recognized member of the ECS, such as a metabolic enzyme or even the cannabinoid receptor (Ortega-Gutierrez et al. 2004). The third mechanism is based on endocytosis; the molecule is delivered across the membrane via caveolae/lipid rafts where it is readily available for FAAH (McFarland et al. 2004).

The activity of the endocannabinoids is ultimately terminated by specific intracellular enzymes. AEA is known to be mainly hydrolyzed by an enzyme called fatty acid amide hydrolase (FAAH). Recently, it has been reported that another enzyme which is a member of choloylglycine hydrolase family, *N*-acylethanolamine-hydrolyzing acid amidase (NAAA), is also able to hydrolyze AEA (Tsuboi et al. 2005). The role of FAAH in the ECS is supported, not only by its distribution in brain which correlates well with that of the CB1 receptors, (Deutsch et al. 2002) but especially by the studies conducted with the recently introduced FAAH(-/-) mice (Ueda et al. 2000; Cravatt et al. 2001). Unlike other known amidases, FAAH is a membrane-bound protein which was known to catalyze the formation and hydrolysis of *N*-acyl ethanolamides long before the connection between FAAH and AEA was discovered (Ueda et al. 2000). Soon after the isolation of AEA, its metabolizing enzyme was first reported to be FAAH, and few years later FAAH was cloned from rat liver plasma membranes (Deutsch et al. 1993; Cravatt et al. 1996). At present, the X-ray crystal structure for FAAH is also available, and this has provided valuable information about the three dimensional and quaternary

structure of FAAH, most importantly, for its active site (Bracey et al. 2002). Mutation studies and crystal structure have revealed that FAAH is a serine hydrolase with a catalytic function based on serine-serine-lysine triad (McKinney and Cravatt 2005). Additionally, it has been proposed that FAAH has a dimeric quaternary structure and pores allowing the enzyme to interact with the membrane and cytoplasmic compartments simultaneously (McKinney and Cravatt 2005). FAAH is also known to possess an esterase activity for monoacyl glycerols, such as 2-AG. However, according to present knowledge, FAAH is not the main metabolizing enzyme for 2-AG (Di Marzo et al. 1998; Dinh et al. 2002a, b). Instead, since it is a monoacylglycerol, 2-AG is mainly metabolized by a specific lipase. Currently, knowledge about this lipase is circumstantial since good pharmacological tools, such as specific inhibitors, are not available. However, one lipase that is believed to be responsible for the degradation is a serine hydrolase called monoacylglycerol lipase (MGL) (Saario et al. 2004). MGL was cloned recently from rat brain in a study where MGL was also shown to be the main metabolizing enzyme of 2-AG since overexpression of MGL resulted an accumulation of 2-AG in cortical neurons, but had no effect on AEA accumulation (Dinh et al. 2002a). In addition, the significance of MGL in the ECS received support when the distribution of MGL mRNA was evaluated. It was found to overlap fully with those brain areas containing the highest CB1 receptor densities (Dinh et al. 2002a, b). A few years later, the intracellular site MGL was localized to presynaptic neuronal compartments. This contrasts with FAAH which has been shown to be primarily a postsynaptic enzyme associated with cytoplasmic membranes (Gulyas et al. 2004). Some data are also available about the active site of MGL. Karlsson et al. (1997) have used site-directed mutagenesis to reveal the existence of a lipase-specific catalytic triad (Ser-Asp-His). Saario et al. (2005) have further identified, by means of sulfhydryl-specific reagents, a cysteine residue critical for an inhibitory activity.

Quite recently, other metabolic pathways for endocannabinoids, especially those associated with cyclooxygenase-2 (COX-2) and lipoxygenases, have been proposed (Lambert and Fowler 2005). For example, it has been shown that a COX-2 selective inhibitor, but not a FAAH inhibitor, can increase endocannabinoid-mediated DSI in

hippocampal slices, pointing to a role for COX-2 in hippocampal endocannabinoid metabolism (Kim and Alger 2004).

Other lipid mediators related to the ECS. In addition to the endocannabinoids mentioned above, other compounds with a lipid structure can affect the function of the ECS. Two of these lipid mediators, dihomono- γ -linolenoyl ethanolamide and docosatetraenoyl ethanolamide bind to the cannabinoid receptors but are not primarily considered to be endocannabinoids (Figure 1.2) (Hanus et al. 1993). Nevertheless, most of these compounds do not have affinity for the cannabinoid receptors even though they possess some cannabimimetic characteristics. This may be a consequence of their ability to compete for the same metabolic enzymes with endocannabinoids, thus increasing endocannabinoid levels in tissues and prolonging their duration of action (Jonsson et al. 2001).

The effects of AEA are potentiated by several lipid derivatives, for example, acyl ethanolamides, such as oleoyl ethanolamide, and linoleoyl ethanolamide (Figure 1.2). However, the most extensively studied of these so called entourage compounds is *N*-palmitoyl ethanolamide (PEA, Figure 1.2) (Bradshaw and Walker 2005). The anti-inflammatory and antinociceptive effects of PEA have been recognized for decades. It has been observed that the antinociceptive effects of PEA could be enhanced by up to 100-fold when it was co-administered with AEA (Calignano et al. 2000). This increased potency is cannabinoid receptor mediated since it can be abolished by cannabinoid receptor antagonists. Similarly as AEA is affected by acyl ethanolamides, 2-AG is potentiated by some acyl glycerols, such as 2-linoleoyl glycerol and 2-palmitoyl glycerol (Figure 1.2). These compounds have been reported to significantly potentiate the biological activity of 2-AG both *in vitro* and *in vivo* (Ben-Shabat et al. 1998).

1.2.3 Physiological significance

There is a body evidence to indicate that the ECS plays an important role as a neuronal modulator. The CB1 receptors are mainly located on axon terminal membranes, where they have been suggested to participate in the regulation of the release of other neurotransmitters (Howlett et al. 2002; Freund et al. 2003). Figure 1.4

provides a simplified image of the ECS and its mechanisms of action. Cannabinoids have been shown to be linked to previously characterized phenomena called depolarization-induced suppression of inhibition (DSI) and depolarization-induced suppression of excitation (DSE) (Kreitzer and Regehr 2001; Wilson and Nicoll 2001). DSI (or DSE) is initiated by depolarization-induced opening of N-type voltage-controlled Ca^{2+} channels which trigger the synthesis and release of endocannabinoids (Maejima et al. 2001). The postsynaptically released endocannabinoids bind to the presynaptic CB1 receptors leading to a reduction of cAMP production, inhibition of voltage-dependent Ca^{2+} channels, and stimulation inwardly rectifying potassium channels. These actions ultimately inhibit the release of GABA (or glutamate) in the presynaptic neurons. In conclusion, via DSI/DSE, the ECS can act as a retrograde modulator system controlling the extent of neuronal excitability. Finally, one must remember, that in addition to being a neuronal modulator, the ECS can affect also other physiological systems, such as the cardiovascular and reproductive systems (Sugiura and Waku 2000).

1.2.4 The endogenous cannabinoid system as a target for a drug discovery

The ECS provides several approaches for the drug discovery. The traditional method has been a direct manipulation of the cannabinoid receptors with an exogenously administered receptor ligand (agonist/antagonist/inverse agonist). Another approach is indirect activation of the cannabinoid receptors 1) by increasing the levels of the endocannabinoids with FAAH, MGL or AT inhibitors, 2) by enhancing the effects of the endocannabinoids (or exogenously administered ligands) by allosteric modulation of the cannabinoid receptors, or 3) modulation of the biosynthesis of endocannabinoids (Pertwee 2005b; Ueda et al. 2005). Indirect activation of the cannabinoid receptors is based on the concept that certain pathological conditions elevate local levels of endocannabinoids, and therefore, compounds that are able to potentiate the effects of endocannabinoids may be able to relieve the symptoms without evoking side-effects (Pertwee 2005b).

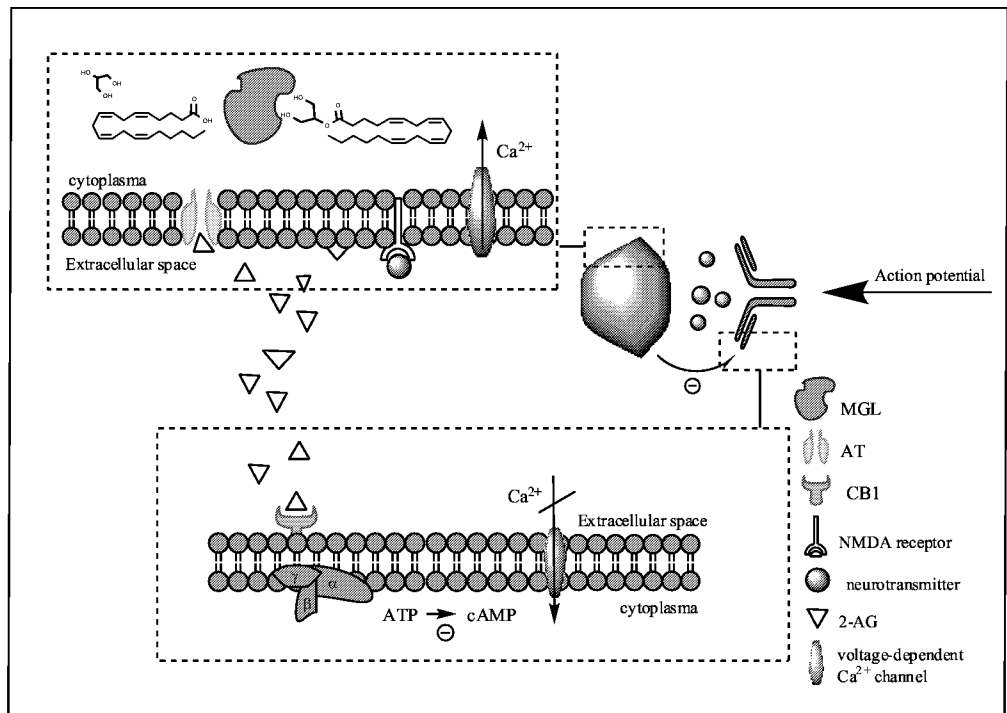


Figure 1.4. Schematic representation of the ECS and its mechanism of action.

1.3 CB1 receptor ligands

Although the word “cannabinoid” is readily associated with plant-derived Δ^9 -THC-like compounds, cannabinoids, or more precisely, cannabinergics, are nowadays a diverse group of compounds acting via the ECS. The subsequent chapters will provide an overview of currently available CB1 receptor ligands. The ligands are reviewed by dividing them into smaller groups according to their pharmacological profile and chemical structure. This literature review does not extend to CB2 ligands or FAAH/MGL inhibitors which will be discussed in more detailed in the doctoral theses of Katri Raitio, M.Sc., and Susanna Saario, M.Sc. (Pharm), respectively.

1.3.1 Agonists

The CB1 receptor agonists are traditionally divided into five different groups:

- a) endocannabinoid related compounds,
- b) classical cannabinoids,
- c) non-classical cannabinoids,
- d) aminoalkylindoles, and
- e) miscellaneous compounds, such as hybrid ligands.

a) Endocannabinoid related compounds (eicosanoids)

These compounds are structural analogues of the endocannabinoids. Their structural relationship to AEA and 2-AG may explain why these kinds of compounds act exclusively as agonists at the CB1 receptors. Structure-activity relationship (SAR) investigations of eicosanoids have been focused mainly on the structural analogues of AEA. A large number of different kinds of derivatives with various modifications on the polar head, the amide group, the lipophilic pentyl tail, and/or the arachidonoyl backbone have been reported.

Modifications on the polar head (Table 1.1)

It is known that a hydroxyl group, and thus a hydrogen-bonding group, is not necessary for CB1 receptor activation. In fact, compounds having *N*-ethyl, *N*-propyl (**1**), cyclopropyl (**2**) or alkenyl/alkynyl instead of hydroxyethyl have even higher affinity for CB1 receptors than AEA (Pinto et al. 1994; Lin et al. 1998; Hillard et al. 1999). In addition, the hydroxyl group can be replaced by halogens (**3,4**) leading to compounds with very high affinity (Adams et al. 1995a, b; Hillard et al. 1999). Methyl alkylations at positions 1 (**5**, (*R*)-metanandamide) and 2 of hydroxyethyl group retain the activity while providing significantly better enzymatic stability (Abadji et al. 1994; Adams et al. 1995a, b). Amino and carboxylic acid groups as well as bulky alkyl, cyclic or aromatic head groups diminish CB1 affinity (Pinto et al. 1994; Adams et al. 1995a, b; Khanolkar et al. 1996; Sheskin et al. 1997; Lin et al. 1998). Finally, only a few polar head modifications for 2-AG and 2-AGE have been reported. Those few attempts have lead

generally to only weak activities compared to the parent compounds (Pinto et al. 1994; Ng et al. 1999; Sugiura et al. 1999; Suhara et al. 2001).

Table 1.1. AEA analogues with modified polar head groups.

| Compound | Structure | $K_i(\text{CB1}) \pm \text{SEM (nM)}^1$ | Reference |
|-----------------|-----------|---|--|
| AEA | | 61 ± 8 72 ± 7.3 89 ± 10 | (Adams et al. 1995b; Lin et al. 1998; Hillard et al. 1999) |
| 1 | | 7.3 | (Pinto et al. 1994) |
| 2 (ACPA) | | 2.2 ± 0.4 | (Hillard et al. 1999) |
| 3 | | 8.6 ± 1.1 | (Adams et al. 1995b) |
| 4 (ACEA) | | 1.4 ± 0.3 | (Hillard et al. 1999) |
| 5 | | 20 ± 1.6 | (Abadji et al. 1994) |

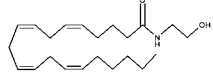
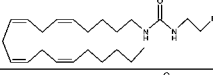
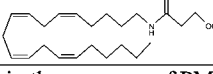
1) Affinity determined in the presence of PMSF

Modifications on the amide/ester/ether group (Table 1.2)

Synthesis and biological evaluation of carbonate, carbamate, thioamide and tertiary amide derivatives of AEA have shown that the amide group of AEA cannot be replaced directly by any other group without affecting the affinity (Khanolkar et al. 1996; Ng et al. 1999; Urbani et al. 2006). However, by modifying other parts of molecule at the same time, appreciable affinities can be achieved (**6**) (Ng et al. 1999). Although the amide bond cannot be replaced, it can be reversed (**7**) (Lin et al. 1998). The reversed amides are slightly weaker agonists than AEA when PMSF is used as an enzyme inhibitor (Lin et al. 1998). However, without PMSF, these compounds show notable activity improvements in comparison to AEA. This is an evidence of their significantly improved enzymatic stability.

There have been a few attempts to replace the ester bond of 2-AG. However, removal of a carbonyl group (i.e. 2-AGE) or replacement of the ester with a ketone resulted in significantly weaker biological activity (Suhara et al. 2001).

Table 1.2. AEA analogues with modified amido groups.

| Compound | Structure | $K_i(\text{CB1}) \pm \text{SEM (nM)}^1$ | Reference |
|-------------------------------|---|---|--|
| AEA |  | 61 ± 8 72 ± 7.3 89 ± 10 | (Adams et al. 1995b; Lin et al. 1998; Hillard et al. 1999) |
| 6 |  | 55 ± 8 | (Ng et al. 1999) |
| 7 (retroanandamide) |  | 115 ± 9 | (Lin et al. 1998) |

1) affinity determined in the presence of PMSF

Modifications on the end pentyl tail (Table 1.3)

The importance of the end pentyl chain for the activity properties of AEA and its derivatives has been examined by branching and changing the length of the chain (Ryan et al. 1997; Selzman et al. 1997). The CB1 affinity properties of AEA can be improved by addition of one to four methylenes into the AEA backbone, with the optimal being 3 methylenes (**8**) (Ryan et al. 1997). It is noteworthy though, that such analogues did not exhibit any significant *in vivo* pharmacological effects (Ryan et al. 1997). The structure of AEA can be further modified by branching the chain. Ryan et al. (1997) showed that methylation(s) at position 16 resulted in high affinity compounds with significantly improved *in vivo* activity (**9**, **10**, **11**). In addition, Di Marzo et al. (2001a) reported that by replacing the end methyl with cyano (**12**) or bromine (**13**) considerable receptor selectivity could be achieved (1000-fold CB1 affinity over CB2 affinity). Recently, Li et al. (2005) described two high affinity covalently binding AEA analogues for the CB1 receptor this being achieved by introducing either a photoactivatable azido group (**14**) or an electrophilic isothiocyanato (**15**) into the terminal carbon of the pentyl chain. Ramazan et al. (2004) published a synthesis method for a group of compounds with an acetylene group at the end pentyl chain. However, no biological data for these compounds is available, but it will be interesting to see whether the effect is similar to that seen in the series of classical cannabinoids.

Table 1.3. AEA analogues with modified pentyl tail.

| Compound | Structure | $K_i(\text{CB1}) \pm \text{SEM (nM)}^1$ | Reference |
|------------|-----------|---|--|
| AEA | | 61 ± 8 72 ± 7.3 89 ± 10 | (Adams et al. 1995b; Lin et al. 1998; Hillard et al. 1999) |
| 8 | | 18 ± 1.7 | (Ryan et al. 1997) |
| 9 | | 1.9 ± 0.6 7.0 ± 0.6 | (Ryan et al. 1997; Selzman et al. 1997) |
| 10 | | 1.0 ± 0.1 | (Ryan et al. 1997) |
| 11 | | 4.8 ± 1.7 | (Ryan et al. 1997) |
| 12 | | 3.4 ± 0.5 without PMSF | (Di Marzo et al. 2001a) |
| 13 | | 2.2 ± 0.2 without PMSF | (Di Marzo et al. 2001a) |
| 14 | | 0.9 ± 0.2 | (Li et al. 2005) |
| 15 | | 1.3 ± 0.2 | (Li et al. 2005) |

1) Affinity determined in the presence of PMSF

Modifications on the arachidonoyl backbone (Table 1.4)

Three different approaches have been taken while studying SAR's of the arachidonoyl backbone; alkylation(s) on the arachidonoyl skeleton, replacement of double bonds, and variation of the saturation degree.

Goutopoulos et al. (2001) demonstrated that methyl alkylation at the α -position of arachidonoyl backbone (**16-18**) provided improved stability against enzymatic degradation. Alkylation resulted in a pair of enantiomers (**16**, **17**) of which (*S*)-form seemed to be somewhat favored while the metabolizing enzyme was inhibited by PMSF. However, it is noteworthy, that when PMSF was not used, the enzyme also favored the (*S*)-form even more than AEA, meaning that under physiological conditions, AEA would probably be a better ligand (Goutopoulos et al. 2001). The best stability and activity data was, however, produced by methylating also the 2' position of ethanolamine moiety. After dimethylation, diastereoselectivity played a very important

role with the (*R,R*)-form (**19**) having clearly the best affinity as well as the best enzymatic stability. Finally, bulkier groups, such as isopropyl, at the α -position resulted in a loss of CB1 affinity (Adams et al. 1995b).

Endocannabinoids have been observed to be sensitive to the changes in saturation degree. Firstly, *cis* double bonds are crucial for the CB1 activity (Sheskin et al. 1997). Secondly, other endogenously occurring *N*-acyl amides (22:4, 20:3, *n*-6, **20**, **21**) have similar affinity for the CB1 receptor as AEA, but otherwise, affinity is lost gradually when adding or removing double bonds or shortening the chain length (Sheskin et al. 1997; Lin et al. 1998; Appendino et al. 2003). In general, there are very few tolerated modifications which can be made in the arachidonoyl backbone. For example, prostaglandin derivatives of AEA do not possess CB1 affinity (Pinto et al. 1994). However, some small changes are allowed. Affinity studies with lipoxygenase metabolites as well as with other hydroxy derivatives of AEA have shown that positions 12 and 20 can be hydroxylated without this causing any significant decrease in CB1 affinity (Edgmond et al. 1998).

Table 1.4. AEA analogues with modified arachidonoyl backbone.

| Compound | Structure | $K_i(\text{CB1}) \pm \text{SEM (nM)}^1$ | Reference |
|------------|-----------|---|--|
| AEA | | 61 \pm 8 72 \pm 7 89 \pm 10 | (Adams et al. 1995b; Lin et al. 1998; Hillard et al. 1999) |
| 16 | | 54 \pm 5 | (Goutopoulos et al. 2001) |
| 17 | | 35 \pm 4 | (Goutopoulos et al. 2001) |
| 18 | | 72 \pm 6 | (Goutopoulos et al. 2001) |
| 19 | | 7.4 \pm 0.9 | (Goutopoulos et al. 2001) |
| 20 | | 34 \pm 3 | (Sheskin et al. 1997) |
| 21 | | 53 \pm 6 | (Sheskin et al. 1997) |

1) affinity determined in the presence of PMSF

b) and c) Classical cannabinoids and non-classical cannabinoids (Table 1.5)

The group of classical and non-classical cannabinoids includes plant-derived cannabinoids and their structural analogues. From the therapeutical point of view, it is worth mentioning that the two cannabinergic compounds that are currently in medical use belong to the group of classical cannabinoids; synthetic Δ^9 -THC (Marinol[®]), and nabilone (**29**, Cesamet[®]). Classical cannabinoids (CC's) are Δ^9 -THC-like (**22**) compounds with a dibenzopyran ring and a hydrophobic chain at position 3 (see Table 1.5). A huge number of CC's have been synthesized, and their SAR's are known relatively well. From the pharmacophoric point of view, there are five important positions in these compounds: 1) the pentyl chain at position 3, 2) position 1 on the phenyl ring, 3) the pyran ring B, 4) position 6 on the pyran ring, and 5) position 9 on ring C.

The significance of a pentyl chain was recognized already in the 1940's when Adams et al. (1949) observed a distinct potency improvement when the pentyl chain was replaced by 1',1'-dimethylheptyl. Subsequently, the pentyl chain has been proven to play a key role in ligand-receptor interactions (see Table 1.5 for a few representative high affinity ligands, **34-37**) (Thakur et al. 2005). Pentyl chain modifications have also been observed to offer some receptor selectivity. Lu et al. (2005) synthesized adamantyl derivatives of Δ^8 -THC with interesting selectivity features; compound **30** is somewhat CB1 selective whereas compounds **31** and **32** show a preference for the CB2, and finally, compound **33** exhibits no selectivity. The phenolic hydroxyl group is important for CB1 activity, but it can be replaced, for example, by a methoxy group (**39**), if CB2 selectivity is desired (Huffman et al. 1996). The pyran ring B does not play an important role in receptor activation. In fact, removal of this ring produces a group of non-classical cannabinoids (NCC's, **28**, **41**). CP55,940 (**28**), the tritiated form of this compound played a significant role in the discovery of the CB1 receptor, is the best known member of the NCC's (Palmer et al. 2002). *gem*-Dimethyls at position 6 can be replaced by longer alkyl chains with a terminal hydroxyl group generating the fourth pharmacophoric feature of the CC's the so-called "southern aliphatic hydroxyl" (**40**) (Drake et al. 1998; Harrington et al. 2000). Both a methyl group and double bond at position 9 are insignificant in the terms of receptor activation. However, by replacing

the methyl with a hydroxymethyl, the most potent CB1 receptor agonist found so far, HU-210 (**23**), was obtained ($-\log EC_{50} = 8.3 \pm 0.1$, unpublished data). One striking example of ligand structure accuracy versus receptor activation is that the enantiomeric isomer of HU-210, HU-211 (**38**), totally lacks affinity for the cannabinoid receptors (Howlett et al. 1990).

Table 1.5. Classical and non-classical cannabinoids.

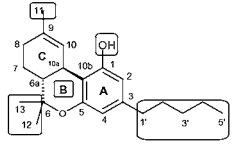
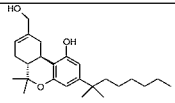
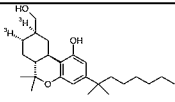
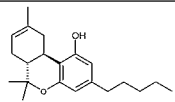
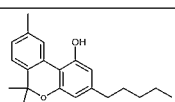
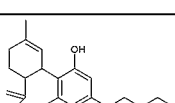
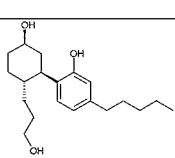
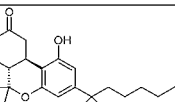
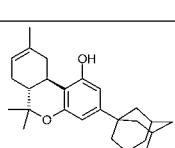
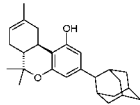
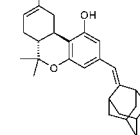
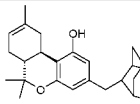
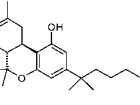
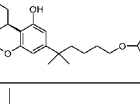
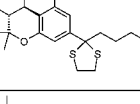
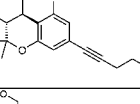
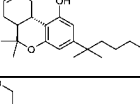
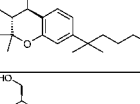
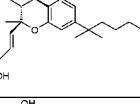
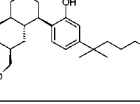
| Compound | Structure | K_i (CB1) nM | Ref. |
|--------------------------------------|---|-----------------------|-----------------------|
| 22 Δ^9 -THC |  | 41 | (Compton et al. 1993) |
| 23 HU-210 |  | 0.2 | (Howlett et al. 1990) |
| 24 [³ H]HU-243 |  | K_D 0.045 | (Pertwee 1999) |
| 25 Δ^8 -THC |  | 126 | (Thakur et al. 2005) |
| 26 CBN |  | 308 | (Pertwee 1999) |
| 27 CBD |  | 4350 | (Compton et al. 1993) |
| 28 CP55,940 |  | 0.9 | (Compton et al. 1993) |
| 29 Nabilone |  | 2.2 | (Palmer et al. 2002) |
| 30 |  | 6.8 K_i (CB2)=52 | (Lu et al. 2005) |

Table 1.5. (Continued).

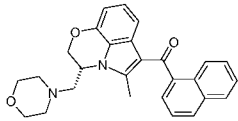
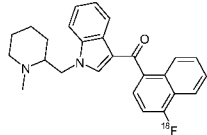
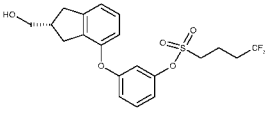
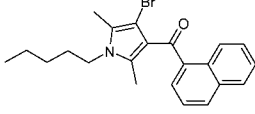
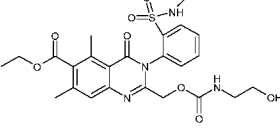
| Compound | Structure | $K_i(\text{CB1})$ nM | Ref. |
|--------------|---|-------------------------------|--------------------------|
| 31 |  | 35 $K_i(\text{CB2})=14$ | (Lu et al. 2005) |
| 32 |  | 49 $K_i(\text{CB2})=9$ | (Lu et al. 2005) |
| 33 |  | 80 $K_i(\text{CB2})=76$ | (Lu et al. 2005) |
| 34 |  | 0.2 | (Thakur et al. 2005) |
| 35 |  | 1.5 | (Thakur et al. 2005) |
| 36 |  | 0.3 | (Thakur et al. 2005) |
| 37 |  | 0.65 | (Thakur et al. 2005) |
| 38 HU-211 |  | no appreciable affinity | (Howlett et al. 1990) |
| 39 |  | 1.2 $K_i(\text{CB2})=0.03$ | (Huffman et al. 1996) |
| 40 |  | 0.7 | (Harrington et al. 2000) |
| 41 |  | 1.4 | (Palmer et al. 2002) |

d) and e) Aminoalkylindoles and other CB1 receptor ligands (Table 1.6)

Already in the early 1990's, aminoalkylindoles (AAI's) were recognized as novel ligands for the cannabinoid receptors (D'Ambra et al. 1992). These derivatives of pravadoline were originally designed as non-ulcerogenic non-steroidal anti-inflammatory drugs, and the discovery of their cannabinergic activity was groundbreaking since their structure was so different from classical cannabinoids (Ward et al. 1990; Bell et al. 1991). The most extensively studied compound of this group is WIN-55,212 (**42**). Stereochemistry plays an important role in the biological activity of WIN-55,212 since only one enantiomer possesses cannabinergic activity. The other enantiomer has been reported to act as a neutral antagonist at the CB2 receptors (Savinainen et al. 2005). The SAR's of AAI's are well known with the key structural features being a bicyclic substituent at the 3-position, a small substituent at the 2-position, and an aminoethyl substituent (morpholino) at the 1-position (Eissenstat et al. 1995). Even minor structural changes have been observed to reverse the activity from agonist to antagonist/inverse agonist (Eissenstat et al. 1995; Palmer et al. 2002). Recently, Willis et al. (2005) reported novel aminoalkylindoles which could serve as PET (positron emission tomography) ligands in animal studies. A racemic mixture of their best compound, **43**, showed high affinity for the CB1 receptor. After the enantiomers were separated and tested in a displacement assay in mouse brain, only one enantiomer was shown to be active.

Currently, a many other types of CB1 receptor ligands have been described. Most of the novel structures have been produced by pharmaceutical companies, and therefore, are only briefly described in patents, such as the compound **46** developed by Novartis (Hertzog 2004). However, a few novel classes of cannabinergics have also been depicted in some scientific papers. In 2002, Mauler et al. (2002) described totally new class of compounds, diarylether sulfonylesters, possessing cannabinergic activity. Their model compound, BAY 38-7271 (**44**), was a high affinity cannabinoid receptor agonist with neuroprotective efficacy *in vivo*. In addition, Tarzia et al. (2003) synthesized a series of pyrrole derivatives, of which compound **45** had comparable activity to WIN-55,212.

Table 1.6. Aminoalkylindoles and other types of CB1 receptor ligands.

| Compound | Structure | K _i (CB1) nM | Reference |
|---------------------------|---|----------------------------|---|
| 42 (WIN-55,212) |  | 1.9 8.7 | (Dutta et al. 1997; Palmer et al. 2002) |
| 43 |  | 0.7 | (Willis et al. 2005) |
| 44 |  | 0.46 | (Mauler et al. 2002) |
| 45 |  | 13.3 ± 0.5 * | (Tarzia et al. 2003) |
| 46 |  | 34 | (Hertzog 2004) |

* [³⁵S]GTPγS binding EC₅₀ ± SEM

1.3.2 Antagonist/inverse agonists (Table 1.7)

The history of CB1 receptor antagonists dates to 1994 when researchers in Sanofi-Synthélabo (currently Sanofi-Aventis) discovered the first selective CB1 antagonist, SR141716A (**47**). In fact, SR141716A is now classified as an inverse agonist rather than a pure antagonist since it produces effects that are opposite to those of agonists (Pertwee 1999). SR141716A, also known as rimonabant or Acomplia[®], belongs to the structural category of diarylpyrazoles the SAR's of which are well established. CB1 activity requires that the pyrazole ring is substituted at the 5-position with a *p*-substituted phenyl ring and at the 1-position with 2-chloro or 2,4-dichlorophenyl ring (Lan et al. 1999). Biological activity can be further improved by substituting the 3-position with a carboxamide group bearing a cyclic group, such as piperidinyl. In addition, a short alkyl chain at position 4 is not only favoured from the activity point of view, but it also provides a longer duration of action (**48**) (Rinaldi-Carmona et al. 2004; Lange and

Kruse 2005; Muccioli and Lambert 2005). Another compound which has been used widely as a valuable pharmacological tool is AM251 (**49**). It has a structure very similar to SR141716A but its phenyl ring is substituted with iodide instead of chloride.

In the group of CC's and NCC's, few compounds have shown antagonistic activity for the CB1 receptor (representative compound **51**). However, biological data for these compounds is more or less contradictory since compounds seem to act as agonists/partial agonists or antagonists depending on the assay used (Pertwee et al. 1996).

Much development work has been done with compounds similar to rimonabant. Several novel CB1 antagonists seem to offer some clinical potential due to the success of rimonabant. A few new compound groups have been described by the academic research groups, such as aryl-imidazoline-2,4-diones (**52**) developed by Didier Lambert and co-workers (Kanyonyo et al. 1999) or the high affinity pyrazole-3-carboxamides (**53**) synthesized by Ruiu et al. (2003). However, most of the novel group of CB1 antagonists are being developed by pharmaceutical companies and are only described in patents, such as the azetidines prepared by Aventis (**54**), the 3,4-diaryl-pyrazolines by Solvay (**55**), the diaryl-pyrazines by AstraZeneca (**56**), the pyrazolotriazines by Pfizer (**57**), and the furo[2,3]pyridines by Merck (**58**) (Muccioli and Lambert 2005). The activity of so many large pharmaceutical companies in the field of CB1 receptor antagonists emphasizes not only the clinical importance of these compounds but also their commercial value.

Table 1.7. CB1 receptor antagonists.

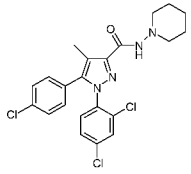
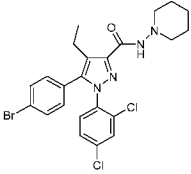
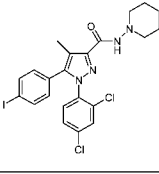
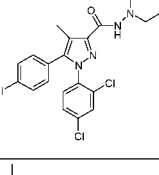
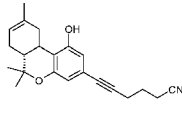
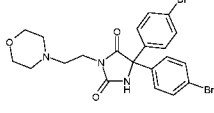
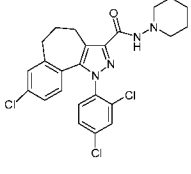
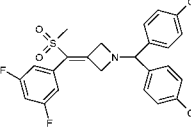
| Compound | Structure | K _i (CB1) nM | Reference |
|---------------------------------------|---|----------------------------|---|
| 47 SR141716A, rimonabant |  | 2.0 | (Rinaldi-Carmona et al. 1995) |
| 48 SR147778 |  | 3.5 | (Rinaldi-Carmona et al. 2004) |
| 49 AM251 |  | 7.5 | (Palmer et al. 2002) |
| 50 AM281 |  | 12 | (Palmer et al. 2002) |
| 51 |  | 0.77 | (Pertwee et al. 1996) |
| 52 |  | 70 | (Kanyonyo et al. 1999) |
| 53 NESS 0327 |  | 0.0035 | (Ruiu et al. 2003) |
| 54 |  | not reported | (Palmer et al. 2002; Muccioli and Lambert 2005) |

Table 1.7. (Continued).

| Compound | Structure | K _i (CB1) nM | Reference |
|----------|-----------|----------------------------|-----------------------------|
| 55 | | 14 | (Muccioli and Lambert 2005) |
| 56 | | not reported | (Muccioli and Lambert 2005) |
| 57 | | not reported | (Muccioli and Lambert 2005) |
| 58 | | not reported | (Muccioli and Lambert 2005) |

1.4 Therapeutic potential of the CB1 receptor ligands

Despite of an apparent therapeutic potential of cannabinoids, their current medical use is exiguous. Synthetic Δ^9 -THC (Marinol[®]), Δ^9 -THC's close structural analogue, nabilone (Cesamet[®]) and Savitex[®], a drug containing cannabis extracts, are available in a few countries for the treatment of chemotherapy-associated nausea and vomiting, the management of AIDS related wasting and MS-disease-associated pain (<http://www.drugs.com/cons/Cesamet.html>); <http://www.marinol.com/>; <http://www.mssociety.ca/en/research/medmmo-marij-sativex-june05.htm>). Research intended to evaluate the therapeutic use of cannabinoids has faced severe political and social pressure due to the recreational use of marijuana. Since many of the beneficial effects of cannabinoids result from the ligand interactions with the CB1 receptors located in the CNS, the psychotropic side-effects are difficult to avoid. The fact that the cannabinoids seem to have some kind of effect on almost all biological systems, can also be considered problematic. There are also other more practical problems, such as dose optimization and correct route of administration, which have hampered their therapeutic use. In spite of these problems, it is hoped that an understanding of the

details of their pharmacology as well as novel methods dispensing will be able to overcome the current problems. It is reasonable to believe that in the future the cannabinoids will achieve more general acceptance as therapeutically significant agents. The following chapters will give a short overview of those disorders where expectations about therapeutic benefits of cannabinoids are the highest.

1.5 Nervous system

The most important and also the most challenging targets for cannabinoid-based drug development are found in the CNS. Since the CB1 receptors are widely expressed in different parts of the brain as well as in the spinal cord, it is to be expected that cannabinoids would have a clear impact on a variety of physiological events. The best-known effects of cannabinoid agonists are their ability to induce hypothermia, stimulate appetite and reduce vomiting mediated mainly through the hypothalamus (Porter and Felder 2001; Berry and Mechoulam 2002; Rawls et al. 2004). As mentioned in the previous chapter, cannabinoids are already in a medical use in several eating related disorders. The CB1 receptors in basal ganglia and hippocampus reflect a functional role of cannabinoids in movement and movement disorders, e.g. Huntington's disease (HD), Tourette's syndrome (TS) and Parkinson's disease (PD) (Muller-Vahl et al. 1998; Sieradzan et al. 2001; Lastres-Becker et al. 2003). The CB1 receptors in the hippocampus, amygdala, and cerebral cortex are believed to be involved in some kinds of spasticity, for example, in multiple sclerosis (MS) and spinal cord injury (Consroe 1998; Baker et al. 2000; Baker et al. 2001). In addition, cannabinoids are proposed to have positive effects on conditions with neuronal loss, e.g. that occurring after a stroke (Fowler 2003). In a variety of animal models, cannabinoids have been reported to be effective against both acute and chronic pain (Walker and Huang 2002). Cannabimimetic-induced analgesia is mediated especially via the spinal cord but also the thalamus and the periaqueductal gray (PAG) as well as the peripheral CB1 receptors are involved. Finally, cannabinoids alter memory and cognition mainly via hippocampal neurons. It has been suggested that drugs inhibiting the metabolic fate of the endocannabinoids as well as CB1 receptor inverse agonist may serve as memory

enhancers in conditions where cognition and memory have deteriorated, e.g. dementia (Consroe 1998; Goutopoulos and Makriyannis 2002).

1.5.1 Appetite and emesis

The appetite-inducing and anti-emetic effects of cannabimimetics have been recognized for centuries, in fact they can be considered as the most extensively studied properties of cannabinoids. In the 1980's, Δ^9 -THC was approved by FDA for clinical use in the treatment of chemotherapy-related nausea, and at the beginning of the 1990's its range was expanded to the treatment of AIDS-induced wasting (Cota et al. 2003a).

To date, emesis is the widest indication for cannabinoid use. It is reasonable to believe that the antiemetic effects of cannabinoids are mediated via the CB1 receptor, since 1) it has been shown that the CB1 receptors are located in those areas involved in emesis; the myenteric plexus of the stomach and duodenum, and in the dorsal vagus complex of the brainstem, and 2) CB1 receptor antagonists evoke vomiting and agonists inhibit emesis in a variety of animal models (Coutts and Izzo 2004). In the year 2001, Tramer et al. reviewed the results of 30 cannabinoid and nausea-related clinical trials observing that cannabinoids were slightly more efficacious than conventional antiemetics for chemotherapy induced nausea and vomiting, and patients chose these compounds over other available drugs even though cannabinoids carried a higher risk of adverse effects. It is noteworthy that some adverse effects, like euphoria, "high" and sedation, were even considered as positive by many chemotherapy patients.

There are several facts supporting the concept that the ECS participates in the control of food intake via the CNS. First, the CB1 receptors as well as the endocannabinoids are densely distributed in the hypothalamus, the main brain area regulating feeding behaviour and body weight control. In addition, the levels of the endocannabinoids in the hypothalamus and the limbic forebrain have been reported to vary in different feeding phases, being highest during fasting and lowest when sated (Kirkham et al. 2002). Secondly, leptin, a hormone playing a key role in modulating food intake and body fat, seems to be linked to the ECS. Leptin-deficient mice are obese and they have increased levels of endocannabinoids in their hypothalamus, however, after leptin administration, feeding decreases and endocannabinoid levels in the hypothalamus are

also reduced (Di Marzo et al. 2001b). Thirdly, the selective CB1 receptor antagonist, rimonabant, has been shown to inhibit the positive feedback normally associated with food consumption (Thornton-Jones et al. 2005). Finally, both the animal studies and the clinical trials with rimonabant, have demonstrated a reduction of food intake, especially that of palatable food (Colombo et al. 1998; Simiand et al. 1998; Di Marzo and Matias 2005). In addition to the CNS regulation of feeding, cannabinoids have also been suggested to participate in lipogenesis and fat accumulation in peripheral level; the CB1 receptors are found in white adipocytes where their stimulation activates lipoprotein lipase (Cota et al. 2003b). Also other organs, such as liver, pancreas and skeletal muscle are believed to regulate energy balance through the endocannabinoid system (Di Marzo and Matias 2005; Lichtman and Cravatt 2005).

One issue closely related to cannabinoids and appetite, is the role of the ECS in the survival newborns. It has been demonstrated that the CB1 receptors are needed for the initiation of the suckling response in neonatal mice (Fride et al. 2001). The 2-AG level in the brain of newborn mice is twice as high as those in adults, however, after suckling is initiated, it has been suggested that feeding is promoted by 2-AG present in maternal milk (Berrendero et al. 1999; Fride 2004).

It took over 20 years from the time when the first cannabinoid was approved for therapeutic use before the next synthetic cannabinoid was seriously applied for clinical approval. In 2006, Sanofi-Aventis (formerly Sanofi-Synthelabo), will probably launch their CB1 receptor antagonist, Acomplia[®] (rimonabant, SR141716A), for the treatment of obesity and smoking cessation (<http://www.newstarget.com/003794.html>). Large phase 4 clinical trials for rimonabant have just been completed (RIO-Europe and RIO-North America), indicating that rimonabant is able to promote modest to significant decrease in bodyweight and waist circumference, and to improve cardiovascular risk factors (Van Gaal et al. 2005; Pi-Sunyer et al. 2006). However, the RIO-North America trial was limited by a high drop-out rate.

1.5.2 Movement disorders

Several facts support the concept that the ECS is involved in the regulation of motor functions. Firstly, the CB1 receptors are abundant in the basal ganglia and the

cerebellum, areas controlling motor activity (Herkenham et al. 1991a; Herkenham et al. 1991b). In addition, the motor depressive effects for various CB1 receptor agonists have been well demonstrated *in vivo*, and they are also a well-recognized side-effects among marijuana smokers (Rodriguez de Fonseca et al. 1998). Lastly, a few clinical studies with patients with neurological disorders and the previous *in vivo* studies with rodents having induced motor dysfunction, have confirmed the impaired function of the CB1 receptors in these conditions (Consroe 1998; Muller-Vahl et al. 1998; Lastres-Becker et al. 2001a, b). The most extensively studied disorders related to the motor dysfunction are dystonia, epilepsy, Huntington's disease (HD), Tourette's syndrome (TS) and Parkinson's disease (PD) the last three of which are presented here in more detail.

Huntington's disease is neurodegenerative disease characterized by motor, cognitive, and psychiatric dysfunctions (Lastres-Becker et al. 2003). Typical symptoms experienced by HD patients are chorea, subcortical dementia, and emotional disturbance. Currently, there is no cure for HD; only the choreic symptoms can be partly treated (Consroe 1998). The link between HD and the ECS was first discovered from the post-mortem studies of HD patients where a massive loss of the CB1 receptors in the basal ganglia, especially in the substantia nigra and the lateral globus pallidus, was detected (Glass et al. 1993; Richfield and Herkenham 1994; Glass et al. 2000). Previously, Glass et al. (2000) demonstrated that loss of the CB1 receptors occurs even before the appearance of the major symptoms, and therefore, the theory was advanced that the CB1 receptors are involved in the pathogenesis of HD. Later, also *in vivo* studies modeling the HD have confirmed the changes in the ECS in HD. In addition, animal models have shown evidence that providing the animals with an "enriched" environment can retard the progress of HD (Lastres-Becker et al. 2003; Glass et al. 2004). In spite of the growing biochemical and pharmacological evidence, it is still partly unclear whether CB1 receptors are involved in emergence and progress of the disease. However, it is strongly believed that in the future, cannabinoids may be useful in the treatment of this difficult disorder not only by decreasing the hyperkinetic symptoms but also via their neuroprotective and anti-inflammatory properties.

Tourette's syndrome is a chronic neurological disorder characterized by motor and vocal tics tied to rapidly fluctuating emotional states (Consroe 1998). The deficiently

functioning dopamine system is believed to be responsible for the disease, since administration of dopamine agonists will worsen the symptoms (Williamson and Evans 2000). The reports of beneficial effects of cannabinoids on TS are mainly anecdotal and based on marijuana smoking. In the study of Muller-Vahl et al. (1998) 64 patients with TS were interviewed for the effects of marijuana on their TS symptoms. A total of 82% of those who reported the prior use of marijuana had experienced a clear reduction of these symptoms (Muller-Vahl et al. 1998). More recently, a randomized double-blind placebo-controlled single-dose trial with Δ^9 -THC has also been conducted, and the results were in good agreement with the former findings (Muller-Vahl et al. 2002). Several theories about the role of the ECS in TS have been proposed but many results, for example about dopamine and cannabinoid receptor interactions in the striatum, are contradictory; earlier it was hypothesized that cannabinoids were able to modulate dopaminergic transmission in the striatum, however, accordingly to the recent findings, cannabinoids act only as modulators of GABAergic and glutamatergic transmission (Kofalvi et al. 2005). Thus, the mechanisms by which cannabinoids act in TS, remain unclear, even if there are clear structural and mechanistic interaction points between TS and the ECS (Consroe 1998).

Recently, Parkinson's disease (PD) has aroused considerable interest as a new therapeutic target for cannabinoids. PD is a progressive neurodegenerative disease characterized by tremor, rigidity, and bradykinesia. In PD, the dopaminergic neurons in the substantia nigra compacta are destroyed which influences the overall activity of the basal ganglia. The actual mechanism behind the denervation is still a mystery. However, it has been suggested that the nerve cell death occurs as a consequence of various factors, such as oxidative stress, mitochondrial defects, glutamate toxicity, genetic factors and apoptosis (Blandini et al. 2000). Post-mortem studies in PD patients as well as animal studies have indicated that the ECS in basal ganglia is overactive as a consequence of the loss of dopaminergic neurons (Romero et al. 2000; Lastres-Becker et al. 2001a). Currently, dopamine receptor agonists and/or L-DOPA and anticholinergics are used for the treatment of PD but since the effect of these compounds tends to fade with time, novel treatment methods are needed. Overall, cannabinoid agonists are considered as ineffective in treating the symptoms of PD, in

fact, they can make them even worse. The documentation about cannabinoid agonists in the treatment of PD is controversial and seems to depend on the target being investigated. There is some evidence that cannabinoid agonists can inhibit glutamate release (Romero et al. 2002), and therefore, the ECS could protect neurons from glutamatergic excitotoxicity. Recently, cannabinoids have been shown to provide neuroprotection against 6-hydroxydopamine caused toxicity both *in vitro* and *in vivo* (Lastres-Becker et al. 2005). The glutamate release inhibition has also been proposed to be effective in compensating for the overactivity of the neurons in the subthalamic nucleus responsible for the tremor characteristic in PD patients (Sanudo-Pena et al. 1998). In 2001 Sieradzan et al. showed that cannabinoid receptor agonist, nabilone, is able to reduce L-DOPA-induced dyskinesia in PD. In contrast, Carroll et al. (2004) reported that orally administrated cannabis extract could not improve dyskinesias or parkinsonism. CB1 receptor antagonists have been considered to offer greater potential for the treatment of PD than cannabinoid agonists, though, at the moment, the results are just as controversial (Di Marzo et al. 2000; Meschler et al. 2001; Mesnage et al. 2004). The recent study of Fernandez-Espajo et al. (2005) indicated that the CB1 receptor antagonist was efficient only in the very severe stages of PD.

1.5.3 MS-disease and spinal cord injury

Multiple sclerosis (MS) is an autoimmune disease causing neuronal damage. Typical symptoms, experienced also by patients with spinal cord injury, are muscle stiffness and spasticity, tremor, fatigue, pain, incontinence, and sexual dysfunction (Croxford 2003). Inflammation is considered to be a main cause of the demyelination; however, recent findings have suggested that oligodendrocyte apoptosis and microglial activation in myelinated tissue may also be of some importance (Barnett and Prineas 2004). Since the denervation starts at a very early state of the disease, the therapeutic approaches are concentrated on the prevention of neuronal loss. It seems that MS shares characteristics with other neurodegenerative diseases, being susceptible to an excess of glutamate, different ions, and nitric oxide (Jackson et al. 2005). As cannabinoids are known to have an impact on oxidative responses as well as glutamate and ion influxes, questions about the neuroprotective effects of cannabinoids in MS arise. No data are available about

changes in the ECS in the post-mortem brain or spinal cord of MS patients, but some evidence exists that there are higher levels of endocannabinoids in the brain and spinal cord of a MS mice (Baker et al. 2001). However, there is a lot of anecdotal evidence of beneficial effect of cannabis, for example, on spasticity, tremor, pain, and anxiety of MS and spinal cord injury patients (Consroe et al. 1997; Pertwee 2002). Recently, Baker et al. (2000; 2001) have also found experimental evidence that cannabinoid receptor agonists as well as inhibitors of endocannabinoid-metabolizing enzymes are able to reduce spasticity and tremor in a mouse model of MS. The effects seemed to be mediated through both the CB1 and CB2 receptors. The results of the most recent clinical studies are controversial; a few trials have reported clear beneficial effects, (Wade et al. 2003; Zajicek et al. 2003) but the majority of the studies failed to demonstrate any therapeutic efficacy (Killestein and Polman 2004). However, these studies suffer from dosing and administration problems, patient heterogeneity, drop-outs and inappropriate masking. Many MS patients seem to believe that there are therapeutic benefits with cannabinoids and they continue to self-medicate in spite of the disappointing results of clinical trials and the intense social arguments and legal pressure they have to face. Therefore, to resolve these political and scientific arguments, the future work of cannabinoids in MS should concentrate on new modes of administration, methods able to separate therapeutic effects from side-effects, and finally, there will need to be better organized clinical trials.

1.5.4 Neuroprotection

There is clear evidence that cannabinoids possess neuroprotective properties. In 1996 Shen et al. showed that cannabinoid agonists act through the CB1 receptors, inhibiting glutamate release in hippocampal cultures (Shen et al. 1996). Based on this finding, they suggested that cannabinoids might reduce glutamate-induced neuronal injuries. Recent studies have confirmed that cannabinoids are able to protect neurons by reducing intracellular Ca^{2+} levels via the inhibition of cAMP/PKA (Kim et al. 2005; Zhuang et al. 2005). In addition, there is some evidence that cannabinoid-induced hypothermia is involved in the neuroprotection (Leker et al. 2003).

The effects of cannabinoids against brain injuries have been examined in various animal models. In the study of Panikashvili et al. (2001) a closed head injury elevated the level of endogenous 2-AG in the mouse brain. In the same study, administration of synthetic 2-AG significantly reduced brain oedema, improved clinical recovery, reduced infarct volume and reduced hippocampal cell death. The CB1 receptor expression has also been reported to be increased in an experimental stroke in rats (Jin et al. 2000). Nagayama et al. (1999) observed that WIN-55,212-2 could protect from hippocampal neuronal loss in global and focal ischemia; in the latter case, both before and up to 30 minutes after the onset of ischemia. WIN-55,212-2 has also been found to protect from both early and delayed neuronal loss in a newborn rat model of acute severe asphyxia *in vivo* (Martinez-Orgado et al. 2003). Some of the effects of cannabinoid agonists are not abolished by the CB1 antagonist, SR141716A, which indicates that also other, CB1-independent, mechanisms of action are involved (Martinez-Orgado et al. 2003).

It is well known that compounds having similar chemical structure to cannabinoids but devoid CB1 receptor activity, like cannabidiol (CBD) and HU-211 (Table 1.5), also act as neuroprotective agents, which complicates understanding of their mechanism of action. Several proposals for the mechanism of action of CBD and HU-211 have been made, such as involvement of some unknown cannabinoid receptor, activity via an additional reaction pathway, enhancement of endocannabinoid levels or possible antioxidant properties (Mechoulam 2002).

Previously, researchers have recognized that there is a need of neuroprotection also in other CNS disorders, e.g. in MS. Several mechanisms for a neuronal damage in MS have been proposed. For example, phagocytosis of myelin increases the production of tumour necrosis factor alpha and nitric oxide, and those are agents which can damage oligodendrocytes and neurons. In addition, nitric oxide can form peroxynitrate when it combines with superoxide anion, leading to mitochondrial damage and adenosine triphosphate (ATP) depletion which in turn affects the sodium/potassiumATPase pumps. An excess of nitric oxide can also lead to overactivation of glutamate receptors. Since cannabinoids are reported to be able to regulate oxidative responses as well as glutamate and ion influxes, they may have therapeutic potential in MS and related neurodegenerative disorders (Jackson et al. 2005).

1.5.5 Pain

Cannabinoids have been used as painkillers for centuries. This analgesic effect of cannabinoids is not surprising since the cannabinoid receptors are densely distributed in those areas modulating pain, such as the PAG, the rostral ventrolateral medulla, the spinal dorsal horn, and the dorsal root ganglion (Martin et al. 1995; Lichtman et al. 1996; Sanudo-Pena et al. 1999). Cannabinoids have been shown to be effective in acute and physiological pain models (thermal, mechanical and chemical pain) as well as in chronic pain models (inflammatory and neuropathic pain) to where cannabinoids may well possess even greater potency and efficacy (Fuentes et al. 1999; Fox et al. 2001; Rice et al. 2002). The participation of the ECS in pain perception is probably rather complex and some effects of cannabinoids are also mediated via other systems, such as vanilloid and opioid receptors (Pertwee 2001). The potency and efficacy of cannabinoids in antinociception have been reported to be higher or at least comparable to opioids (Cichewicz 2004). Recent findings have indicated that the cannabinoid and opioid signaling pathways may even be connected, since some analgesic effects of Δ^9 -THC have been shown to be mediated through delta and kappa opioid receptors (Fuentes et al. 1999). Some data also indicate that the analgesic effect of Δ^9 -THC and morphine given in combination is even greater-than-additive (Cichewicz 2004). This finding may provide an interesting new approach to treatment of pain; a low dose of cannabinoids and opioids in a combination could offer effective analgesia with fewer side-effects, especially for pain that is resistant to opioids alone. Some previous trials have provided clinical evidence for the antinociceptive effects of cannabinoids. In the preliminary study of Karst et al. (2003), 1',1'-dimethylheptyl- Δ^8 -tetrahydrocannabinol-11-oic acid (CT-3), a potent analog of THC-11-oic acid, was proved to be effective in reducing chronic neuropathic pain and the drug did not cause any significant side-effects. The recent clinical study of Svendsen et al. (2004) showed that dronabinol had a modest but clinically relevant analgesic effect on central pain in patients with MS, however, adverse effects typical for cannabinoids were observed. In contrast, a clinical study attempting to demonstrate an analgesic effect of orally administered Δ^9 -THC in postoperative pain in humans failed to demonstrate the benefits of cannabinoids,

however, one needs to keep in mind the poor oral bioavailability of Δ^9 -THC (Buggy et al. 2003).

1.5.6 Cognition and memory

One of the best-known effects of cannabimimetics is their ability to affect cognition and memory. The hippocampus, an area rich in CB1 receptors, has a very important role in memory processing. Therefore, after cannabinoid agonist administration, the disruption of working memory can be observed more sensitively than any other pharmacological effects, while long-term memory remains largely intact (Varvel et al. 2001; Lichtman et al. 2002). Even though the effects of exogenous CB1 receptor activation in normal brain produces most probably undesirable effects on memory and cognition, it has been suggested that drugs that could interfere with other components of the ECS, such as inhibitors of FAAH and MGL, may have some therapeutic potential (Goutopoulos and Makriyannis 2002). Endocannabinoids are believed to be responsible for the depolarization-induced suppression of inhibition (DSI) and excitation, and since DSI enhances memory in the hippocampus, a compound which was able to modify DSI could well have beneficial effects on memory.

As the population ages, the diseases associated with aging are becoming a major health care problem in western countries. Alzheimer's disease (AD), the most common type of dementia, is characterized by deterioration of memory, cognition, personality, language, and visual-spatial skills (Consroe 1998). In AD especially the cholinergic neurons in the hippocampus and cerebral cortex are damaged. There is some evidence that CB1 receptor inverse agonists might serve as memory enhancers both in the defective memory in normal-aged people and in patients with dementias (Terranova et al. 1996).

1.6 Cancer

Already in the 70's, it was observed that cannabinoids could suppress Lewis lung carcinoma cell growth in mice, and inhibit DNA synthesis in isolated Lewis lung carcinoma cells, L1210 leukemia cells, and bone marrow cells (Munson et al. 1975;

Carchman et al. 1976; White et al. 1976). Subsequently, it has been shown that cannabinoids possess antineoplastic effects against other types of growths, such as gliomas, thyroid epithelioma, lymphomas, skin carcinomas, uterine carcinoma, breast cancer, prostate carcinoma, and neuroblastoma (De Petrocellis et al. 1998; Sanchez et al. 1998; Galve-Roperh et al. 2000; Bifulco et al. 2001; Casanova et al. 2003; Contassot et al. 2004; Flygare et al. 2005; McAllister et al. 2005; Sarfaraz et al. 2005). Several, complex biochemical events seem to be involved in the inhibition of apoptosis and cell growth. After cannabinoid receptor activation, various cellular pathways become activated, many of which can affect the cell's fate, such as ceramide accumulation, extracellular signal-regulated kinase (ERK) activation, Akt inhibition, and enhanced cox-2 expression (Guzman 2003). Reports about the role of the ECS in the growth of tumour cells are somewhat contradictory, and although both the CB1 and CB2 have been shown to be involved in these antitumoral effects, it has been suggested that other, cannabinoid receptor-independent, systems, such as vanilloid receptor activation, may be involved (Derocq et al. 1998; Maccarrone et al. 2000; Hall et al. 2005).

Although various *in vitro* and *in vivo* studies have provided evidence that cannabinoids may be viewed as cancer therapy agents, without reliable clinical trial data they cannot achieve wide-ranging acceptance. However, it is tempting to speculate that cannabinoids might offer a relatively comprehensive medical treatment for cancer patients; it is possible that they could inhibit tumor cell proliferation, induce appetite, and reduce inflammation and pain.

1.7 Gastrointestinal system

Autoradiographical and immunohistochemical studies have pointed to the presence of the CB1 receptors in the gastrointestinal tract in a variety of animal species (Coutts et al. 2004). The receptor localization has been confirmed by examining endocannabinoid levels and FAAH expression in intestine, colon, and ileum after noxious stimuli, food deprivation or clinically diagnosed colorectal cancer (Coutts et al. 2004). Cannabinoids have been shown to decrease gastric acid secretion in the rat stomach via the CB1 receptors located in the pre- and postganglionic cholinergic pathways (Adami et al. 2002). In addition, AEA has been found to inhibit secretion in mice intestine after

cholera toxin administration (Izzo et al. 2003). Cannabimimetics have also been reported to reduce motility in different regions of the gastrointestinal tract mainly through inhibition of acetylcholine release (Coutts et al. 2004).

The therapeutic potential of cannabimimetics in gastrointestinal diseases is still largely unexplored. A protective role of cannabinoids in intestinal inflammation has been suggested, however, unambiguous conclusions are difficult to draw since the results are controversial (Crocì et al. 2003; Massa et al. 2004). Some evidence does exist that endocannabinoid levels are enhanced in colorectal carcinoma cells, and therefore, it has been suggested that cannabinoids could reduce the proliferation of these malignancies (Ligresti et al. 2003). Finally, emesis, already discussed in chapter 1.5.1., is also considered as a gastrointestinal therapeutic application of cannabinoids.

1.8 Cardiovascular system

The ECS system seems to play some role in the modulation of many vascular functions, since marijuana smoking evokes tachycardia and induces noradrenaline release, however, tolerance to this effect rapidly appears after repeated administration (Jones 2002). Huestis et al. (2001) demonstrated that CB1 receptors mediated the cardiovascular effects of marijuana since SR141716A produced dose-dependent blockade of marijuana-induced subjective intoxication and tachycardia.

Of all the many therapeutic applications of cannabinoids, it is most difficult to summarize their cardiovascular effects. Even though there is an extensive amount of data available and evidence of the ECS-related cardiovascular regulation exists, it is impossible to draw any unambiguous conclusions. The results obtained vary extensively, depending on what study conditions, species and ligands are used, whether the test animals are anaesthetized or conscious, what part of the vascular system is being studied, and whether the actions of possible metabolites and other receptors (e.g. vanilloid receptor) have been taken into account.

1.9 Respiratory system

CB1 receptors can be found in the lungs in the noradrenergic terminals of airway nerves (Vizi et al. 2001). The study of Calignano et al. 2000 indicated that AEA has a dual effect in the airways of rodents. After chemical irritation with capsaicin, AEA inhibits bronchospasm and cough, probably by inhibiting the prejunctional release of excitatory neurotransmitters and neuropeptides. However, AEA itself can cause bronchospasm when the muscle is in a relaxed state after vagotomization and atropine treatment. Both effects were reported to be mediated via the CB1 receptor. Since AEA is also an agonist for the vanilloid receptor, the possible effects occurring through that mechanism need to be taken into account. In the study of Calignano et al. (2000) this possibility was excluded since the vanilloid receptor antagonist, capsazepine, was not able to attenuate the responses. In contrast, Tucker et al. (2001) postulated that in isolated guinea-pig bronchial smooth muscle AEA induces a modest contractile response, involving vanilloid receptors rather than cannabinoid receptors. Since the data on the role of the ECS in the respiratory tract is currently relatively scarce, it remains to be clarified whether cannabinoids offer any therapeutic potential, for example, as antitussive or anti-asthmatic agents.

1.10 Reproductive system

Marihuana smoking as well as exposure to its active constituent, Δ^9 -THC, are known to have adverse effects on the function of the reproductive system possibly as a consequence of altered hormonal regulation in hypothalamus, pituitary gland, and gonadal glands (Murphy et al. 1998). Cannabinoids have been proposed to act through other neurotransmitters and neuropeptides that are responsible for the regulation of a release of gonadotropin-releasing hormone (GnRH). Suppression of GnRH release results in lowered luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels, leading to the impaired release of gonadal steroids, e.g. estradiole, progesterone, and testosterone. This impaired sex hormone release ultimately disturbs ovulation and spermatogenesis, and thus, affects fertility. In addition to LH and FSH, cannabinoids also inhibit the release of prolactin, the hormone responsible for milk production.

Although, high concentrations of the CB1 agonists seem to have a severe impact on reproduction, the CB1 receptors as well as their endogenous ligands are still abundantly expressed in male and female reproductive systems implying that the ECS has an important role in the ultimate success of fertilization (Lynn and Herkenham 1994; Schuel et al. 2002; Rossato et al. 2005). It is particularly interesting, how cannabinoids seem to regulate two critical phases of the pregnancy; low endocannabinoid levels are needed for embryonal implantation, however, as mentioned in chapter 1.5.1, activation of the CB1 receptors and elevated 2-AG levels are needed for survival of newborn (Fride 2004). A role in fertilization is not the only role that the ECS seems to play in reproduction. There is substantial evidence that the ECS participates in the development of the embryonic brain (Fernandez-Ruiz et al. 2000).

1.11 Eye

The CB1 receptors are widely distributed in the human anterior eye and retina (Straiker et al. 1999b). Also other components of the ECS have been identified in the ocular tissues (Bisogno et al. 1999; Straiker et al. 1999a; Lu et al. 2000). The CB1 receptor localization in the eye has been suggested to indicate that endocannabinoids may participate in the regulation of trabecular and uveoscleral aqueous humour outflow and aqueous humour production (Tomida et al. 2004). These hypotheses are supported by the fact that already in the 70's, it was observed that cannabinoids are able to lower the IOP (Hepler and Frank 1971). The involvement of the CB1 receptor in the IOP reducing effects of cannabinoids are further endorsed by the findings that the IOP lowering effects of cannabinoid receptor agonists are abolished by the CB1 receptor antagonist, SR141716A, whereas the CB2 receptor agonist, JWH-133, has no effect on the IOP of normotensive rabbits (Pate et al. 1998; Song and Slowey 2000; Laine et al. 2003). The actual mechanism underlying the IOP decrease is not fully understood, however, cannabinoids have been claimed to mediate their IOP-lowering effects both locally and via the CNS (Jarvinen et al. 2002).

The combination of the IOP lowering properties and possible neuroprotective effects make cannabinoids very interesting from the therapeutic point of view. Cannabinoids could represent a breakthrough in the treatment for glaucoma, one of the leading cause

of blindness all over the world. However, in spite of numerous anecdotal reports of IOP-lowering effects of marijuana, and the encouraging *in vivo* results, the clinical trials conducted so far have failed to demonstrate the efficacy of cannabinoids in the treatment of glaucoma. However, the research in this area has not disappeared completely; the recent study of Chen et al. (2005) is a good example of on-going work. Their results showed that the levels of 2-AG, AEA, and PEA are decreased in glaucomatous eye compared to those of normal eye concluding that glaucoma and altered functioning of the ECS may be connected.

1.12 The CB1 receptor agonists as therapeutics –useful or harmful?

Therapeutic effects of CB1 receptor agonist may be a double-edged sword. Although individuals suffering from severe diseases, such as MS, endorse the medical use of CB1 agonists, possible psychotropic side-effects of cannabinoids complicate their progress in clinical trials. Indeed, cannabinoid drug discovery is clearly moving towards the “softer” mechanisms, such as enzyme inhibitors. It remains to be seen whether these novel targets will be ultimately preferred or will CB1 receptor agonists exhibit superiority in some situations. One example may be in the treatment of neurotraumas where CNS side-effects do not play any role or in the treatment of glaucoma where such low drug concentrations are used that they should not produce CNS effects. Meanwhile, it might be useful to focus on (Lambert and Fowler 2005; Pertwee 2005b)

- 1) the design of CB1 agonists as valuable pharmacological tool,
- 2) ligands that do not cross the blood-brain barrier,
- 3) high potency and low efficacy ligands for the disease states where CB1 receptor sensitivity is increased,
- 4) allosteric modulation of the CB1 receptor, and finally
- 5) novel administration strategies, such as co-administration of CB1 agonists with an opiate receptor agonist or ways to achieve topical administration of CB1 agonist.

1.13 References

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2 AIMS OF THE STUDY

At the time this thesis study was initiated, subsequently developed 3D model of the CB1 receptor was not available. Therefore, the CB1 ligand design was focused on endocannabinoid type compounds due to their good activity properties and wide knowledge of their SAR's. Although endocannabinoids possess good activity properties, they still are prone to a few crucial disadvantages, hindering their use as pharmacological tools not to mention as pharmaceuticals. First, the endocannabinoids are metabolized soon after their release into the synaptic cleft which results in a short duration of action. Secondly, the endocannabinoids are metabolized to AA, the precursor of inflammation mediators. Thirdly, the endocannabinoids have poor "drug-like" properties since they are very lipophilic, and therefore, poorly water soluble. Finally, we need to keep in mind that high potency is required to ensure that the concentration of active drug is not excessively high. Thus, although the endocannabinoids are reasonably efficacious CB1 ligands, they still need to be more potent if they are to become potential drug candidates.

The specific aims of this thesis were as follows:

1. To design and synthesize a CB1 agonist which, after been enzymatically or chemically hydrolyzed, would not liberate arachidonic acid, the precursor of inflammation mediators.
2. To design and synthesize metabolically more stable, and thus, more long-lasting analogues of the endogenous cannabinoids by reversing the amide bond of *N*-arachidonoyl ethanolamide and by introducing a methyl group at the α -position of 2-arachidonoyl glycerol.
3. To study the SAR's of 2-arachidonoyl glycerol and 2-arachidonoyl glyceryl ether by varying the chain length and by replacing the pentyl tail with a dimethylheptyl structure.

4. To determine the cannabinergic activity (potency $-\log EC_{50}$ and efficacy E_{max}) of the novel CB1 ligands by the [35 S]GTP γ S binding assay at rat cerebellar membranes.

5. To determine the chemical and enzymatic stability of novel CB1 ligands in rat brain homogenate or rat cerebellar membranes and membrane free buffer.

3 GENERAL EXPERIMENTAL PROCEDURES

3.1 Synthesis

Solvents and reagents were of the highest purity, and they were used without further purification, unless a reaction required dry conditions when the solvents were dried according to common procedures. The petroleum ether used in flash chromatography was 60/95 grade. Arachidonyl alcohol and arachidonic acid were purchased from Nu Chek prep, Inc.

The syntheses were generally performed under dry conditions. The glassware was flame dried or dried in the oven at 140 °C. Reactions were performed under Ar or N₂ atmosphere, dry solvents were used, and when the starting materials were not easily volatile, they were dried *in vacuo*.

Reactions were monitored by thin layer chromatography (TLC). TLC was performed on aluminium sheets coated with silica gel 60 F₂₅₄ (Merck, Germany), and bands were visualized by UV-light and staining with ninhydrin, anisaldehyde or vanillin. In general, intermediate and final products were purified by flash chromatography using 30–60 µm silica gel (J.T.Baker). Finally, if the product would tolerate the procedure, the remaining volatile solvents were removed at high vacuum.

3.2 Analysis

¹H NMR and ¹³C NMR were recorded on a Bruker Avance 500 spectrometer operating at 500.1 MHz and 125.8 MHz, respectively. CDCl₃ was used as a solvent, and tetramethylsilane (TMS) was used as an internal standard. The spectra were processed from the recorded FID files with MestRe-C software (version 2.3a, Departamento Química Orgànica, Universidade de Santiago de Compostela, Spain). Chemical shifts (δ) are reported in parts per million (ppm) downfield from TMS. The following abbreviations are used: s = singlet, br s = broad singlet, d = doublet, t = triplet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dq = doublet of quintets, tq = triplet of quintets, q = quartet, qn = quintet, m = multiplet, st = sextet, and sep = septet. Coupling constants are reported in Hz and letter *J* indicates ³*J* if not otherwise noted. ESI-MS spectra were acquired using a LCQ ion trap mass spectrometer equipped with

an electrospray ionization source (Finnigan MAT, San Jose, CA, USA). Gas chromatography mass spectrum was obtained on a HP6890 GC mass spectrometer with electron-ionisation detector. The free hydroxyl groups of the sample (0.1 mg/mL) in methanol were coated with silicon-containing groups. Elemental analyses for C, H and N were performed on a ThermoQuest CE Instruments EA1110-CHNS-O elemental analyser (ThermoQuest, Italy).

The analytical HPLC system consisted of a Merck Hitachi (Hitachi Ltd.) L-7100 pump, D-7000 interface module, L-7455 diode-array detector (190–800 nm, set at 211 nm) and L-7250 programmable autosampler. The separations were performed with Zorbax SB-C18 endcapped reversed-phase precolumn (4.6 mm×12.5 mm, 5 µm) and column (4.6 mm×150 mm, 5 µm) (Agilent). The injection volume was 50 µL. A mobile phase mixture of 28% phosphate buffer (30 mM, pH 3.0) in acetonitrile at a flow rate of 2.0 mL min⁻¹ was used.

3.3 *In vitro* assay for the CB1 receptor activity

The maximal responses (E_{\max} , % basal) and potencies ($-\log EC_{50}$) to all compounds, as well as their CB1 receptor-dependent activity, were determined by the [³⁵S]GTP γ S binding assay. These studies were conducted using four-week-old male Wistar rats. All animal experiments were approved by the local ethics committee. The animals lived in a 12-h light/12-h dark cycle (lights on at 07:00 h) with water and food available *ad libitum*. The rats were decapitated 8 h after lights on (15:00 h), whole brains were removed, dipped in isopentane on dry ice and stored at -80 °C. Rat cerebellar membranes were prepared as previously described (Savinainen et al. 2001). The [³⁵S]GTP γ S binding assay protocol was improved during the preparation of this doctoral thesis, and therefore, two different determination methods for the CB1 receptor activity were used.

The CB1 receptor activity for the compounds described in chapter 4 were determined as previously reported (method A) (Savinainen et al. 2001). The method B, used for the activity studies of the final compounds presented in chapters 5, 6, and 7, is an optimized [³⁵S]GTP γ S binding assay protocol where noise due to tonic adenosine A₁ receptor

activity and to enzymatic degradation of endocannabinoids has been eliminated (Savinainen et al. 2003). Maximal agonist responses (E_{\max} , %basal) and potencies ($-\log EC_{50}$) were determined from dose-response curves, and the results are presented as mean \pm SEM of at least three independent experiments, performed in duplicate. The CB1 dependent activity was confirmed by antagonizing half-maximal responses with the CB1 selective antagonist AM251 (10^{-6} M). Data-analysis for dose-response curves were calculated as non-linear regressions by GraphPad Prism 3.0 or 4.0 for Windows.

3.4 References

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4 SYNTHESIS AND CB1 RECEPTOR ACTIVITIES OF NOVEL ARACHIDONYL ALCOHOL DERIVATIVES*

4.1 Introduction

The endogenous cannabinoids are susceptible to enzymatic degradation. Regardless of this fact, they have an undisputable role as the pharmacological tools, which is the reason for attempts to develop novel, metabolically stable structure analogues. So far, several attempts have been made to improve the stability properties of AEA and 2-AG. Best results have been achieved by introducing methyl group(s) to a carbon adjacent to an amide bond, by reversing the amide bond or replacing ester or amide with more stable linkages, such as ether or ketone (Abadji et al. 1994; Adams et al. 1995a, b; Lin et al. 1998; Hanus et al. 2001; Suhara et al. 2001). Despite the intensive ligand development in this area, derivatives of arachidonyl alcohol had not been previously investigated. Therefore, inspired by the instability aspects of endogenous cannabinoids as well as the problems arising from their unwanted metabolite, AA, we designed and synthesized previously unpublished ester, carbonate and carbamate derivatives of arachidonyl alcohol (Figure 4.1).

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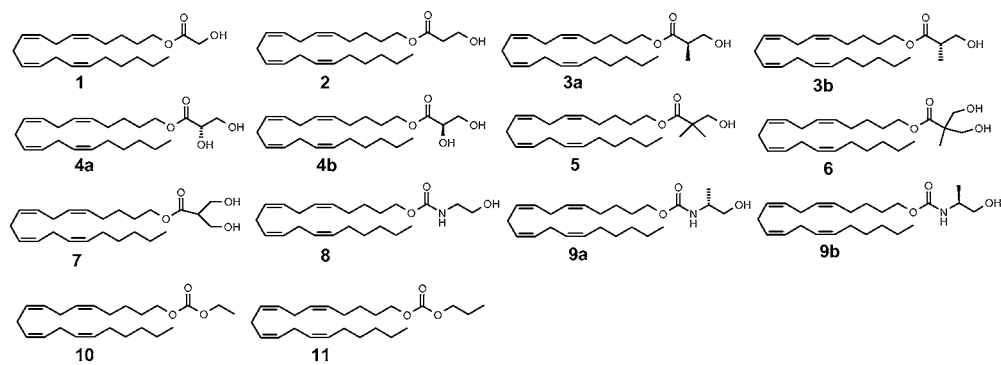
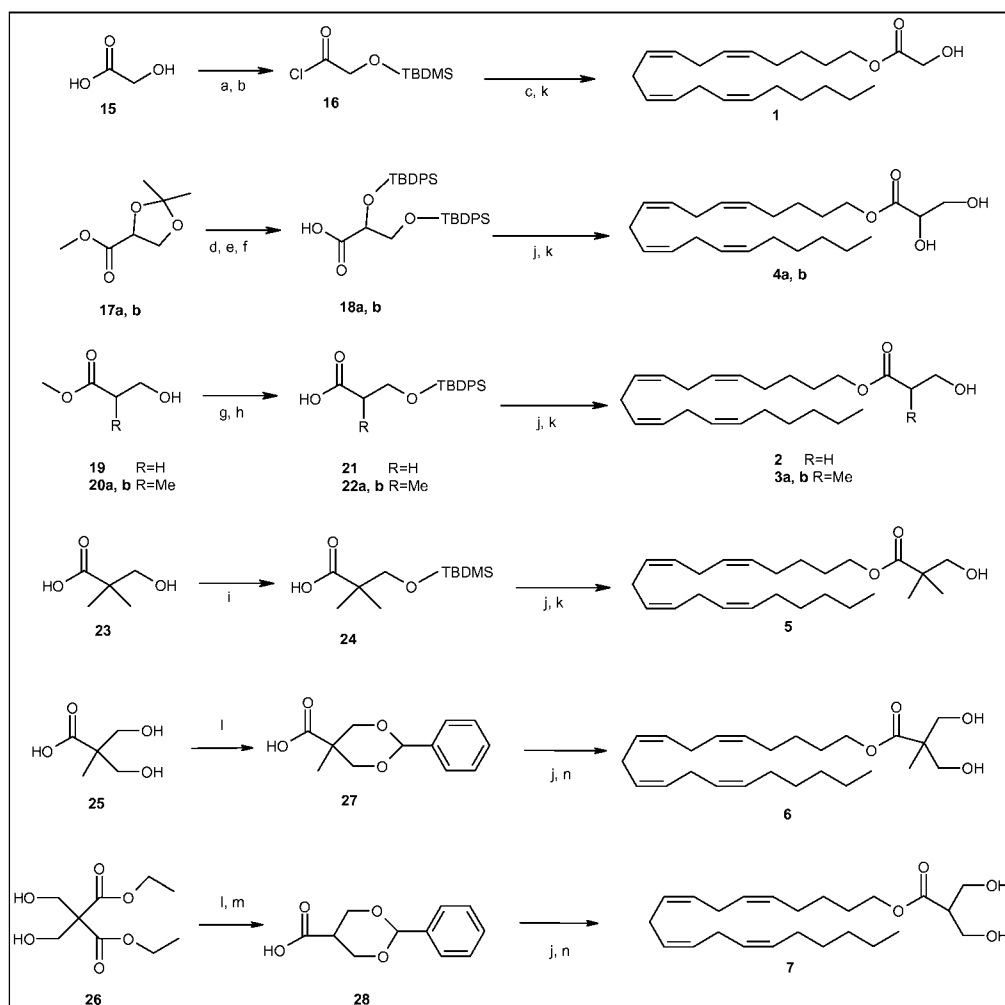


Figure 4.1. Chemical structures for derivatives of arachidonyl alcohol.

4.2 Synthesis

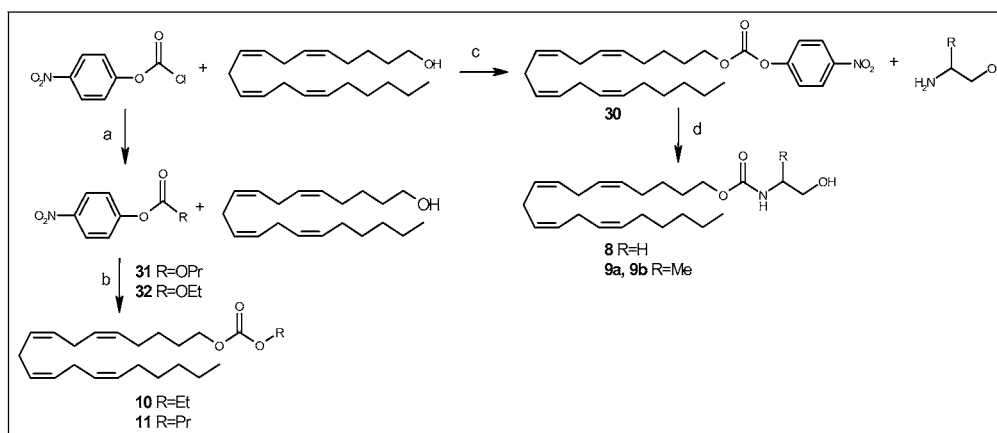
Syntheses of arachidonyl alcohol esters **1-7** faced several problems, such as self degradation and polymerization. However, Scheme 4.1 summarizes the synthesis methods used. Compound **1** was prepared by attaching the protected and activated compound **15** to arachidonyl alcohol in the presence of triethylamine (Et_3N) (Gu et al. 1989; Glabe et al. 1996). The final products **4a** and **4b** were produced from enantiomerically pure starting materials. Compound **7** was first deprotected under basic conditions, followed by protection of carboxylic acid group with benzyl (Bn), and then hydroxyl groups with *t*-butyldiphenylsilyl group (TBDPS) (Oliver et al. 2000; Shin et al. 2000). The benzyl group was removed by hydrogenolysis, and finally, compound **18** was coupled with arachidonyl alcohol (Shin et al. 2000). The final products **3a** and **3b** were also obtained from enantiomerically pure starting materials. Compounds **19-20** were first protected with TBDPS following the hydrolysis of a methyl ester and coupling with arachidonyl alcohol. All silyl protected intermediates were deprotected using tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF), leading to the final products **1-5** (Lal et al. 1998; Oliver et al. 2000). The intermediates for the synthesis routes of final products **6** and **7** were prepared by protecting hydroxyl groups of compounds **25** and **26** with benzylidene acetal. After protection, **26** was decarboxylated, leading to **28** (Scriba 1993). Finally, compounds **27** and **28** were attached to arachidonyl alcohol, and deprotected with concentrated HCl/MeOH (1:1) solution.



Scheme 4.1. (a) TBDMSCl, imidazole, DMF; (b) 2 M oxalyl chloride, CH_2Cl_2 ; (c) arachidonyl alcohol, Et_3N , CH_2Cl_2 ; (d) 1. 2 M NaOH, 2. 2 M HCl, 3. BnBr; (e) TBDPSCl, imidazole, THF; (f) Pd/C, H_2 ; (g) TBDPSCl, THF; (h) LiOH; (i) 1. TBDMSCl, imidazole, THF, 2. THF/ H_2O ; (j) arachidonyl alcohol, DCC, DMAP; (k) TBAF; (l) benzaldehyde, toluene, *p*-TSA; (m) 1. KOH, 2. HCl, 3. Et_3N , Δ ; (n) HCl/MeOH.

Synthesis methods for the final compounds **8** – **11** are outlined in Scheme 4.2. Carbamates **8-9** were prepared by attaching the *p*-nitrophenyl chloroformate activated arachidonyl alcohol to aminoalcohol in the presence of Et_3N (Andrews et al. 1999; Hay et al. 2000). Carbonates **10** – **11** were also obtained by *p*-nitrophenyl chloroformate

activation method. However, in this case, the carboxylic acid residue was activated and attached to arachidonyl alcohol.



Scheme 4.2. a) Ethanol or propanol, pyridine, THF; (b) DMAP, CH₂Cl₂; (c) pyridine, THF; (d) Et₃N, THF.

4.3 Results and discussion

Of all the ligands synthesized, only compounds **4a** and **4b** showed dose-dependent CB1-activity (Table 4.1). For both of these compounds, responses at 5×10^{-5} M were reversed by the CB1 receptor antagonist, AM251 (data not shown). However, both efficacy and potency values of **4a** and **4b** were weaker than those of AEA (Table 4.1).

Table 4.1. Comparison of efficacy (E_{\max}) and potency ($-\log EC_{50}$) values of AEA, **4a** and **4b** at rat cerebellar membranes ($n=3$).

| Compound | CB1 activation | | |
|-----------|-------------------------------|-------------------------|----------------------|
| | E_{\max} (%basal \pm SEM) | $-\log EC_{50} \pm$ SEM | EC_{50} (μ M) |
| AEA | 380 ± 6 | 4.8 ± 0.0 | 16 |
| 4a | 278 ± 21 | 4.4 ± 0.2 | 40 |
| 4b | 301 ± 8 | 4.6 ± 0.1 | 25 |

The hydroxypropyl esters and carbonate derivatives did not stimulate [³⁵S]GTP γ S binding. The problems encountered during the synthesis development, especially during the column chromatography, give reason to suspect that the lack of activity of the hydroxypropyl esters most probably results from their unstable structures. In addition,

the carbonate derivatives had so low aqueous solubilities that their biological activity could not be reliably determined. The carbamate derivatives exhibited some CB1 activity, however, the activity achieved was not attenuated by 1 μ M AM251, which indicates that it is not mediated via the CB1 receptors.

4.4 Conclusions

The ester, carbamate, and carbonate derivatives of arachidonyl alcohol were synthesized, and their CB1 activity was determined by the [35 S]GTP γ S binding assay with rat cerebellar membranes. Within the series, only the compounds possessing a structure very close to 2-AG were able to induce weak CB1 activation, therefore, it can be concluded that this type of arachidonyl alcohol derivatives are not able to act as good CB1 receptor ligands.

4.5 Experimental

4.5.1. Synthesis

Glycolic acid icoso-5,8,11,14-tetraenyl ester (1). *t*-Butyldimethylchlorosilane (3.1 g, 21 mmol) and glycolic acid (0.85 g, 11.2 mmol) were dissolved in dry DMF (4 mL), and the reaction mixture was stirred at RT under N₂ for 21 hours. The solution was poured into water (45 mL) and extracted with petroleum ether (3x20 mL). The combined organic layers were washed with saturated NaHCO₃, dried over Na₂SO₄, filtered and evaporated. Yield was 2.2 g (63%). ¹H NMR (CDCl₃): δ 0.10 (s, 6 H), 0.28 (s, 6H), 0.92 (s, 9H), 0.93 (s, 9H) 4.19 (s, 2H). *t*-Butyldimethylsilyl (*t*-butyldimethylsilyloxy)acetate (1.0 g, 3.4 mmol) was dissolved in dry CH₂Cl₂ (5 mL) and two drops of dry DMF was added. 2 M oxalyl chloride (2.2 mL) in dry CH₂Cl₂ was added slowly through a dropping funnel. After the addition, stirring was continued for 1 hour. The solvent was evaporated. Yield was 0.6 g (84%). (*t*-Butyldimethylsilyloxy)acetyl chloride (310 mg, 1.5 mmol) in dry CH₂Cl₂ (5 mL) was added dropwise to the precooled solution of arachidonyl alcohol (350 mg, 1.2 mmol) and Et₃N (0.35 mL, 2.5 mmol) in dry CH₂Cl₂ (5 mL). After the addition, the stirring was continued at RT for 3 hours. The solution was washed with water and brine, and the

combined organic layers were dried over Na_2SO_4 , filtered and evaporated. The crude product was purified by flash chromatography eluting with 0–4% EtOAc in PE. Evaporation of solvents yielded oily product (190 mg, 34 %; after purification some impurities remained). $R_f=0.84$ (PE:EtOAc; 4:1); $^1\text{H NMR}$ (CDCl_3): δ 0.28 (s, 6H), 0.89 (t, $J=7.0$, 3H), 0.93 (s, 9H), 1.26–1.46 (m, 8H), 1.67 (qn, $J=6.8$, 2H), 2.04–2.12 (m, 4H), 2.80–2.85 (m, 6H), 4.14 (t, $J=6.7$, 2H), 4.19 (s, 2H), 5.31–5.43 (m, 8H). To the stirred solution of arachidonyl [*t*-butyldimethylsilyloxy]acetate (190 mg, 0.4 mmol) 1 M TBAF in THF (0.45 mL) was added. Stirring was continued for 1 hour and the solvent was evaporated. The crude product was purified by flash chromatography eluting with hexane:EtOAc; 4:1. Evaporation of solvents yielded a colorless, oily product (100 mg, 70%). $R_f=0.24$ (PE:EtOAc; 4:1); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=7.0$, 3H), 1.26–1.48 (m, 8H), 1.69 (qn, $J=6.8$, 2H), 2.04–2.13 (m, 5H), 2.80–2.85 (m, 6H), 4.15 (s, 2H), 4.21 (t, $J=6.7$, 2H), 5.31–5.43 (m, 8H); $^{13}\text{C NMR}$ (CDCl_3): δ 14.1, 22.6, 25.7, 25.8, 26.7, 27.2, 28.2, 29.3, 31.5, 60.6, 65.6, 127.5, 127.9, 128.2, 128.2, 128.5, 128.6, 129.4, 130.5, 173.4; elemental analysis: calculated for ($\text{C}_{22}\text{H}_{36}\text{O}_3$): C 75.82%, H 10.41%; found C 75.66%, H 10.50%.

[3-(*t*-Butyldiphenylsilyloxy)propanoic acid (21). Methyl 3-hydroxypropanoate **19** (510 mg, 4.9 mmol) and *t*-butyldiphenylchlorosilane (1.6 g, 5.9 mmol) were dissolved in dry THF. The reaction mixture was stirred at RT overnight, and finally, quenched with water (45 mL). Water layer was separated and extracted with diethyl ether (3x20 mL). Organic layers were combined, washed with saturated NaHCO_3 , and dried over Na_2SO_4 . The crude product was purified by flash chromatography eluting with PE:EtOAc; 40:1. Evaporation of solvents yielded 1.3 g (76%) of white crystals. Mp 101.6 °C. $^1\text{H NMR}$ (CDCl_3): δ 1.04 (s, 9H), 2.56 (t, 2H, $J=6.4$), 3.68 (s, 3H), 3.95 (t, 2H, $J=6.3$), 7.37–7.44 (m, 6H), 7.65–7.67 (m, 4H). The solution of methyl [3-(*t*-butyldiphenylsilyloxy)propanoate (1.3 g, 3.9 mmol) in methanol (6 mL/mmol) and water (2 mL/mmol) was cooled on the ice-bath, and LiOH (470 mg, 11 mmol) was added slowly. The reaction mixture was stirred until the starting material was consumed. The solution was poured into the ice cold water (30 mL), and the water phase was extracted with diethyl ether and hexane (1:1). The water phase was acidified with 2 M KHSO_4 and extracted successfully with diethyl ether and hexane (1:1). The

combined organic phases were dried over Na₂SO₄ and evaporated. The yield was 1.0 g (77 %). ¹H NMR (CDCl₃): δ 1.09-1.01 (m, 9H), 2.60 (t, *J*=6.3, 2H), 3.95 (t, *J* = 6.3, 2H), 7.35-7.44 (m, 6H), 7.65-7.72 (m, 4H).

Arachidonyl 3-hydroxypropanoate (2). A mixture of **21** (230 mg, 0.70 mmol), arachidonyl alcohol (140 mg, 0.49 mmol), DCC (150 mg, 0.72 mmol) and DMAP (8 mg, 0.06 mmol) in dry CH₂Cl₂ was stirred at RT for 18 hours. The white precipitate was filtered and the filtrate was washed with 5% NaHCO₃ and water. The organic layers were combined and dried over Na₂SO₄. The crude product was purified by flash chromatography eluting with 1.5% EtOAc in PE. The evaporation of solvents yielded 170 mg (59%) of a clear, oily product. R_f 0.75 (EtOAc:PE; 1:4); ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.03 (s, 9H), 1.26-1.39 (m, 6H), 1.40-1.46 (m, 2H), 1.62-1.68 (m, 2H), 2.03-2.11 (m, 4H), 2.55 (t, *J*=6.3, 2H), 2.80-2.85 (m, 6H), 3.95 (t, *J*=6.4, 2H), 4.09 (t, *J*=6.7, 2H), 5.31-5.42 (m, 8H), 7.36-7.44 (m, 6H), 7.65-7.67 (m, 4H); ¹³C NMR (CDCl₃): δ 14.1, 19.2, 22.6, 25.7, 26.0, 26.8, 26.8, 27.2, 28.3, 29.3, 31.5, 38.0, 60.0, 64.4, 127.6, 127.7, 127.9, 128.2, 128.2, 128.6, 129.6, 129.7, 130.5, 133.6, 135.6, 171.8. Arachidonyl [3-(*t*-butyldiphenylsilyloxy)propanoate (340 mg, 0.60 mmol) was dissolved in dry THF, and 1 M TBAF in THF (700 μL) was added. Stirring was continued at RT for 40 min. The solvent was evaporated, and the crude product was purified by flash chromatography eluting with 1.5-20% EtOAc in PE. Evaporation of solvents yielded 60 mg (30%) of the desired product. ¹H NMR (CDCl₃): δ 0.88-0.92 (m, 3H), 1.26-1.36 (m, 6H), 1.42-1.45 (m, 2H), 1.64-1.69 (m, 2H), 2.04-2.13 (m, 4H), 2.57 (t, *J*=5.6, 2H), 2.80-2.85 (m, 6H), 3.86 (s, 1H), 4.12 (t, *J* = 6.7), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 22.6, 25.7, 25.9, 26.6, 26.8, 27.3, 28.2, 29.3, 29.7, 31.5, 36.8, 58.3, 64.7, 127.6, 127.9, 128.2, 128.2, 128.4, 128.6, 130.5, 134.8, 173.0; ESI-MS 363.3 [MH]⁺; elemental analysis: calculated for (C₂₃H₃₈O₃): C 76.20%, H 10.56%; found: C 76.25%, H 10.60%.

(2*S*)-[3-(*t*-butyldiphenylsilyloxy)-2-methylpropanoic acid (22b). The mixture of methyl ((2*S*)-3-hydroxy-2-methyl)propanoate (420 mg, 3.5 mmol), TBDPSCl (970 mg, 3.5 mmol) and imidazole (720 mg, 11 mmol) in dry THF was stirred under N₂ at RT overnight. The reaction mixture was poured into water (45 mL) and extracted with diethyl ether (3x20 mL). The combined organic layers were washed with saturated

NaHCO₃, dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with 2% EtOAc in PE. Evaporation of solvents yielded white crystals (640 mg, 51%). ¹H NMR (CDCl₃): δ 1.04 (s, 9H), 1.16 (d, 3H, *J* = 7.1), 2.69-2.75 (m, 1H), 3.68 (s, 3H), 3.71-3.74 (m, 1H), 3.84-3.85 (m, 1H), 7.38-7.43 (m, 6H), 7.64-7.66 (m, 4H). The solution of methyl propanate (640 mg, 1.8 mmol) in H₂O/THF (40/70 v/v per mmol) and 2 M NaOH (1.9 mL, 3.8 mmol) was stirred at RT for 50 hours. The reaction mixture was treated with Amberlite. THF was evaporated and the water phase was extracted with diethyl ether (5x20 mL). The solvents were evaporated and the crude product was dried in vacuo. The yield of the crystalline product was 520 mg (79%). ¹H NMR (CDCl₃): δ 1.03 (s, 9H), 0.21-1.07 (m, 3H), 2.69-2.72 (m, 1H), 3.73-3.77 (m, 1H), 3.82-3.89 (m, 1H), 7.36-7.43 (m, 6H), 7.65-7.66 (m, 4H).

Methyl (2*R*)-[3-(*t*-butyldiphenylsilyloxy)-2-methylpropanate. The yield of the crystalline product was 680 mg (50%). ¹H NMR (CDCl₃): δ 1.03 (s, 9H), 1.15 (d, *J*=7.1, 3H), 2.68-2.75 (m, 1H), 3.68 (s, 3H), 3.71-3.74 (m, 1H), 3.81-3.84 (m, 1H), 7.36-7.44 (m, 6H), 7.65-7.66 (m, 4H).

(2*R*)-[3-(*t*-butyldiphenylsilyloxy)-2-methylpropionic acid (22a). The yield of the crystalline product was 540 mg (83%). ¹H NMR (CDCl₃): δ 1.04 (s, 9H), 1.17-1.19 (m, 3H), 2.71-2.76 (m, 1H), 3.74-3.77 (m, 1H), 3.82-3.85 (m, 1H), 7.36-7.43 (m, 6H), 7.64-7.67 (m, 4H).

Arachidonyl (2*S*)-3-hydroxy-2-methylpropanate (3b). Arachidonyl alcohol (130 mg, 0.46 mmol), compound **22b** (220 mg, 0.64 mmol), DCC (140 mg, 0.67 mmol) and DMAP (7 mg, 0.05 mmol) were dissolved in dry CH₂Cl₂. The reaction mixture was stirred under Ar, at RT for 80 hours. The white precipitate was filtered, and the filtrate was washed with saturated NaHCO₃ and water. The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified with flash chromatography eluting with 3% EtOAc in PE. Evaporation of solvents yielded colorless, oily product (130 mg, 45%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.03 (s, 9H), 1.14-1.16 (m, 3H), 1.26-1.36 (m, 6H), 1.41-1.46 (m, 2H), 1.62-1.67 (m, 2H), 2.03-2.11 (m, 4H), 2.66-2.73 (m, 1H), 2.80-2.85 (m, 6H), 3.70-3.74 (m, 1H), 3.81-3.85 (m, 1H), 4.07-4.11 (m, 2H), 5.31-5.42 (m, 8H), 7.36-7.43 (m, 6H), 7.64-7.66 (m, 4H); ¹³C

NMR (CDCl₃): δ 13.6, 14.1, 19.3, 22.6, 25.7, 26.0, 26.8, 26.8, 27.2, 28.3, 29.3, 31.5, 42.6, 64.4, 66.0, 127.6, 127.7, 127.9, 128.2, 128.3, 128.6, 129.6, 129.7, 130.5, 133.5, 133.6, 135.6, 135.6, 175.0. Arachidonyl (2*S*)-[3-(*t*-butyldiphenylsilyloxy)-2-methylpropanoate (130 mg, 0.21 mmol) and 1 M TBAF (300 μ L) in dry THF were stirred under Ar for 7 minutes. The solvent was evaporated, and the crude product was purified with flash chromatography using 1.5–10% EtOAc in PE as an eluent. The yield of the oily product was 51 mg (66%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=6.9, 3H), 1.18 (d, *J*=7.3, 3H), 1.25-1.39 (m, 6H), 1.41-1.47 (m, 2H), 1.63-1.69 (m, 2H), 2.03-2.12 (m, 4H), 2.61-2.85 (m, 1H), 2.80-2.85 (m, 6H), 3.67-3.74 (m, 2H), 4.08-4.15 (m, 2H), 5.31-5.42 (m, 8H); ¹³C NMR (CDCl₃): δ 13.5, 14.1, 22.6, 25.7, 25.9, 26.6, 26.8, 27.2, 28.3, 29.3, 31.5, 41.8, 64.6, 64.7, 127.6, 127.7, 127.9, 128.4, 128.6, 129.5, 130.5, 134.8, 175.9; elemental analysis: calculated for (C₂₄H₄₀O₃·¼H₂O): C 75.64%, H 10.71%; found C 75.80%, H 10.56%.

Arachidonyl (2*R*)-[3-(*t*-butyldiphenylsilyloxy)-2-methylpropanoate. The yield of the colorless, oily product was 190 mg (69%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.03 (s, 9H), 1.15 (d, *J*=7.1, 3H), 1.26-1.37 (m, 6H), 1.40-1.46 (m, 2H), 1.62-1.67 (m, 2H), 2.03-2.11 (m, 4H), 2.67-2.73 (m, 1H), 2.80-2.85 (m, 6H), 3.68-3.73 (m, 1H), 3.82-3.85 (m, 1H), 4.06-4.10 (m, 2H), 5.31-5.42 (m, 8H), 7.35-7.44 (m, 6H), 7.64-7.66 (m, 4H); ¹³C NMR (CDCl₃): δ 13.6, 14.1, 19.3, 22.6, 25.7, 26.0, 26.8, 26.8, 27.2, 28.3, 29.3, 31.5, 42.6, 64.4, 66.0, 127.9, 127.2, 128.3, 128.3, 128.6, 129.7, 129.6, 130.5, 133.5, 133.6, 135.6, 135.6, 175.0.

Arachidonyl (2*R*)-3-hydroxy-2-methylpropanoate (3a). The yield of the oily, colorless product was 50 mg (42%). ¹H NMR (CDCl₃): δ 0.74-0.87 (m, 3H), 1.09-1.32 (m, 6H), 1.34-1.40 (m, 2H), 1.57-1.63 (m, 2H), 2.56-2.63 (m, 1H), 2.73-2.78 (m, 6H), 3.60-3.68 (m, 2H), 4.04-4.07 (m, 2H), 5.24-5.35 (m, 8H); ¹³C NMR (CDCl₃): δ 13.5, 14.0, 22.5, 25.6, 25.9, 26.7, 27.2, 28.2, 29.3, 31.5, 41.7, 64.6, 64.6, 127.5, 127.9, 128.4, 128.2, 128.2, 128.6, 129.5, 130.5, 175.7; ESI-MS 377.2 (M+H); elemental analysis: calculated for (C₂₄H₃₀O₃·¼H₂O): C 75.64%, H 10.71%; found C 76.49%, H 10.66%.

(2*R*)-2,3-di-[(*t*-butyldiphenylsilyloxy)propanoic acid (18b). Methyl (4*R*)-2,2-dimethyl-1,3-dioxolane-4-carboxylate (500 mg, 3 mmol) was dissolved in THF/H₂O (160

mL/90 mL), and the solution was cooled on an ice bath. 2 M NaOH was added slowly. After the addition, the cooling bath was removed and stirring was continued for 1 hour. The reaction mixture was acidified with 2 M HCl and stirred at RT for 2 hours. The solvent was evaporated. (2*R*)-2,3-dihydroxypropanoic acid (330 mg, 3.1 mmol) was dissolved in MeOH/H₂O (5 mL/0.5 mL) and the solution was neutralized with 20% NaHCO₃. The solvent was evaporated. The residue was dissolved in dry DMF, and benzyl bromide was added. The reaction mixture was stirred under Ar at RT for 2 days. The solvent was evaporated, the residue was dissolved in EtOAc and washed with brine. The organic layer was dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with EtOAc:PE; 3:1. The yield of the colorless oil was 140 mg (23%). R_f=0.35 (EtOAc:PE; 4:1). The mixture of benzyl (2*R*)-2,3-dihydroxypropanoate (140 mg, 0.71 mmol), TBDMSCl (430 mg, 1.6 mmol) and imidazole (150 mg, 2.2 mmol) in DMF (3 mL) was stirred under Ar at RT for 5 hours. The solvent was evaporated, and the residue was dried in vacuo. Benzyl (2*R*)-2,3-di-[(*t*-butyldiphenylsilyl)oxy]propanoate (480 mg, 0.71 mmol) was dissolved in dry THF (5 mL) and Pd/C (386 mg) was added. The reaction mixture was stirred under an H₂-balloon for 3 days. The catalyst was filtered, and the solvent was evaporated. After the purification with flash chromatography, some impurities remained (eluent PE:EtOAc; 5:1), but the product was used in the next reaction without further purification. Evaporation of solvents yielded colorless oil. R_f=0.56 (PE:EtOAc;4:1).

(2*R*)-2,3-dihydroxypropanoic acid icosa-5,8,11,14-tetraenyl ester (4b). (2*R*)-2,3-di-[(*t*-butyldiphenylsilyl)oxy]propanoic acid (**18b**) (410 mg, 0.71 mmol), was dissolved in dry CH₂Cl₂ (10 mL) and arachidonyl alcohol (170 mg, 0.59 mmol), DCC (240 mg, 1.2 mmol) and DMAP (43 mg, 0.35 mmol) were added. The reaction mixture was stirred under Ar at RT overnight. The white precipitate was filtered, and the filtrate was washed with 10% citric acid, 5% NaHCO₃ and water. The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with 0–4 % EtOAc in PE. Evaporation of solvents yielded colorless oil (320 mg, 57%). The solution of (2*R*)-2,3-di-[(*t*-butyldiphenylsilyl)oxy]propanoic acid icosa-5,8,11,14-tetraenyl ester (170 mg, 0.20 mmol) and 1 M TBAF (300 μL) in dry THF (3 mL) was stirred under Ar at RT for 2

hours. THF was evaporated, and the crude product was purified by flash chromatography eluting with PE:EtOAc; 3:1. Evaporation of solvents yielded 59 mg of an oily product (78%). $R_f=0.11$ (PE:EtOAc; 3:1); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=6.8$, 3H), 1.26-1.39 (m, 6H), 1.44 (qn, $J=7.7$, 2H), 1.70 (qn, $J=6.8$, 2H), 2.06 (q, $J=7.1$, 2H), 2.11 (q, $J=7.1$, 2H), 2.25 (br s, 1H), 2.80-2.85 (m, 6H), 3.23 (br s, 1H), 3.87 (dd, 2H), 4.23 (t, $J=6.6$, 2H), 4.26 (br s, 1H), 5.31-5.43 (m, 8H); $^{13}\text{C NMR}$ (CDCl_3): δ 14.1, 22.6, 25.7, 25.7, 26.6, 26.7, 27.2, 28.1, 29.3, 31.5, 64.1, 66.1, 71.5, 127.5, 127.9, 128.2, 128.2, 128.5, 128.6, 129.4, 130.5, 173.1; elemental analysis: calculated for $\text{C}_{23}\text{H}_{38}\text{O}_4 \cdot 1/10\text{H}_2\text{O}$: C 72.63%, H 10.12%; found: C 72.49%, H 10.18%.

(2S)-2,3-dihydroxypropanoic acid icoso-5,8,11,14-tetraenyl ester (4a). $R_f=0.11$ (PE:EtOAc; 3:1); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=6.9$, 3H), 1.26-1.39 (m, 6H), 1.44 (qn, $J=7.6$, 2H), 1.70 (qn, $J=6.7$, 2H), 2.06 (q, $J=7.2$, 2H), 2.11 (q, $J=7.2$, 2H), 2.25 (br s, 1H), 2.80-2.85 (m, 6H), 3.86 (dd, 2H), 4.23 (t, $J=6.6$, 2H), 4.26 (br s, 1H), 5.31-5.43 (m, 8H); $^{13}\text{C NMR}$ (CDCl_3): δ 14.1, 22.6, 25.7, 25.8, 26.6, 26.7, 27.2, 28.1, 29.3, 31.5, 64.1, 66.1, 71.6, 127.5, 127.9, 128.2, 128.2, 128.5, 128.6, 129.4, 130.5, 173.1; ESI-MS 400.3 (M+Na); elemental analysis: calculated for $\text{C}_{23}\text{H}_{38}\text{O}_4 \cdot 1/10\text{H}_2\text{O}$: C 72.63%, H 10.12%; found: C 72.30%, H 10.30%.

3-(*t*-butyldimethylsilyloxy)-2,2-dimethylpropanoic acid 24. The solution of 2,2-dimethyl-3-hydroxypropanoic acid **23** (500 mg, 4.23 mmol), TBDMSCl (1.2 g, 7.6 mmol) and imidazole (870 mg, 12.7 mmol) in dry THF was stirred under Ar at RT for 20 hours. Water (45 mL) was added and the solution was extracted with diethyl ether (3x20 mL). The organic phase was washed with saturated NaHCO_3 , dried over Na_2SO_4 , and evaporated. The yield was 1.1 g (71%). $^1\text{H NMR}$ (CDCl_3): δ 0.02 (s, 6H), 0.25 (s, 6H), 0.87 (s, 9H), 0.94 (s, 9H), 1.13 (s, 6H), 3.57 (s, 2H). *t*-Butyldimethylsilyl 3-[(*t*-butyldimethylsilyloxy)-2,2-dimethylpropanoate (830 mg, 1.53 mmol) was dissolved in water (30 mL) and THF (25 mL), and the solution was stirred until the starting material was consumed. The solvents were evaporated, and the product was dried in vacuo. The yield was 170 mg (48%). $^1\text{H NMR}$ (CDCl_3): δ 0.06 (s, 6H), 0.88 (s, 9H), 1.27 (s, 6H), 3.67 (s, 2H).

Arachidonyl 2,2-dimethyl-3-hydroxypropanoate (5). Arachidonyl alcohol (150 mg, 0.51 mmol), 3-[(*t*-butyldiphenylsilyloxy)-2,2-dimethylpropanoic acid **24** (170 mg,

0.73 mmol), EDCI (200 mg, 1.0 mmol), and DMAP (18 mg, 0.15 mmol) were dissolved in dry CH₂Cl₂ (7 mL). The reaction mixture was stirred at RT for 6 days after which the mixture was washed with 5% NaHCO₃ and water. The crude product was purified by flash chromatography eluting with 2% EtOAc in PE. R_f=0.60 (PE:EtOAc; 9:1). Arachidonyl [3-(*t*-butyldiphenylsilyloxy)-2,2-dimethylpropanoate (250 mg, 0.50 mmol) was dissolved in dry THF (2 mL), and 1 M TBAF (1 mmol) in THF was added. Mixture was stirred for 24 hours. THF was evaporated, and diethyl ether (15 mL) and 1 M HCl (5 mL) were added. The organic layer was separated, dried over Na₂SO₄, and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc; 6:1. Evaporation of solvents yielded 100 mg (52%) of oily product. R_f=0.30 (PE:EtOAc; 4:1); ¹H NMR (CDCl₃): δ 0.89 (t, *J*=6.9, 3H), 1.19 (s, 6H), 1.26-1.39 (m, 6H), 1.44 (qn, *J*=7.6, 2H), 1.67 (qn, *J*=7.2, 2H), 2.04-2.13 (m, 4H), 2.39 (t, *J*=6.8, 1H), 2.80-2.85 (m, 6H), 3.55 (d, *J*=6.5, 2H), 4.11 (t, *J*=6.6, 2H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 22.1, 22.6, 25.7 (2C), 25.9, 26.8, 27.2, 28.2, 29.3, 31.5, 44.2, 64.6, 69.7, 127.5, 127.9, 128.2 (2C), 128.4, 128.6, 129.5, 130.5, 177.5.

5-Methyl-2-phenyl-1,3-dioxane-5-carboxylic acid (27). The mixture of 2,2-bis(hydroxymethyl)propionic acid (1.6 g, 7.5 mmol), *p*-TSA (0.04 g, 0.2 mmol) and benzaldehyde (1 mL, 9.9 mmol) in toluene (20 mL) was refluxed using a Dean-Stark trap for 24 hours. The solution was washed with saturated NaHCO₃ and water. The solvent was evaporated and the residue was used in the next reaction without further purification (yield 0.85 g).

3-Hydroxy-2-hydroxymethyl-2-methylpropanoic acid icoso-5,8,11,14-tetraenyl ester (6). DCC (110 mg, 0.55 mmol) and DMAP (30.0 mg, 0.25 mmol) were added to the precooled solution of **27** (110 mg, 0.49 mmol) and arachidonyl alcohol (120 mg, 0.41 mmol) in dry CH₂Cl₂ (2 mL). After the addition, the cooling bath was removed and stirring was continued for 3 days. The white precipitate was filtered and the filtrate was evaporated to dryness. The crude product was purified by flash chromatography eluting with 10% EtOAc in PE. Evaporation of solvents yielded a colorless, oily product (130 mg, 64%). The product (130 mg, 0.26 mmol) was stirred in HCl/MeOH solution (1:1) at RT for 3 hours. MeOH was evaporated, and the residue was dissolved in EtOAc (40 mL). The solution was neutralized with saturated NaHCO₃ and washed with brine.

The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc; 3:2. Evaporation of solvents yielded an oily product (78 mg, 74%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=6.9, 3H), 1.06 (s, 3H), 1.26-1.39 (m, 6H), 1.45 (qn, *J*=7.6, 2H), 1.69 (qn, *J*=6.6, 2H), 2.04-2.13 (m, 4H), 2.43 (s, 2H), 2.78-2.85 (m, 6H), 3.71 (d, *J*=11.2, 2H), 3.90 (d, *J*=11.2, 2H), 4.17 (t, *J*=6.6, 2H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 17.1, 22.6, 25.7 (2C), 25.9, 26.7, 27.2, 28.2, 29.3, 31.5, 49.1, 65.0, 68.5, 127.6, 127.9, 128.2 (2C), 128.5, 128.6, 129.5, 130.5, 176.0.

2-phenyl-1,3-dioxane-5-carboxylic acid (28). The mixture of diethylbis(hydroxymethyl)malonate (5.00 g, 23 mmol), concentrated H₂SO₄ (4 drops), benzaldehyde (4.8 mL, 48 mmol) and toluene (60 mL) was refluxed using a Dean-Stark trap for 18 hours. The reaction mixture was cooled and evaporated and the residue was dissolved in diethyl ether. The solution was washed with saturated NaHCO₃ and water. The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc; 10:1. The yield was 4.7 g (67%). ¹H NMR (CDCl₃): δ 1.26 (t, *J*=7.0, 3H), 1.32 (t, *J*=7.0, 3H), 4.15 (d, *J*=11.5, 2H), 4.20 (q, *J*=7.0, 2H), 4.33 (q, *J*=7.0, 2H), 4.85 (d, *J*=11.7, 2H), 5.48 (s, 1H), 7.32-7.36 (m, 3H), 7.42-7.45 (m, 2H). The mixture of diethyl 2-phenyl-1,3-dioxane-5,5-dicarboxylic acid ester (4.7 g, 15 mmol) and KOH (3.6 g, 65 mmol) in EtOH (33 mL) was reflux for 30 minutes. The solvent was evaporated after which CH₂Cl₂ and water were added and the solution was acidified with ice cold 10% HCl. The organic phase was separated, dried over Na₂SO₄ and evaporated. The crude product was purified by recrystallizing from hexane:diethyl ether 3:2. The resulting white crystals (2.00 g, 7.8 mmol) were refluxed in dry Et₃N (8 mL) for 1.5 hours. The solvent was evaporated, and CH₂Cl₂ was added. The solution was acidified with ice cold 10% HCl. The combined organic layers were dried over Na₂SO₄, filtered and evaporated (yield 0.97 g, 31%). ¹H NMR (CDCl₃) (*trans*): δ 3.17-3.23 (m, 1H), 3.99-4.04 (t, *J*=11.5, 2H), 4.71 (d, *J*=11.0, 2H), 5.44 (s, 1H), 7.25-7.48 (m, 5H), 9.28 (s, 1H). ¹H NMR (CDCl₃) (*cis*): δ 2.49 (br s, 1H), 4.11-4.14 (m, 2H), 4.49 (q, *J*=4.6, *J*=11.8, 2H), 5.53 (s, 1H), 7.25-7.48 (m, 5H), 9.28 (s, 1H).

3-Hydroxy-2-hydroxymethylpropanoic acid icoso-5,8,11,14-tetraenyl ester (7). Compound **28** (150 mg, 0.73 mmol) and arachidonyl alcohol (150 mg, 0.52 mmol) in dry CH₂Cl₂ (5 mL) were cooled on an ice bath and DCC (160 mg, 0.77 mmol) and DMAP (7 mg, 0.06 mmol) were added. After 1 hour stirring, the cooling bath was removed and stirring was continued at RT overnight. The white precipitate was filtered, and the filtrate was washed with water. The crude product was purified by flash chromatography eluting with PE: EtOAc; 4:1. Evaporation of solvents yielded colorless oil (200 mg, 79%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.25-1.39 (m, 6H), 1.43 (qn, *J*=7.6, 2H), 1.66 (qn, *J*=6.7, 2H), 2.06 (q, *J*=7.0, 2H), 2.11 (q, *J*=7.0, 2H), 2.81-2.86 (m, 6H), 3.15 (sep, *J*=4.9, 1H), 3.97 (t, *J*=11.6, 2H), 4.11 (t, *J*=6.7, 2H), 4.46 (dd, 2H), 5.31-5.43 (m, 8H), 5.43 (s, 1H), 7.46-7.48 (m, 2H), 7.32-7.39 (m, 3H). 2-Phenyl-1,3-dioxane-5-carboxylic acid icoso-5,8,11,14-tetraenyl ester (93 mg, 0.19 mmol) was dissolved in MeOH/H₂O (7 mL/3 mL) and the solution was stirred at RT for 1.5 hours. MeOH was evaporated and the residue was dissolved in EtOAc. The solution was washed with 5% NaHCO₃ (3x10 mL) and brine (3x10 mL). The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc; 2:1. The yield of the oily product was 43 mg (56%). ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.26-1.39 (m, 6H), 1.45 (qn, *J*=7.5, 2H), 1.69 (qn, *J*=6.8, 2H), 2.04-2.13 (m, 4H), 2.72 (qn, *J*=4.9, 1H), 2.80-2.85 (m, 6H), 3.99 (ddd, 4H), 4.17 (t, *J*=6.7, 2H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 22.6, 25.6, 25.8, 25.9, 26.7, 27.2, 28.2, 29.3, 31.5, 48.7, 62.2, 65.0, 127.6, 127.9, 128.2, 128.5, 128.6, 129.5, 130.5, 173.4; ESI-MS 393.2 (M+H); elemental analysis: calculated for C₂₄H₄₀O₄*1/3H₂O: C 72.32%, H 10.28%; found: C 72.55%, H 10.18%.

(2-Hydroxyethyl)carbamic acid icoso-5,8,11,14-tetraenyl ester (8). Arachidonyl alcohol (100 mg, 0.34 mmol), pyridine (0.03 mL) and *p*-nitrophenyl chloroformate (73 mg, 0.36 mmol) were dissolved in dry THF (4 mL). The reaction mixture was stirred at RT under Ar for 24 hours. EtOAc (20 mL) and water (10 mL) were added and the organic layer was separated. The organic layer was washed with saturated NaHCO₃ (3x20 mL), dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with 20-50% CH₂Cl₂ in hexane. Evaporation of solvents yielded an oily product (150 mg, 86 %). R_f=0.38 (PE:EtOAc; 4:1); ¹H NMR

(CDCl₃): δ 0.89 (t, $J=6.9$, 3H), 1.25-1.39 (m, 6H), 1.52 (qn, $J=7.3$, 2H), 1.79 (qn, $J=6.7$, 2H), 2.05 (q, $J=7.2$, 2H), 2.15 (q, $J=7.1$, 2H), 2.80-2.86 (m, 6H), 4.30 (t, $J=6.6$, 2H), 5.31-5.44 (m, 8H), 7.38 (d, $J=9.1$, 2H), 8.23 (d, $J=9.1$, 2H). Compound **30** (140 mg, 0.31 mmol), ethanolamine (0.02 mL, 0.3 mmol) and Et₃N (0.04 mL, 0.3 mmol) in dry THF were stirred at RT for 2 hours. Diethyl ether was added, and the solution was washed with saturated NaHCO₃ (3x20 mL) and brine. The combined organic layers were dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc; 2:1. Evaporation of solvents yielded an oily product (80 mg, 68 %). $R_f=0.18$ (PE:EtOAc; 2:1); ¹H NMR (CDCl₃): δ 0.89 (t, $J=7.0$, 3H), 1.25-1.29 (m, 6H), 1.43 (qn, $J=7.6$, 2H), 1.64 (qn, $J=13.3$, 2H), 2.04-2.10 (m, 5H), 2.80-2.85 (m, 6H), 3.34 (qn, $J=5.3$, 2H), 3.72 (br s, 2H), 4.07 (t, $J=6.6$, 2H), 5.02 (br s, 1H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 22.6, 25.7, 25.8, 25.9, 26.8, 27.2, 28.7, 29.3, 31.5, 43.5, 62.6, 65.1, 127.6, 127.9, 128.2, 128.3, 128.3, 128.6, 129.7, 130.5, 194.0; ESI-MS 378.0 (M+H); elemental analysis: calculated for C₂₃H₃₉NO₃·1/10H₂O: C 72.82%, H 10.42%, N 3.69%; found: C 72.72%, H 10.34%, N 3.77%.

(1S)-(2-Hydroxy-1-methylethyl)-carbamic acid icoso-5,8,11,14-tetraenyl ester (9b). The synthesis was similar to the synthesis of compound **8**. The crude product was purified by flash chromatography eluting with 20–25% EtOAc in PE. Yield was 80%. $R_f=0.09$ (PE:EtOAc; 3:1); ¹H NMR (CDCl₃): δ 0.89 (t, $J=7.0$, 3H), 1.17 (d, $J=6.7$, 3H), 1.25-1.38 (m, 6H), 1.42 (qn, $J=7.6$, 2H), 1.63 (qn, $J=6.7$, 2H), 2.03-2.11 (m, 4H), 2.44 (br s, 1H), 2.80-2.85 (m, 6H), 3.53 (br s, 1H), 3.65 (br s, 1H), 3.81 (br s, 1H), 4.06 (t, $J=6.5$, 2H), 4.78 (br s, 1H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.1, 17.3, 22.6, 25.7, 25.8, 25.9, 26.8, 27.2, 28.6, 29.3, 31.5, 48.9, 65.1, 67.1, 127.6, 127.9, 128.2, 128.3, 128.3, 128.6, 129.7, 130.5, 157.0; elemental analysis: calculated for C₂₄H₄₁NO₃·1/8H₂O: C 73.19%, H 10.56%, N 3.56%; found: C 73.16%, H 10.62%, N 3.41%.

(1R)-(2-Hydroxy-1-methylethyl)-carbamic acid icoso-5,8,11,14-tetraenyl ester (9a). The synthesis was similar to the synthesis of compound **8**. The crude product was purified by flash chromatography eluting with 20–25% EtOAc in PE. The yield was quantitative. $R_f=0.15$ (PE:EtOAc; 3:1); ¹H NMR (CDCl₃): δ 0.89 (t, $J=7.0$, 3H), 1.17 (d,

$J=6.8$, 3H), 1.26-1.39 (m, 6H), 1.43 (qn, $J=7.6$, 2H), 1.63 (qn, $J=6.8$, 2H), 2.12-2.04 (m, 4H), 2.40 (br s, 1H), 2.80-2.85 (m, 6H), 3.53 (br s, 1H), 3.66 (br s, 1H), 3.81 (br s, 1H), 4.06 (t, $J=6.6$, 2H), 4.78 (br, 1H), 5.31-5.43 (m, 8H); ^{13}C NMR (CDCl_3): δ 14.1, 17.3, 22.6, 25.7, 25.8, 25.9, 26.8, 27.2, 28.6, 29.3, 31.5, 35.1, 48.9, 67.1, 127.6, 127.9, 128.2, 128.3, 128.3, 128.6, 129.7, 130.5, 157.0; ESI-MS 392.1 (M+H); elemental analysis: Calculated for $\text{C}_{24}\text{H}_{41}\text{NO}_3$: C 73.61%, H 10.55%, N 3.58%; found: C 73.62%, H 10.56%, N 3.68%.

***p*-Nitrophenyl propyl carbonate (31).** *p*-Nitrophenyl chloroformate (590 mg, 2.9 mmol) in dry THF (8.9 mL) was added dropwise to the cooled solution of 1-propanol (130 mg, 2.2 mmol), pyridine (0.8 mL) and THF (8.9 mL). After the addition, the mixture was stirred at RT for 18 hours. The solvent was evaporated and the residue was dissolved in EtOAc (25 mL), washed twice with 10% citric acid and brine. The combined organic layers were dried over Na_2SO_4 , filtered and the solvent was evaporated. The crude product was purified by flash chromatography using 2% EtOAc in PE as an eluent. The evaporation of solvents yielded cream colored crystals (414 mg, 83%). ^1H NMR (CDCl_3): 1.03 (t, 3H, $J=7.5$), 1.76-1.83 (m, 2H), 4.26 (t, 2H, $J=6.7$), 7.39 (d, 2H, $J=9.4$), 8.28 (d, 2H, $J=9.4$); ^{13}C NMR (CDCl_3): δ 10.1, 21.9, 71.7, 121.8, 125.3, 145.4, 152.6, 155.7.

***p*-Nitrophenyl ethyl carbonate (32).** The synthesis was similar to the synthesis of compound 31. Yield was 77%. ^1H NMR (CDCl_3): δ 1.41 (t, 3H, $J=7.1$), 4.36 (q, 2H, $J=7.1$), 7.38 (d, 2H, $J=9.4$), 8.28 (d, 2H, $J=9.4$); ^{13}C NMR (CDCl_3): δ 14.2, 65.6, 121.8, 125.3, 145.4, 152.5, 155.6.

Arachidonyl propyl carbonate (11). The mixture of arachidonyl alcohol (150 mg, 0.53 mmol), compound 31 (210 mg, 0.93 mmol) and DMAP (67 mg, 0.55 mmol) in dry CH_2Cl_2 (14 mL) was stirred for 48 hours at RT. The reaction mixture was poured into EtOAc (20 mL) and washed with saturated NaHCO_3 and brine. The organic phase was dried over Na_2SO_4 , filtered and the solvent was evaporated. The crude product was purified by flash chromatography eluting with 0–2% EtOAc in hexane. The yield of the oily product was 180 mg (91%). ^1H NMR (CDCl_3): δ 0.89 (t, 3H, $J=6.8$), 0.96 (t, 3H, $J=7.4$), 1.25-1.39 (m, 6H), 1.39-1.48 (m, 2H), 1.66-1.73 (m, 4H), 2.05 (q, 2H, $J=7.2$), 2.10 (q, 2H, $J=6.9$), 2.80-2.85 (m, 6H), 4.09 (t, 2H, $J=6.7$), 4.13 (t, 2H, $J=6.6$), 5.31-

5.43 (m, 8H); ^{13}C NMR (CDCl_3): δ 10.2, 14.1, 22.1, 22.6, 25.6, 25.6, 25.6, 25.7, 26.8, 27.2, 28.3, 29.3, 31.5, 67.8, 69.5, 127.6, 127.9, 128.2, 128.3, 128.4, 128.6, 129.5, 130.5, 155.5; ESI-MS 377.2 (M+H); elemental analysis: calculated for $\text{C}_{24}\text{H}_{40}\text{O}_3$: C 76.55%, H 10.71%; found: C 76.75%, H 10.68%.

Arachidonyl ethyl carbonate (10). The synthesis was similar to the synthesis of compound **11**. The crude product was purified by flash chromatography eluting with 1% EtOAc in PE. The yield was 77%. ^1H NMR (CDCl_3): δ 0.89 (t, 3H, $J=7.0$), 1.26-1.39 (m, 6H), 1.31 (t, 3H, $J=7.1$), 1.46 (qn, 2H, $J=7.6$), 1.69 (qn, 2H, $J=7.3$), 2.05 (q, 2H, $J=7.2$), 2.10 (q, 2H, $J=6.9$), 2.80-2.85 (m, 6H), 4.13 (t, 2H, $J=6.7$), 4.19 (q, 2H, $J=7.1$), 5.31-5.43 (m, 8H); ^{13}C NMR (CDCl_3): δ 14.1, 14.3, 22.6, 25.6, 25.6, 25.6, 25.7, 26.8, 27.2, 28.3, 29.3, 31.5, 63.8, 67.8, 127.6, 127.9, 128.2, 128.3, 128.4, 128.6, 129.5, 130.5, 155.3; ESI-MS 363.2 (M+H); elemental analysis: calculated for $\text{C}_{23}\text{H}_{38}\text{O}_3$: C 76.20 %, H 10.56 %; found: C 76.01 %, H 10.56 %.

4.6 References

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5 SYNTHESIS, CANNABINOID RECEPTOR ACTIVITIES AND ENZYMATIC STABILITY FOR REVERSED AMIDE DERIVATIVES OF ARACHIDONOYL ETHANOLAMIDE*

5.1 Introduction

Several approaches to overcome the poor enzymatic stability of AEA have been suggested. For example, introduction of a methyl group at an α -position or adjacent to nitrogen ((*R*)-methanandamide) gives rise to enzymatically more stable, high affinity CB1 receptor ligands (Abadji et al. 1994; Adams et al. 1995a, b). However, a methyl group addition involves a stereoselective synthesis which can be considered as an extra challenge. Therefore, more practical methods are needed. Lin et al. (1998) observed indirectly that reversal of the carbonyl and NH in the amide group could provide high metabolic stability. The hypothesis is based on the affinity studies with and without a FAAH inhibitor. Based on the findings of Lin et al. (1998), we synthesized reversed amides with various head groups and different chain lengths (C₂₁ vs. C₂₂) to study their cannabinoid receptor activity in rat cerebellar membranes and hCB2 receptors, and to determine their enzymatic stability using rat brain homogenate.

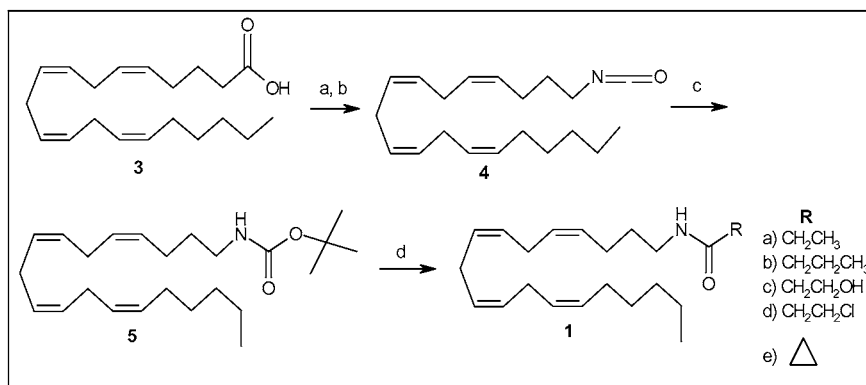
*Adapted from: Teija Parkkari, Juha R. Savinainen, Katri H. Raitio, Susanna M. Saario, Laura Matilainen, Tuomas Sirviö, Jarmo T. Laitinen, Tapio Nevalainen, Riku Niemi, and Tomi Järvinen. Synthesis, cannabinoid receptor activities and enzymatic stability of reversed amide derivatives of arachidonoyl ethanolamide.

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5.2 Synthesis

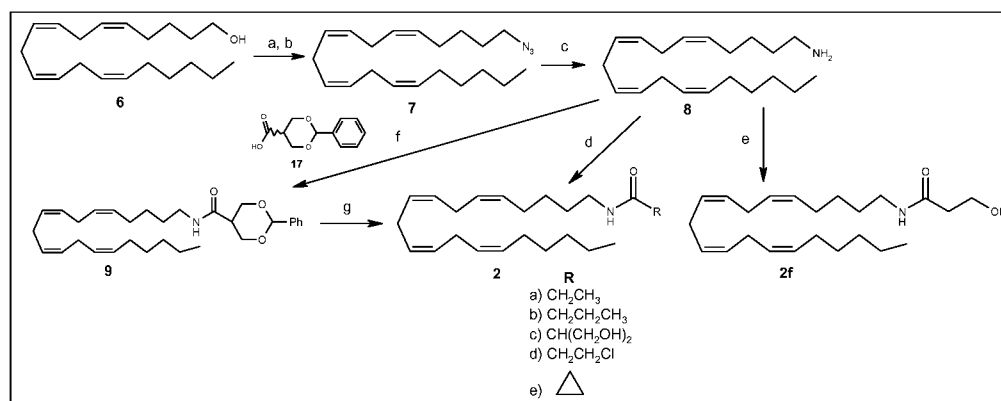
Norarachidonyl amides **1a-e** (C₁₉ series) were prepared by the method published by Nazih and Heissler (2002) (Scheme 5.1). This method allows one-pot conversion of *t*-butyl carbamates to amides. It is based on a cleavage of a *t*-butoxycarbonyl group with hydrogen iodide generated *in situ* by reaction of acyl halide with methanol and on an acylation of an intermediate amine with excess acyl halide in the presence of diisopropylethylamine (DIPEA). The key intermediate, norarachidonyl *N*-*t*-butylcarbamate **5**, was synthesized from arachidonic acid which is first converted to acyl azide with diphenyl phosphoryl azide (DPPA). The acyl azide undergoes the Curtius rearrangement to norarachidonyl isocyanate **4** upon heating (Ng et al. 1999). Compound **5** is finally obtained by refluxing the isocyanate **4** with *t*-butanol for several days.



Scheme 5.1. a) DPPA, Et₃N, benzene; (b) toluene, Δ; (c) *t*-BuOH; (d) 1. RCOCl, NaI, acetonitrile, MeOH; 2. DIPEA.

Synthesis methods for compounds **2a-f** (C₂₀ series) are summarized in Scheme 5.2. Synthesis of a key intermediate, arachidonyl amine **8**, was performed by converting commercially available arachidonyl alcohol to arachidonyl azide **7** via the more reactive mesylate and further reducing the azide to amine with LiAlH₄ (LAH) (Lin et al. 1998). Compounds **2a-b** and **2d-e** were synthesized from arachidonyl amine and appropriate acid halides. Retroanandamide **2f** was synthesized as previously described (Lin et al. 1998, Sidler et al. 1994). Compound **2c** was prepared by coupling *cis/trans*-2-phenyl-

1,3-dioxane-5-carboxylic acid **17** (Juaristi et al. 1997) with arachidonyl amine using *N*'-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDCI) and 4-dimethylaminopyridine (DMAP) as coupling reagents. The desired product was obtained from compound **9** by removing the benzylidene acetal protective group with concentrated HCl/MeOH solution.



Scheme 5.2. (a) MsCl, pyridine; (b) NaN_3 ; (c) LAH; (d) RCOCl , Et_3N ; (e) $\text{Al}(\text{CH}_3)_3$, β -propiolactone, CH_2Cl_2 ; (f) EDCI, DMAP, CH_2Cl_2 ; (g) HCl (aq)/MeOH.

5.3 Results and discussion

Table 5.1 summarizes the results from the CB1 and CB2 receptor activation studies. All the synthesized compounds, except **2c**, showed dose-dependent CB1 activity, and their responses at 5×10^{-5} M were reversed by the CB1 receptor antagonist, AM251 at 10^{-6} M concentration (data not shown).

In the $[^{35}\text{S}]\text{GTP}\gamma\text{S}$ binding assay with rat cerebellar membranes, the reversed amides, as well as AEA, acted as partial agonists. As the previous affinity studies of retroanandamide suggested (Lin et al. 1998), AEA has a higher E_{max} value than **1c** and **2f** (retroanandamide) in the study conditions where activity of FAAH is blocked with an enzyme inhibitor (MAFP). However, the compounds appear to have comparable potency to AEA in the reversed amide series where the hydroxyethyl group is replaced with an alkyl chain (**1a-b**, **1d-e** and **2a-b**, **2d-e**); the results are in good agreement with

findings of Pinto et al. (1994) and Sheskin et al. (1997) who observed that by increasing the lipophilicity, the CB1 receptor affinity could be improved. It has been shown that naturally occurring anandamides, such as (22:4, *n*-6) and (20:4, *n*-6) i.e. AEA, possess similar affinities for the CB1 receptor (Sheskin et al. 1997). Reports about reversed amides with different chain lengths or different degree of double bonds have not been published. In our reversed amide series, a longer chain length (i.e. C₂₀) is preferred although the differences are not very significant.

Table 5.1. Comparison of efficacy (E_{\max}) and potency ($-\log EC_{50}$) values of 2-AG, AEA, **1a-e** (C₁₉ series) and **2a-f** (C₂₀ series) at rat cerebellar membranes and at CHO-hCB2 cells.

| Compound | CB1 activation | | | CB2 activation | | |
|---------------|---|---------------------------------------|----------------------|---|---------------------------------------|----------------------|
| | Relative E_{\max} (% 2-AG $E_{\max} \pm$ SEM) | Potency ($-\log EC_{50} \pm$ SEM) | EC_{50} (μ M) | Relative E_{\max} (% HU-210 E_{\max} \pm SEM) | Potency ($-\log EC_{50} \pm$ SEM) | EC_{50} (μ M) |
| 2-AG | 100 | 6.0 \pm 0.0 | 1.0 | - | - | - |
| HU-210 | - | - | - | 100 | 8.3 \pm 0.1 | 0.005 |
| AEA | 67 \pm 2 | 5.3 \pm 0.1 | 5.0 | 48 \pm 5 | 6.5 \pm 0.2 | 0.3 |
| 1a | 45 \pm 4 | 5.4 \pm 0.1 | 4.0 | 54 \pm 3 | 6.7 \pm 0.1 | 0.2 |
| 1b | 36 \pm 3 | 5.4 \pm 0.2 | 4.0 | 53 \pm 4 | 7.1 \pm 0.2 | 0.07 |
| 1c | 46 \pm 6 | 4.4 \pm 0.1 | 40 | 54 \pm 15 | 5.8 \pm 0.3 | 1.6 |
| 1d | 38 \pm 12 | 5.3 \pm 0.3 | 5.0 | 59 \pm 10 | 6.6 \pm 0.5 | 0.3 |
| 1e | 37 \pm 4 | 6.1 \pm 0.1 | 0.8 | 55 \pm 4 | 6.7 \pm 0.2 | 0.2 |
| 2a | 49 \pm 5 | 5.7 \pm 0.1 | 2.0 | 64 \pm 3 | 7.1 \pm 0.2 | 0.07 |
| 2b | 39 \pm 2 | 5.6 \pm 0.1 | 2.5 | 73 \pm 3 | 7.0 \pm 0.2 | 0.1 |
| 2c | NA | NA | NA | ND | ND | ND |
| 2d | 34 \pm 1 | 5.8 \pm 0.2 | 1.6 | 64 \pm 4 | 6.8 \pm 0.2 | 0.2 |
| 2e | 40 \pm 4 | 5.6 \pm 0.3 | 2.5 | 67 \pm 4 | 6.7 \pm 0.2 | 0.2 |
| 2f | 55 \pm 7 | 4.9 \pm 0.1 | 13 | 75 \pm 7 | 6.6 \pm 0.2 | 0.3 |

NA = no detectable CB1 activation at 10^{-4} M concentration. ND = not determined. The CB1 data represents the mean (\pm s.e.m.) of [³⁵S]GTP γ S binding as percentage of the maximal response evoked by 2-AG, and the CB2 data represents the mean (\pm s.e.m.) of [³⁵S]GTP γ S binding from the maximal response evoked by HU-210. Data are produced from at least three independent experiments performed in duplicate.

As shown in Table 5.1, all the studied compounds behaved as partial agonists and had comparable efficacy and potency values to AEA on hCB2-CHO lysates. The most interesting finding in both the C₁₉ and C₂₀ series was that compounds with a butanoyl tail (**1b**, **2b**) possess a potency that equals that of 2-AG (7.4 ± 0.1). In addition, compound **2b** is relatively efficacious. Although, Lin et al. (1998) reported that retroanandamide has only weak affinity for the CB2 receptor our findings suggest that reversed amides are able to activate both cannabinoid receptors.

Our enzymatic stability studies in rat brain homogenate showed that the synthesized compounds are stable (no degradation during 90 minutes) whereas AEA is almost fully degraded in 90 minutes. The degradation of AEA in the rat brain homogenate was studied by a potent FAAH inhibitor URB597. URB597 was able to inhibit the AEA degradation with an IC₅₀ value of 3.8 ± 0.6 nM ($n=3$) which is comparable to the previously reported results, and it confirms that in the rat brain homogenate, AEA is mainly metabolized by FAAH (Mor et al. 2004).

5.4 Conclusions

In this study, a novel synthesis method for fatty acid derivatives having a chain length of C₁₉ was developed, and the method was used for the preparation of reversed amide analogues of AEA. The cannabinergic activity of the synthesized compounds was determined by the [³⁵S]GTP_γS binding assay using the rat cerebellar membranes and hCB2-CHO lysates. Finally, the metabolic stability of the novel compounds was studied in rat brain homogenate. The CB1 activation studies showed that the reversal of the amide bond of AEA results in weaker CB1 activity properties, however, they can be slightly improved by increasing lipophilicity and chain length. In contrast, all the reversed amides studied had similar or higher activity properties for the CB2 receptor compared to AEA. Thus, our results indicate that the reversed amides are able to activate both cannabinoid receptors. Finally, the stability studies showed that the reversed amides remain intact in the studied conditions, whereas AEA is almost fully degraded. Therefore, it can be concluded that by reversing the amide bond of AEA it is possible to develop good cannabinergic compounds with excellent stability against FAAH or other types of enzyme mediated metabolism.

5.5 Experimental

5.5.1 Synthesis

***t*-Butyl norarachidonylcarbamate (5).** Arachidonic acid (500 mg, 1.6 mmol) and dry Et₃N (0.34 mL, 2.5 mmol) were dissolved in dry benzene (6 mL) and stirred for 10 minutes. DPPA (0.53 mL, 2.5 mmol) was added, and stirring was continued at RT for 2 hours. Benzene was evaporated, the residue was dissolved in PE:EtOAc 10:1 and filtered through a pad of silica gel. Solvents were evaporated and the oily residue was dissolved in dry toluene (3 mL). *t*-BuOH (0.31 mL, 3.3 mmol) in dry toluene (3 mL) was added, and the reaction mixture was stirred at 65 °C for 5 days. The reaction mixture was cooled to RT, water (20 mL) and EtOAc (20 mL) were added and the solution was washed with brine. The organic layers were combined, dried over Na₂SO₄ and evaporated. The crude product was purified by flash chromatography eluting with 2.5% EtOAc in PE. Evaporation of solvents yielded 240 mg of a yellowish oily product (39%). R_f=0.45 (PE:EtOAc 6:1); ¹H NMR δ 0.89 (t, *J*=7.0, 3H), 1.26–1.40 (m, 6H), 1.44 (s, 9H), 1.56 (qn, *J*=7.4, 2H), 2.04–2.12 (m, 4H), 2.80–2.85 (m, 6H), 3.12–3.13 (m, 2H), 4.51 (br s, 1H), 5.31–5.43 (m, 8H).

General Synthesis Procedure for the Compounds 1a-b and 1d-e. An appropriate acid halide (4 eq) was added into the mixture of *t*-butyl arachidonylcarbamate (5) (1 eq), MeOH (2 eq) and NaI (2 eq) in dry ACN (10 mL/mmol). The reaction mixture was stirred for 0.5 hour. The yellow solution was cooled on an ice-bath and DIPEA (4 eq) was added. Stirring was continued at RT for 1 hour. 10% HCl was added and the solution was extracted with diethyl ether. The organic layer was washed with saturated NaHCO₃ and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc 5:1.

***N*-norarachidonylpropanamide (1a).** Colourless oil (65%). R_f=0.59 (PE:EtOAc 1:1); ¹H NMR: δ 0.89 (t, *J*=7.0, 3H), 1.15 (t, *J*=7.6, 3H), 1.26–1.40 (m, 6H), 1.59 (qn, *J*=7.2, 2H), 2.03–2.13 (m, 4H), 2.2 (q, *J*=7.6, 2H), 2.80–2.85 (m, 6H), 3.25–3.29 (m, 2H), 5.31–5.43 (m, 8H); ¹³C NMR: δ 9.9, 14.1, 22.6, 24.7, 25.7 (2C), 27.3, 29.3, 29.6, 29.8, 31.6, 39.2, 127.6, 127.8, 128.1, 128.3, 128.7 (2C), 129.1, 130.6, 173.6; ESI-MS

[M+H] 332.2; elemental analysis: calculated for $C_{22}H_{37}NO \cdot 1/3H_2O$: C 78.28%, H 11.25%, N 4.15%; found: C 78.30%, H 11.48%, N 4.07%.

***N*-norarachidonylbutanamide (1b)**. Colourless oil (52%). $R_f=0.60$ (PE:EtOAc 1:1); 1H NMR: δ 0.89 (t, $J=7.0$, 3H), 0.95 (t, $J=7.3$, 3H), 1.26–1.39 (m, 6H), 1.58 (qn, $J=7.3$, 2H), 1.66 (st, $J=7.4$, 2H), 2.03–2.15 (m, 6H), 2.80–2.58 (m, 6H), 3.25–3.29 (m, 2H), 5.31–5.43 (m, 8H), 5.51 (br s, 1H); ^{13}C NMR: δ 13.8, 14.1, 19.2, 22.6, 24.7, 25.6, 25.7, 27.3, 29.3, 29.6, 31.5, 38.8, 39.2, 127.6, 127.8, 128.1, 128.3, 128.7 (2C), 129.1, 130.5, 172.9; ESI-MS [M+H] 346.2; elemental analysis: calculated for $C_{23}H_{39}NO \cdot 1/2H_2O$: C 77.91%, H 11.37%, N 3.95%; found: C 77.50%, H 11.43%, N 3.99%.

3-Chloro-*N*-norarachidonylpropanamide (1d). Yellowish oil (43%). $R_f = 0.49$ (PE:EtOAc 1:1); 1H NMR: δ 0.89 (t, $J=6.9$, 3H), 1.26–1.39 (m, 6H), 1.61 (qn, $J=7.4$, 2H), 2.04–2.15 (m, 4H), 2.60 (t, $J=6.4$, 2H), 2.80–2.85 (m, 6H), 3.30 (q, $J=6.9$, 2H), 3.81 (t, $J=6.5$, 2H), 5.32–5.44 (m, 8H), 5.60 (bs, 1H); ^{13}C NMR: δ 14.1, 22.6, 24.6, 25.6, 25.7, 27.2, 29.3, 29.4, 31.5, 39.4, 39.8, 40.3, 127.5, 127.8, 128.1, 128.4, 128.7, 128.8, 129.0, 130.5, 169.3; ESI-MS [M+H⁺] 366.0.

***N*-norarachidonylcyclopropanecarboxamide (1e)**. Yellowish oil (99%). $R_f=0.15$ (PE:EtOAc 6:1); 1H NMR: δ 0.69–0.73 (m, 2H), 0.89 (t, $J=6.9$, 3H), 0.94–0.97 (m, 2H), 1.26–1.39 (m, 7H), 1.59 (qn, $J=7.4$, 2H), 2.03–2.14 (m, 4H), 2.79–2.87 (m, 6H), 3.28 (q, $J=7.0$, 2H), 5.31–5.43 (m, 8H), 5.68 (br s, 1H); ^{13}C NMR: δ 7.0, 14.1, 14.8, 22.6, 24.7, 25.6, 25.7, 27.2, 29.3, 29.6, 31.5, 39.4, 71.5, 127.5, 127.8, 128.2, 128.3, 128.6, 128.7, 129.1, 130.5, 173.4; ESI-MS [M+H⁺] 344.1; elemental analysis: calculated for $C_{23}H_{37}NO \cdot 1/10H_2O$: C 79.99%, H 10.86%, N 4.06%; found: C 79.83%, H 10.88%, N 4.11%.

Methyl 3-hydroxypropanoate (10). β -propiolactone (5.0 g, 69 mmol) was added dropwise into the precooled solution of NaOH (150 mg) in MeOH (50 mL). After the addition, stirring was continued on an ice-bath for 10 minutes. The solution was neutralized with 1 M HCl, and most of the solvent was evaporated. The remaining solution was extracted with EtOAc, the organic layers were combined, washed with brine, dried over Na_2SO_4 and evaporated. The crude product was purified by vacuum

distillation (12 mbar, 67-72 °C). ¹H NMR: δ 2.57–2.59 (m, 3H), 3.72 (s, 3H), 3.87 (q, *J*=5.9, 2H).

3-(*t*-butyldiphenylsilyloxy)propanoic acid (12). The mixture of methyl 3-hydroxypropanoate **10** (1 g, 9.6 mmol), *t*-butyldiphenylchlorosilane (3.2 g, 12 mmol) and imidazole (2.0 g, 29 mmol) in dry THF (18 mL) were stirred at RT for 3 hours. The reaction mixture was washed with saturated NaHCO₃, organic layers were combined, dried over Na₂SO₄ and evaporated. The crude product was purified by flash chromatography eluting with 2.5% EtOAc in PE. Evaporation of solvents yielded 2.78 g of the desired product (85%). *R*_f = 0.38 (PE:EtOAc 9:1). Methyl 3-(*t*-butyldiphenylsilyloxy)propanoate **11** was dissolved in MeOH/H₂O (100 mL/20 mL), the solution was cooled on an ice-bath and LiOH (1.0 g, 24 mmol) was added slowly. After the addition, stirring was continued at RT for 5 hours. The reaction mixture was poured into ice-cold water and extracted with diethyl ether and hexane (1:1). The water phase was acidified with 0.5 M KHSO₄ and extracted successfully with diethyl ether and hexane (1:1). The organic layers were combined, washed with water, dried over Na₂SO₄ and evaporated. The crude product was recrystallized from hexane. Yield 1.8 g (68%). *R*_f=0.14 (PE:EtOAc 9:1); ¹H NMR: δ 1.04 (s, 9H), 3.05 (t, *J*=5.9, 2H), 3.97 (t, *J*=5.9, 2H), 7.36–7.46 (m, 6H), 7.64–7.67 (m, 4H).

3-hydroxy-*N*-norarachidonylpropanamide (1c). 3-(*t*-Butyldiphenylsilyloxy)propanoic acid **12** was dissolved in benzene (12 mL) and oxalyl chloride (1.2 mL) and a few drops of DMF were added. The mixture was stirred at RT for 1.5 hours, after which unreacted oxalyl chloride and benzene were distilled off. Benzene (12 mL) was added and the distillation was repeated. 3-(*t*-Butyldiphenylsilyloxy)propanoyl chloride **13** (291 mg, 0.84 mmol) was added into the mixture of *t*-butyl norarachidonylcarbamate **5** (80 mg, 0.21 mmol), MeOH (0.018 mL, 0.42 mmol) and NaI (60 mg, 0.042 mmol) in dry ACN (2 mL). The reaction mixture was stirred for 0.5 hour. The yellow solution was cooled on an ice-bath and DIPEA (0.14 mL, 0.84 mmol) was added. Stirring was continued at RT for 1 hour. 10% HCl was added and the solution was extracted with diethyl ether. The organic layer was washed with saturated NaHCO₃ and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc 5:1. Evaporation of solvents yielded 110 mg of oily product (86%). *R*_f=0.22 (PE:EtOAc

6:1). 3-(*t*-butyldiphenylsilyloxy)-*N*-norarachidonylpropanamide (110 mg, 0.18 mmol) and TBAF (86 mg, 0.27 mmol) in dry THF (3 mL) were stirred at RT for 2 hours. The solvent was evaporated, and the crude product was purified by flash chromatography eluting 33–100% EtOAc in PE. Reaction proceeded quantitatively. $R_f=0.10$ (PE:EtOAc 1:1); $^1\text{H NMR}$: δ 0.89 (t, $J=7.0$, 3H), 1.26–1.39 (m, 6H), 1.60 (qn, $J=7.3$, 2H), 2.06 (q, $J=7.1$, 2H), 2.12 (q, $J=7.1$, 2H), 2.41 (t, $J=5.4$, 2H), 2.80–2.85 (m, 6H), 3.26–3.30 (m, 2H), 3.87 (t, $J=5.4$, 2H), 5.31–5.43 (m, 8H), 5.93 (br s, 1H); $^{13}\text{C NMR}$: δ 14.01, 22.6, 24.7, 25.6, 25.7, 27.2, 29.3, 29.5, 31.5, 38.0, 39.1, 59.0, 127.5, 127.8, 128.1, 128.4, 128.7, 128.8, 128.9, 130.6, 172.4; ESI-MS $[\text{M}+\text{H}]$ 348.1; elemental analysis: calculated for $\text{C}_{22}\text{H}_{37}\text{NO}_2 \cdot \frac{1}{2}\text{H}_2\text{O}$: C 74.11%, H 10.74%, N 3.93%; found: C 74.47%, H 10.77%, N 3.76%.

Arachidonyl amine (8). Methanesulfonyl chloride (0.16 mL, 2.1 mmol) was added slowly into the precooled solution of arachidonyl alcohol (400 mg, 1.4 mmol) in dry pyridine (8 mL). The reaction mixture was stirred on an ice-bath for 5 hours, after which the solution was poured into ice-cold water and extracted with diethyl ether. The organic layers were combined, washed with 0.5 M H_2SO_4 and saturated NaHCO_3 , dried over Na_2SO_4 and evaporated. The residue was dissolved in dry DMF (10 mL) and NaN_3 (450 mg, 6.9 mmol) in dry DMF (30 mL) was added dropwise. After the addition, the reaction mixture was stirred at 90 °C overnight. The solution was cooled to RT, poured into icecold water and extracted with diethyl ether. The organic layers were combined, dried over Na_2SO_4 and evaporated. The crude product was purified by flash chromatography eluting with 1% EtOAc in PE. The evaporation of solvents yielded 350 mg of colourless oil (80%). $^1\text{H NMR}$: δ 0.89 (t, $J=7.0$, 3H), 1.26–1.39 (m, 6H), 1.42–1.49 (m, 2H), 1.59–1.65 (m, 2H), 2.04–2.13 (m, 4H), 2.80–2.85 (m, 6H), 3.27 (t, $J=6.9$, 2H), 5.31–5.43 (m, 8H). Arachidonyl azide (**7**) (110 mg, 0.33 mmol) in diethyl ether (3 mL) was added to the stirred solution of LAH (13 mg, 0.33 mmol) in dry THF (0.35 mL). The reaction mixture was reflux for 4 hours, cooled to RT and diethyl ether was added. The mixture was filtered and evaporated. The crude product was purified by flash chromatography eluting with 10–100% MeOH in CH_2Cl_2 . Evaporation of solvents yielded 60 mg of oily product (64%). $^1\text{H NMR}$: δ 0.89 (t, $J=6.9$, 3H), 1.26–1.51 (m,

10H), 1.81 (br s, 2H), 2.07 (m, 4H), 2.69–2.72 (m, 2H), 2.80–2.84 (m, 6H), 5.31–5.43 (m, 8H).

General Synthesis Procedure for the Compounds 2a-b and 2d-e. Propionyl chloride (0.06 mL, 0.7 mmol) in dry CH₂Cl₂ (2 mL) was added into the precooled solution of arachidonyl amine (**8**) (100 mg, 0.35 mmol) and Et₃N (0.10 mL, 0.70 mmol) in dry CH₂Cl₂ (2 mL). After the addition, the cooling bath was removed and stirring was continued at RT for 2.5 hours. CH₂Cl₂ was added and the solution was washed with water and brine. The organic layers were combined, dried over Na₂SO₄ and evaporated. The crude product was purified by flash chromatography eluting with PE: EtOAc 4:1.

N-arachidonylpropanamide (2a). Yellowish oil (27%). ¹H NMR: δ 0.89 (t, *J*=6.9, 3H), 1.15 (t, *J*=7.6, 3H), 1.26–1.42 (m, 8H), 1.49–1.55 (m, 2H), 2.03–2.17 (m, 4H), 2.20 (q, *J*=7.5, 2H), 2.81–2.58 (m, 6H), 3.25 (t, *J*=7.0, 2H), 5.31–5.43 (m, 8H), 5.50 (s, 1H); ¹³C NMR: δ 10.0, 14.1, 22.6, 25.7, 26.9 (2C), 27.2, 29.3 (2C), 29.7, 29.8, 31.5, 39.4, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 173.8; ESI-MS [M+H⁺] 346.2; elemental analysis: calculated for C₂₃H₃₉NO·1/3H₂O: C 78.58%, H 11.37%, N 3.98%; found: C 78.17%, H 11.54%, N 3.70%.

N-arachidonylbutanamide (2b). Yellowish oil (83%). R_f=0.12 (PE:EtOAc 4:1); ¹H NMR: δ 0.89 (t, *J*=6.9, 3H), 0.95 (t, *J*=7.4, 3H), 1.26–1.43 (m, 8H), 1.49–1.55 (m, 2H), 1.66 (qn, *J*=7.4, 2H), 2.04–2.11 (m, 4H), 2.13 (t, *J*=7.5, 2H), 2.80–2.85 (m, 6H), 3.25 (q, *J*=7.1, 2H), 5.31–5.43 (m, 8H); ¹³C NMR: δ 13.8, 14.1, 19.2, 22.6, 25.7 (2C), 26.9 (2C), 27.2, 29.3, 29.4, 31.5, 38.8, 39.4, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 172.9; ESI-MS [M+H⁺] 360.2; elemental analysis: calculated for C₂₄H₄₁NO: C 80.16%, H 11.49%, N 3.90%; found: C 80.49%, H 11.77%, N 3.81%.

3-Chloro-N-arachidonylpropanamide (2d). Yellowish oil (69%). R_f=0.18 (PE:EtOAc 4:1); ¹H NMR: δ 0.89 (t, *J*=6.9, 3H), 1.26–1.44 (m, 8H), 1.54 (qn, *J*=7.2, 2H), 2.03–2.12 (m, 4H), 2.60 (t, *J*=6.6, 2H), 2.80–2.85 (m, 6H), 3.30 (q, *J*=7.1, 2H), 3.81 (t, *J*=6.4, 2H), 5.31–5.43 (m, 8H), 5.59 (br s, 1H); ¹³C NMR: δ 14.1, 22.6, 25.7, 26.8 (2C), 27.2, 29.2, 29.3, 31.5, 39.6, 39.8, 40.3, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.6, 130.5, 169.3; ESI-MS [M+H⁺] 380.0.

***N*-arachidonylcyclopropanecarboxamide (2e).** Yellowish oil (82%). $R_f=0.14$ (PE:EtOAc 4:1); $^1\text{H NMR}$: δ 0.69–0.73 (m, 2H), 0.89 (t, $J=6.8$, 3H), 0.94–0.97 (m, 2H), 1.26–1.44 (m, 9H), 1.49–1.56 (m, 2H), 2.04–2.12 (m, 4H), 2.80–2.87 (m, 6H), 3.27 (q, $J=6.9$, 2H), 5.31–5.43 (m, 8H), 5.58 (br s, 1H); $^{13}\text{C NMR}$: δ 7.0, 14.1, 14.8, 22.6, 25.7, 26.9 (2C), 27.2, 29.3, 29.4, 29.7, 31.5, 39.6, 127.6, 127.9, 128.2, 128.3 (2C), 128.6, 129.7, 130.5, 173.4; ESI-MS $[\text{M}+\text{H}^+]$ 358.1.

3-hydroxy-2-hydroxymethyl-*N*-arachidonylpropanamide (2c). Arachidonyl amine (**8**) (120 mg, 0.41 mmol), *cis/trans*-2-phenyl-1,3-dioxane-5-carboxylic acid **17** (130 mg, 0.62 mmol), EDCI (160 mg, 0.83 mmol) and DMAP (15 mg, 0.12 mmol) were dissolved in dry CH_2Cl_2 (8 mL) and stirred at RT for 20 hours. The solvent was evaporated and the residue was dissolved in EtOAc. The solution was washed with water and brine, dried over Na_2SO_4 and evaporated. The crude product was purified by flash chromatography eluting with PE:EtOAc 4:1. The evaporation of solvents yielded 50 mg of a white waxy product (25%). $^1\text{H NMR}$: δ 0.89 (t, $J=7.0$, 3H), 1.26–1.42 (m, 8H), 1.50–1.55 (m, 2H), 2.04–2.12 (m, 4H), 2.80–2.89 (m, 7 H), 3.25 (q, $J=7.3$, 2H), 4.13–4.17 (m, 2H), 4.31–4.34 (m, 2H), 5.31–5.43 (m, 8H), 5.51 (s, 1H), 5.53 (br s, 1H), 7.33–7.39 (m, 3H), 7.46–7.48 (m, 2H). $R_f=0.33$ (PE:EtOAc 6:1). Compound **9** was dissolved in concentrated HCl/MeOH (3 mL/7 mL) and the mixture was stirred at RT for 3 hours. The solvents were evaporated, and the crude product was purified with flash chromatography eluting with PE:EtOAc 1:2. Evaporation of solvents yielded 40 mg of a yellowish oil (98%). $R_f=0.2$ (CH_2Cl_2 :MeOH 9:1); $^1\text{H NMR}$: δ 0.89 (t, $J=6.8$, 3H), 1.26–1.43 (m, 8H), 1.54 (qn, $J=7.4$, 2H), 2.03–2.11 (m, 4H), 2.48–2.52 (m, 1H), 2.81–2.85 (m, 6H), 3.20 (s, 2H), 3.27 (q, $J=6.9$, 2H), 3.84–3.93 (m, 4H), 5.31–5.43 (m, 8H), 6.64 (s, 1H); $^{13}\text{H NMR}$: δ 14.1, 22.6, 25.7, 26.8, 26.9, 27.2, 29.1, 29.3, 29.7, 31.5, 39.3, 49.1, 62.0, 127.6, 127.9, 128.2, 128.3, 128.4, 128.6, 129.6, 130.5, 173.9; ESI-MS $[\text{M}+\text{H}^+]$ 392.2; elemental analysis: calculated for $\text{C}_{24}\text{H}_{41}\text{NO}_3 \cdot 1/10\text{H}_2\text{O}$: C 73.28%, H 10.56%, N 3.56%; found: C 73.24%, H 11.00%, N 3.24%.

3-hydroxy-*N*-arachidonylpropanamide (2f). Arachidonyl amine (**8**) (140 mg, 0.49 mmol) and 2 M $\text{Al}(\text{CH}_3)_3$ (in heptane, 0.25 mL, 0.49 mmol) were dissolved in dry CH_2Cl_2 (6 mL) and stirred at RT for 20 minutes. β -propiolactone (0.03 mL, 0.5 mmol) was added and the reaction mixture was refluxed for 4 hours. The solution was cooled

to RT, 1 M HCl was added and the mixture was extracted with CH₂Cl₂. The organic layers were combined, dried over Na₂SO₄ and evaporated. The crude product was purified by flash chromatography eluting with EtOAc. Evaporation of solvents yielded 70 mg of a colourless oil (40 %). ¹H NMR: δ 0.89 (t, *J*=6.9, 3H), 1.26–1.43 (m, 8H), 1.43 (qn, *J*=7.5, 2H), 2.04–2.12 (m, 4H), 2.42 (t, *J*=5.4, 2H), 2.80–2.85 (m, 6H), 3.27 (q, *J*=6.7, 2H), 3.88 (t, *J*=5.4, 2H), 5.31–5.43 (m, 8H), 5.80 (s, 1H); ¹³C NMR: δ 14.5, 23.0, 26.0, 27.2, 27.3, 27.6, 29.6, 29.7, 32.0, 38.3, 40.0, 59.4, 127.9, 128.3, 128.6, 128.6, 128.7, 129.0, 130.0, 131.0, 172.8; ESI-MS 362.2; elemental analysis: calculated for C₂₃H₃₉NO₂*1/3H₂O: C 75.16%, H 10.88%, N 3.81%; found: C 75.22%, H 10.99%, N 3.62%.

5.5.2 Enzymatic stability

Eight-week-old Wistar rats (200 g weight) were decapitated, whole brains without cerebellum were removed and homogenized in one times the volume (v/w) of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) with a Potter-Elvehjem homogenizer (Heidolph). The homogenate was centrifuged at 10 000 x *g* for 20 min at 4 °C and the resulting supernatant was used as a source of FAAH activity. The protein concentration of the supernatant (7.2 mg/mL) was determined by the method of Bradford with bovine serum albumin as a standard (Bradford 1976). Aliquots of the supernatant were stored at -70 °C until use. The experiments were initiated by a preincubation of a mixture (1380 μl, 37°C, 10 min) containing 929 μg of a rat brain homogenate, 81 mM potassium phosphate buffer (pH 7.4) and 0.5 % (w/v) BSA. The actual experiment were carried out by taking 1188 μl of the preincubated brain homogenate protein, adding 12 μl of studied compound (5 mM in ethanol, final volume 1200 μl), and incubating the mixture for 90 min at 37°C. The final incubation mixture contained 800 μg rat brain homogenate protein, 80 mM potassium phosphate buffer (pH 7.4), 0.5 % (w/v) BSA and 50 μM of studied compound. At time points 0 and 90 min, 300 μl samples were taken and 600 μl of cold acetonitrile was added in order to stop the enzymatic reaction. The samples were centrifuged at 23,700 *g* for 4 minutes at RT and the supernatant was analyzed for remaining compound by HPLC. Retention times for AEA, **1a**, **1c**, **1e**, **2b**,

2e, and **2f** were 4.5 min, 10.5 min, 4.9 min, 10.0 min, 17.2 min, 14.7 min, and 6.5 min, respectively.

5.5.3 *In vitro* assay for the CB2 activity

The CB2 receptor activities for the compounds **1a-e** (C₁₉ series) and **2a-f** (C₂₀ series) were determined at the human CB2 (hCB2) receptor expressed in Chinese hamster ovary (CHO) cells as previously described (Savinainen et al. 2005).

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6 SYNTHESIS AND CB1 RECEPTOR ACTIVITIES OF DIMETHYLHEPTYL DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL (2-AG) AND ARACHIDONYL GLYCERYL ETHER (2-AGE)*

6.1 Introduction

Our interest focused on the pentyl tail of an arachidonic backbone as a consequence of a factor analysis performed for 59 commercially available endocannabinoid-type compounds, and previous reports of significantly enhanced biological activity of AEA when the pentyl tail is replaced with a dimethylheptyl (DMH) structure (Ryan et al. 1997; Selzman et al. 1997). Unlike the DMH analogues of 2-AG and 2-AGE, the DMH derivatives of AEA have been studied extensively although it is widely recognized that 2-AG is the main endogenous ligand for the cannabinoid receptors (Sugiura et al. 1999; Gonsiorek et al. 2000; Sugiura et al. 2000; Savinainen et al. 2001, Savinainen et al. 2003). Based on the status of 2-AG in the ECS, and the knowledge of the SAR's of AEA we synthesized a DMH derivative of 2-AG (**1**) in order to explore whether the potency and efficacy of 2-AG could be increased. In addition, a DMH analogue of 2-AGE (**3**) was synthesized aiming at a potent and enzymatically more stable analogue for *in vitro* studies. Finally, the chain length effect on the activity properties (**1** vs **3**, **2** vs **4**) as well as the CB1 activity for a 2-AG derivative possessing more stable and more polar urea linkage (**5**) were studied.

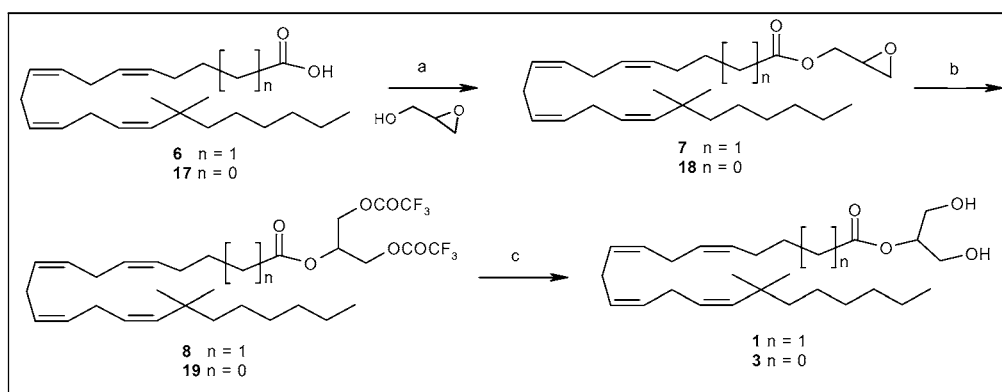
*Adapted from: Teija Parkkari, Outi M.H. Salo, Kristiina M. Huttunen, Juha R. Savinainen, Jarmo T. Laitinen, Antti Poso, Tapio Nevalainen, and Tomi Järvinen. Synthesis and CB1 receptor activities of dimethylheptyl derivatives of 2-arachidonoyl glycerol (2-AG) and arachidonyl glyceryl ether (2-AGE).

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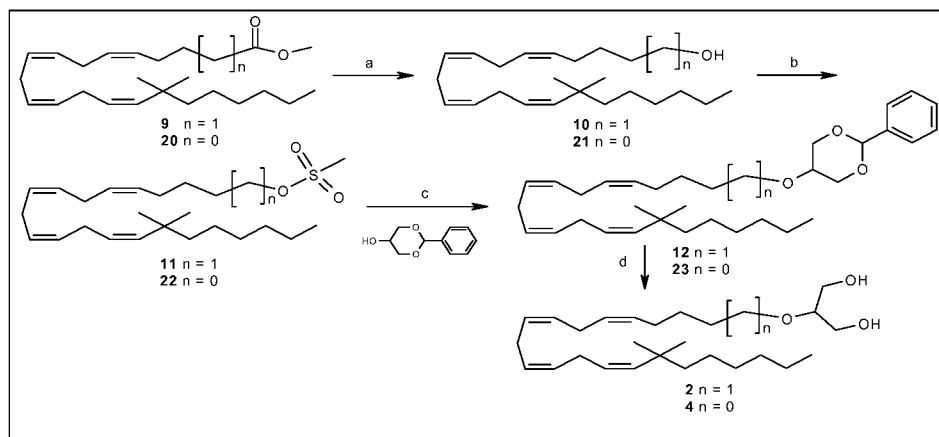
6.2 Synthesis

The synthesis route for the final products **1** and **3** is illustrated in Scheme 6.1. The key synthons **6** and **17** were prepared as described in the literature (Dasse et al. 2000). The synthesis methods for 2-AG and its analogues are limited since the ester bond of 2-AG is very easily isomerized from 2-AG to 1(3)-AG, and secondly, the double bond system is extremely sensitive to auto-oxidation. The final products **1** and **3** were prepared by a method recently developed by Stamatov and Stawinski (2002). The corresponding carboxylic acids **6** and **17** were esterified with (\pm)-glycidol in the presence of EDCI and DMAP to give the glycidyl derivatives **7** and **18**, which were treated with TFAA giving trifluoroacetate esters **8** and **19**. These were further converted into the desired products by transesterification using pyridine and methanol in CH_2Cl_2 -hexane.



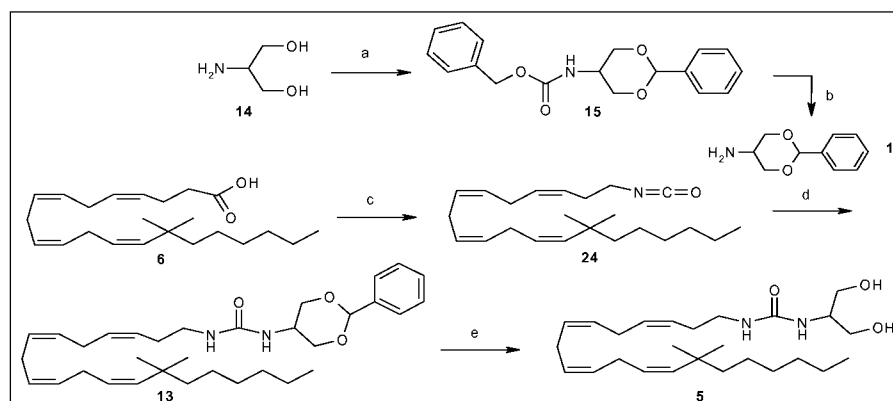
Scheme 6.1. (a) EDCI, DMAP, CH_2Cl_2 ; (b) TFAA, CH_2Cl_2 ; (c) pyridine, MeOH, CH_2Cl_2 /hexane.

Scheme 6.2 illustrates the synthesis method for the 2-AGE analogues **2** and **4**. The key synthons **9** and **20** were prepared as described in the literature (Dasse et al. 2000). In the first step corresponding ester (**9** or **20**) was reduced to alcohol with lithium aluminium hydride (LAH), and alcohol was activated by methanesulfonyl chloride (mesyl chloride, MsCl) prior the addition of 2-phenyl-1,3-dioxan-5-ol. Finally, the benzylidene protective group was cleaved by concentrated HCl in methanol (Mechoulam et al. 1999).



Scheme 6.2. (a) LAH, diethyl ether; (b) MsCl, Et₃N, CH₂Cl₂; (c) KOH, benzene; (d) HCl (aq)/MeOH.

The urea **5** was synthesized by converting the acid **6** to a corresponding azide with DPPA, and further rearranging the acyl azide to an isocyanate **24** by heating (Scheme 6.3) (Ng et al. 1999). 2-Phenyl-1,3-dioxan-5-amine **16** was produced by protecting an amino group of 2-amino-1,3-propanediol with benzyl chloroformate, which enabled a selective protection of hydroxyl groups with benzaldehyde (Wharton et al. 2002). The benzyl carbamate **15** was deprotected by catalytic hydrogenation at elevated pressure, then treated with isocyanate **24** (Ng et al. 1999). Finally, the protecting group of the 1,3-diol was removed with concentrated HCl in methanol.



Scheme 6.3. (a) 1. CbzCl, EtOH, Et₃N, 2. benzaldehyde, toluene, *p*-TSA; (b) Pd/C, H₂, 300 psi, 60°C; (c) 1. DPPA, Et₃N, benzene, 2. toluene, Δ; (d) toluene; (e) HCl/MeOH.

6.3 Factor analysis

The structures of 59 commercially available endocannabinoid-type compounds were examined. To select a representative group of molecules for the activity studies, a factor analysis was performed on the calculated steric and electrostatic fields of the molecules. The first three components were graphed and 11 molecules from the different areas of the field-property space were chosen to be tested for their CB1 receptor activity.

Compound **9** presented in Table 1.3 was found to be as effective and potent as 2-AG. Therefore, four additional molecules were chosen from the structural space surrounding that compound and also tested for their CB1 receptor activity. Another molecule with a similar DMH side chain to that of **9** was found to have a comparable potency and efficacy with **9** and 2-AG. Consequently, we focused on the design and synthesis of new endocannabinoid derivatives with a DMH side chain in order to evaluate structure-activity relationships and to discover novel active CB1 lead structures.

6.4 Results and discussion

The CB1 activation data for 2-AG, 2-AGE and compounds **1-5** is summarized in Table 6.1. All the synthesized compounds showed dose-dependent CB1 activity, and their responses at 5×10^{-5} M were reversed by the CB1 receptor antagonist, AM251 (10^{-6} M, data not shown).

Table 6.1. Comparison of efficacy (E_{\max}) and potency ($-\log EC_{50}$) values of 2-AG, 2-AGE and **1-5**, $n=3$.

| Compound | CB1 activation | | |
|--------------|-------------------------------|--------------------------|----------------------|
| | E_{\max} (%basal \pm SEM) | $-\log EC_{50} \pm$ SEM) | EC_{50} (μ M) |
| 2-AG | 620 \pm 5 | 6.0 \pm 0.0 | 1.0 |
| 2-AGE | 484 \pm 7 | 5.2 \pm 0.0 | 6.3 |
| 1 | 521 \pm 35 | 4.6 \pm 0.1 | 25 |
| 2 | 475 \pm 2 | 4.6 \pm 0.0 | 25 |
| 3 | 590 \pm 76 | 4.3 \pm 0.1 | 50 |
| 4 | 548 \pm 27 | 4.5 \pm 0.1 | 32 |
| 5 | 309 \pm 8 ($n=2$) | 4.0 \pm 0.1 ($n=2$) | 100 |

Surprisingly, the DMH derivatives of 2-AG (**1** and **3**) acted as much weaker CB1 receptor agonists than 2-AG. Although good efficacy values were achieved, potency values remained relatively poor. It was also interesting to notice that the derivative having a shorter chain length (**3**) was more efficient than compound **1** having the structure closer to 2-AG. 2-AGE derivatives **2** and **4** also acted as very low potency agonists for the CB1 receptor, however, they were equally or even more efficacious than 2-AGE. In this series, a similar effect of the chain length on the CB1 receptor activities was observed; the shorter chain length leads to higher efficacy values. It seems that the ligand-receptor interactions of ester derivatives of endocannabinoids are substantially more sensitive to the structural changes than the amide derivatives. This is in good agreement with previous findings of Suhara et al. (2001) who synthesized metabolically stable analogues of 2-AG and determined their CB1 receptor activities by measuring the ability of the compounds to induce Ca^{2+} transients in NG108-15 cells. Few of their compounds showed comparable activity to that of 2-AGE, however, their agonistic activities were approximately 100 times lower than that of 2-AG. Razdan and co-workers published results on carbamate and urea analogues of endocannabinoids aiming at achieving improved enzymatic stability (Ng et al. 1999). In that series, the carbamate derivatives showed only weak activity properties, whereas some urea derivatives were clearly more potent than AEA both *in vitro* and *in vivo*. One important finding of this study was that the enzymatic stability can indeed be increased by substituting the amide bond with the urea bond. Interestingly, a compound where the amide bond of AEA was replaced for urea bond showed only weak binding affinity ($K_i = 347 \pm 33$ nM) but in several pharmacological *in vivo* studies (tail flick test, spontaneous activity, immobility) it was more potent than AEA or the other urea derivatives synthesized. The authors were not able to explain this observation but it resembles data published on 2-AG (Sugiura et al. 1995; Sugiura et al. 1999; Hillard 2000; Savinainen et al. 2001). In our DMH series, the addition of the urea bond (**5**) decreased the activation parameters dramatically. The weak activity properties of compound **5** are most likely a result of its hydrophilic structure; in the flash chromatography, the compound requires clearly more polar solvents for elution, and the state of the product is more like a wax than an oil. In addition, the unusually sharp urea

and hydroxyl group peaks in the ^1H NMR spectrum may refer to the strong hydrogen bonding which may affect the activation properties.

6.5 Conclusions

In the present study, DMH derivatives of 2-AG and 2-AGE have been synthesized and their ability to activate the CB1 receptor have been determined by the $[\text{}^{35}\text{S}]\text{GTP}_\gamma\text{S}$ binding assay using the rat cerebellar membranes. The DMH modification for 2-AG led to a dramatic potency decrease, while the impact on the efficacy was much weaker. A similar modification for 2-AGE, resulted in a loss of potency, whereas the efficacy remained comparable to 2-AGE. Shortening of the chain length did not improve the potency values but, interestingly, led to agonists with increased efficacy. Introducing a more hydrophilic and stable urea bond produced only weak agonistic activity. Based on these results, it can be concluded that unlike AEA-type compounds and classical cannabinoids, the functional properties of 2-AG and 2-AGE, cannot be improved by the replacement of the end pentyl chain with the DMH structure.

6.6 Experimental

6.6.1 Synthesis

Method A: EDCI coupling. The corresponding acid (**6** or **17**) (1 eq), glycidol (1.2 eq), EDCI (1.8 eq) and DMAP (0.3 eq) in dry CH_2Cl_2 (15 mL/mmol) were stirred at room temperature for 20 h. The solvent was evaporated under reduced pressure.

16,16-Dimethyl-docosa-all-cis-5,8,11,14-tetraenoic acid oxiranylmethyl ester (7). The crude product was purified by flash chromatography eluting with PE:EtOAc 8:1. ^1H NMR: δ 0.88 (t, 3H, $J=7.0$), 1.09 (s, 6H), 1.26-1.38 (m, 10H), 1.73 (qn, 2H, $J=7.5$), 2.00-2.17 (m, 3H), 2.34-2.39 (m, 2H), 2.65 (dd, 1H, $^2J=4.8$, $J=2.6$), 2.79-2.86 (m, 4H), 2.88-2.95 (m, 2H), 3.19-3.22 (m, 1H), 3.92 (dd, 1H, $^2J=12.3$, $J=6.3$), 4.41 (dd, 1H, $^2J=12.3$, $J=3.1$), 5.13-5.27 (m, 2H), 5.31-5.43 (6H).

15,15-Dimethyl-henicoso-all-cis-4,7,10,13-tetraenoic acid oxiranylmethyl ester (18). The crude product was purified by flash chromatography eluting with PE:EtOAc; 10:1 yielding a colourless oil (52%). ^1H NMR: δ 0.88 (t, 3H, $J=7.0$), 1.09 (s, 6H), 1.26-

1.36 (m, 10H), 2.36-2.42 (m, 4H), 2.64 (dd, 1H, $^2J=4.9$, $J=2.6$), 2.82-2.95 (m, 7H), 3.19-3.22 (m, 1H), 3.92 (dd, 1H, $^2J=12.3$, $J=6.1$), 4.41 (dd, 1H, $^2J=12.3$, $J=3.1$), 5.14-5.45 (m, 8H).

Method B: TFAA promoted stereoselective transformation of glycidyl ester. The corresponding oxiranylmethyl ester (**7** or **18**) (1 eq) in dry CH_2Cl_2 (5 mL/mmol) was cooled to -20°C and TFAA (6 eq) in dry CH_2Cl_2 (5 mL/mmol) was added. The cooling bath was removed, and the mixture was allowed to warm to room temperature. After 2 hours, the reaction was stopped by evaporating the solvent under vacuum. The residue was purified by flash chromatography eluting with toluene.

1,3-Bis-(trifluoroacetyl)-2-(16,16-dimethyl-docosa-all-cis-5,8,11,14-tetraenoic acid) propyl ester (8). Yield 83%. ^1H NMR: δ 0.88 (t, 3H, $J=6.9$), 1.09 (s, 6H), 1.25-1.38 (m, 10H), 1.70 (qn, 2H, $J=7.5$), 1.98-2.24 (m, 3H), 2.33-2.38 (m, 2H), 2.79-2.95 (m, 5H), 4.46 (dd, 2H, $^2J=11.9$, $J=5.5$), 4.63 (dd, 2H, $^2J=12.0$, $J=4.3$), 5.13-5.27 (m, 2H), 5.32-5.44 (m, 7H).

1,3-Bis-(trifluoroacetyl)-2-(15,15-dimethyl-henicoso-all-cis-4,7,10,13-tetraenoic acid) propyl ester (19). Light yellow oil; yield 81%. ^1H NMR: δ 0.88 (t, 3H, $J=6.9$), 1.09 (s, 6H), 1.23-1.34 (m, 10H), 2.33-2.45 (m, 4H), 2.81-2.95 (m, 6H), 4.46 (dd, 2H, $^2J=11.9$, $J=5.4$), 4.63 (dd, 2H, $^2J=11.9$, $J=4.2$), 5.13-5.46 (m, 9H).

Method C: Cleavage of trifluoroacetyl groups. The corresponding trifluoroacetyl propyl ester (**8** or **19**) (1 eq) was dissolved in dry hexane/ CH_2Cl_2 (2/1, 15 mL/mmol) solution and the mixture was cooled to -20°C . Dry pyridine (12.5 eq) and methanol (18.5 eq) in dry hexane/ CH_2Cl_2 solution (2/1, 15 mL/mmol) were added slowly into the reaction mixture. The cooling bath was removed and the solution was stirred at room temperature for 2 h. The reaction was stopped by evaporating solvents under reduced pressure without a heating bath.

1,3-Bis-(hydroxy)-2-(16,16-dimethyl-docosa-all-cis-5,8,11,14-tetraenoic acid) propyl ester (1). ^1H NMR: δ 0.88 (t, 3H, $J=6.8$), 1.09 (s, 6H), 1.23-1.36 (m, 10H), 2.04-2.14 (br s, 2H), 2.36-2.53 (m, 4H), 2.82-2.96 (m, 6H), 3.82 (d, 4H, $J=4.7$), 4.93 (qn, 1H, $J=4.7$), 5.13-5.46 (m, 8H); ^{13}C NMR: δ 14.1, 17.6, 22.7, 24.8 (2C), 25.6, 25.7, 26.5, 26.8, 29.0, 30.2, 31.9, 33.7, 44.3 (2C), 62.6 (2C), 75.2, 127.0, 127.8, 128.1, 128.3,

128.8, 129.1 (2C), 139.22, 173.3; elemental analysis: calculated for $C_{27}H_{46}O_4 \cdot 1/4 H_2O$: C 73.84%, H 10.62%; found: C 73.50%, H 10.80%.

1,3-Bis-(hydroxy)-2-(15,15-dimethyl-henicoso-all-cis-4,7,10,13-tetraenoic acid) propyl ester (3). Yellowish oil; yield 95%. 1H NMR: δ 0.88 (t, 3H, $J=6.8$), 1.09 (s, 6H), 1.23-1.36 (m, 10H), 2.04-2.14 (br s, 2H), 2.36-2.53 (m, 4H), 2.82-2.96 (m, 6H), 3.82 (d, 4H, $J=4.7$), 4.93 (qn, 1H, $J=4.7$), 5.13-5.46 (m, 8H); ^{13}C NMR: δ 14.1, 22.7, 22.8, 24.8, 25.6, 25.7, 26.7, 29.0 (2C), 30.2, 31.9, 34.2, 36.4, 44.3, 62.5 (2C), 75.3, 127.0, 127.7, 127.8, 127.9, 128.5, 129.1, 129.7, 139.2, 173.3; GC-MS(EI): $m/z=565$; elemental analysis: calculated for $C_{26}H_{44}O_4 \cdot 1/7 H_2O$: C 73.79%, H 10.55%; found: C 73.77% H 10.82%.

Method D: $LiAlH_4$ reduction and alcohol activation with methanesulfonyl chloride. $LiAlH_4$ (1 eq) was added in several portions into dry THF (1.7 mL/mmol), and the solution was cooled to $0^\circ C$. Corresponding ester (**9** or **20**) (1 eq) in dry diethyl ether (1.7 mL/mmol) was added slowly into the reaction mixture, and stirring was continued overnight at room temperature. The solution was cooled to $0^\circ C$ and quenched sequentially with water, 15% NaOH solution and water. The cooling bath was removed, and the mixture was allowed to precipitate at room temperature for half an hour. The solid was filtered and washed with diethyl ether. The aqueous layer was separated from the filtrate and extracted several times with diethyl ether. The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated under reduced pressure to obtain a yellowish, oily alcohol. Alcohol (**10** or **21**) (1 eq) and triethylamine (2.7 eq) were dissolved in dry CH_2Cl_2 (10 mL/mmol). The reaction mixture was cooled to $0^\circ C$ and methanesulfonyl chloride (1.3 eq) was added to the mixture. The cooling bath was removed and the mixture was stirred for 2 hours at room temperature. The reaction mixture was quenched with cold water and the separated aqueous layer was extracted with ether. The combined organic layers were acidified with 0.5 M H_2SO_4 , washed with saturated $NaHCO_3$ and finally dried over $MgSO_4$. The solvent was evaporated under vacuum. The product was used in the next reaction without further purification.

Methanesulfonic acid 16,16-dimethyl-docosa-all-cis-5,8,11,14-tetraenyl ester (11). 1H NMR: δ 0.88 (t, 3H, $J=6.9$), 1.10 (s, 6H), 1.22-1.43 (m, 10H), 1.45-1.57 (m, 2H),

1.72-1.80 (m, 2H), 2.02-2.24 (m, 3H), 2.80-2.95 (m, 5H), 3.00 (s, 3H), 4.23 (t, 2H, $J=6.5$), 5.13-5.43 (m, 8H).

Methanesulfonic acid 15,15-dimethyl-henicoso-all-*cis*-4,7,10,13-tetraenyl ester (22). ^1H NMR: δ 0.88 (t, 3H, $J=6.9$), 1.10 (s, 6H), 1.24-1.30 (m, 10H), 1.83 (qn, 2H, $J=6.9$), 2.14-2.23 (m, 2H), 2.80-2.95 (m, 6H), 3.00 (s, 3H), 4.23 (t, 2H, $J=6.5$), 5.13-5.47 (m, 8H).

Method E: Addition of 5-hydroxy-2-phenyl-1,3-dioxane. KOH (12 eq) and 5-hydroxy-2-phenyl-1,3-dioxane (12 eq) were crushed together and dissolved in dry benzene (50 mL/mmol). The mixture was stirred at 50°C for half an hour. The corresponding alcohol activated with methanesulfonyl group (**11** or **22**) (1 eq) in dry benzene (50 mL/mmol) was added slowly to the reaction mixture and the solution was stirred at 50°C for 3 days. Another portion of crushed KOH (6 eq) and 5-hydroxy-2-phenyl-1,3-dioxane (6 eq) was added to the reaction mixture and stirring was continued overnight at 50°C. After the heating bath was removed, the mixture was diluted with ether and acidified with 10% HCl to pH 1. The separated aqueous layer was extracted several times with diethyl ether, and the combined organic layers were washed with brine and dried over MgSO_4 . The solvent was evaporated under vacuum.

5-(16,16-Dimethyl-docosa-all-*cis*-5,8,11,14-tetraenyloxy)-2-phenyl-1,3-dioxane (12). The crude product was purified by flash chromatography eluting with 10-20% diethyl ether in PE yielding a colorless oil (60%). ^1H NMR: δ 0.88 (t, 3H, $J=6.7$), 1.09 (s, 6H), 1.26-1.40 (m, 10H), 1.43-1.52 (m, 2H), 1.64-1.71 (m, 2H), 1.97-2.17 (m, 3H), 2.80-2.98 (m, 5H), 3.25-3.26 (m, 1H), 3.56 (t, 2H, $J=6.6$) 4.04 (dd, 2H, $^2J=12.6$, $J=1.7$), 4.33 (dd, 2H, $^2J=12.5$, $J=1.4$), 5.13-5.43 (m, 8H), 5.55 (s, 1H), 7.30-7.36 (m, 3H), 7.49-7.52 (m, 2H).

5-(15,15-Dimethyl-henicoso-all-*cis*-4,7,10,13-tetraenyloxy)-2-phenyl-1,3-dioxane (23). The crude product was purified by flash chromatography eluting with 10% diethyl ether in PE yielding a yellow oil (50%). ^1H NMR: δ 0.88 (t, 3H, $J=6.8$), 1.10 (s, 6H), 1.24-1.32 (m, 10H), 1.74 (qn, 2H, $J=7.0$), 2.13-2.21 (m, 2H), 2.79-2.94 (m, 6H), 3.24-3.27 (m, 1H), 3.56 (t, 2H, $J=6.6$) 4.04 (dd, 2H, $^2J=12.4$, $J=1.8$), 4.33 (dd, 2H, $^2J=12.4$, $J=1.5$), 5.13-5.45 (m, 8H), 5.55 (s, 1H), 7.30-7.36 (m, 3H), 7.47-7.52 (m, 2H).

Method F: Deprotection of a diol. Corresponding 2-phenyl-1,3-dioxane derivative (**12** or **23**) was dissolved in solution of concentrated HCl/MeOH (1/2) and stirred at room temperature for 3 hours. MeOH was evaporated under reduced pressure, and the residue was diluted with ethyl acetate. The separated organic phase was washed with 5% NaHCO₃ followed by brine and finally dried over MgSO₄. The solvent was evaporated under reduced pressure.

2-(16,16-Dimethyl-docosa-all-cis-5,8,11,14-tetraenyloxy)-propane-1,3-diol (2).

The crude product was purified by flash chromatography eluting with 30-50% EtOAc in PE yielding a yellowish oil (68%). ¹H NMR: δ 0.88 (t, 3H, *J*=6.8), 1.10 (s, 6H), 1.26-1.39 (m, 10H), 1.44 (qn, 2H, *J*=7.6), 1.59-1.66 (m, 2H), 1.93 (bs, 2H), 2.02-2.21 (m, 3H), 2.74-2.95 (m, 5H), 3.46 (qn, 1H, *J*=4.8), 3.58 (t, 2H, *J*=6.6) 3.68 (dd, 2H, ²*J*=11.5, *J*=5.1), 3.76 (dd, 2H, ²*J*=11.6, *J*=4.4), 5.13-5.42 (m, 8H); ¹³C NMR: δ 14.1, 22.7, 24.8, 25.7 (2C), 26.2, 26.7, 27.0 (2C), 29.0, 29.7, 30.2, 31.9, 36.4, 44.3 (2C), 62.3, 70.0, 79.6, 127.0, 127.9, 128.1, 128.2, 128.3, 129.0, 129.8, 139.2; elemental analysis: calculated for C₂₇H₄₈O₃·1/10 H₂O: C 76.76%, H 11.50%; found: C 76.62%, H 11.80%.

2-(15,15-Dimethyl-henicoso-all-cis-4,7,10,13-tetraenyloxy)-propane-1,3-diol (4).

The crude product was purified by flash chromatography eluting with EtOAc:PE; 1:1 solution yielding a brownish oil (37%). ¹H NMR: δ 0.88 (t, 3H, *J*=6.9), 1.10 (s, 6H), 1.24-1.32 (m, 10H), 1.69 (qn, 2H, *J*=6.8), 2.01-2.30 (m, 4H), 2.78-2.96 (m, 6H), 3.46 (qn, 1H, *J*=4.7), 3.59 (t, 2H, *J*=6.6) 3.69 (dd, 2H, ²*J*=11.6, *J*=5.0), 3.77 (dd, 2H, ²*J*=11.6, *J*=4.4), 5.12-5.43 (m, 8H); ¹³C NMR: δ 14.1, 22.7, 23.8, 24.8, 25.6, 25.7, 26.8, 29.0 (2C), 29.3, 29.9, 30.2, 31.9, 44.3, 62.3 (2C), 69.5, 79.6, 127.0, 127.8, 128.2, 128.2, 128.6, 129.1, 129.3, 139.2; elemental analysis: calculated for C₂₆H₄₆O₃·¼ H₂O: C 75.95%, H 11.40%; found: C 75.87%, H 11.36%.

Benzyl (2-phenyl-1,3-dioxan-5-yl)carbamate (15). 2-Amino-1,3-propanediol **14** (1.0 g, 11 mmol) and Et₃N (2.3 mL, 17 mmol) were dissolved in dry EtOH (40 mL). The reaction mixture was cooled to 0°C and benzyl chloroformate (4.7 mL, 33 mmol) was added slowly to the mixture. The cooling bath was removed and the mixture was stirred for 1 hour at room temperature. The reaction was stopped by evaporating the solvent under vacuum, and the residue was purified by flash chromatography eluting with 2% MeOH in CH₂Cl₂ to yield benzyl (1,3-dihydroxypropyl)carbamate as a white

solid compound (2.0 g, 81%). $^1\text{H NMR}$: δ 3.36-3.46 (m, 5H), 4.53 (t, 2H, $J=5.5$), 5.01 (s, 2H), 6.79 (d, 1H, $J=7.8$), 7.28-7.36 (m, 5H). Benzyl (1,3-dihydroxypropyl)carbamate (2.0 g, 8.9 mmol), benzaldehyde (1.2 mL, 12 mmol) and *p*-TSA (20 mg, 0.1 mmol) in dry toluene (40 mL) were refluxed under a Dean-Stark water separator for 2 hours. After enough water (0.16 mL) was separated from the reaction mixture, the mixture was allowed to cool to room temperature and the solvent was evaporated under reduced pressure. The product was purified by flash chromatography eluting with EtOAc:PE; 1:5 solution to yield a mixture of the *cis* and *trans* isomers as a white solid product (2.0 g, 72%). $^1\text{H NMR}$: (*trans*) δ 3.56-3.60 (m, 2H ax), 4.07 (bs, 1H), 4.34 (dd, 2H eq, $^2J=10.9$, $J=4.8$), 4.60 (bs, 1H), 5.13 (s, 2H), 5.43 (s, 1H), 7.32-7.47 (m, 10H). $^1\text{H NMR}$: (*cis*) δ 3.74 (d, 1H, $J=8.9$ Hz), 4.13-4.18 (m, 4H), 5.13 (s, 2H), 5.54 (s, 1H), 5.81 (d, 1H, $J=9.5$), 7.30-7.47 (m, 10H).

2-Phenyl-1,3-dioxan-5-amine (16). Benzyl (2-phenyl-1,3-dioxan-5-yl)carbamate **15** (2.0 g, 6.4 mmol) was dissolved in dry EtOH (150 mL) and 10% Pd/C (0.1 g) was added. The reaction mixture was placed in a high-pressure hydrogenation reactor (300 psi, 60°C). After 3 hours, the mixture was filtered through a pad of Celite and the solvent was evaporated under vacuum. The product was purified by flash chromatography eluting with 5% MeOH in CH_2Cl_2 to obtain a white solid amine containing both isomers. (1.0 g, 92%). $^1\text{H NMR}$: (*trans*) δ 1.01 (br s, 2H), 3.21 (m, 1H, $J=5.1$), 3.43-3.48 (m, 2H ax), 4.28 (dd, 2H, eq, $^2J=11.3$, $J=4.8$), 5.39 (s, 1H), 7.32-7.49 (m, 5H). $^1\text{H NMR}$: (*cis*) δ 1.79 (s, 2H), 2.81 (qn, 1H, $J=1.8$), 4.05 (dq, 2H ax, $^2J=11.7$, $J=1.5$), 4.17 (dq, 2H, eq, $^2J=11.7$, $J=1.6$), 5.52 (s, 1H), 7.33-7.51 (m, 5H).

1-(14,14-Dimethyl-icosa-all-*cis*-3,6,9,12-tetraenyl)-3-(2-phenyl-1,3-dioxan-5-yl)urea (13). 15,15-Dimethyl-henicoso-all-*cis*-4,7,10,13-tetraenoic acid **6** (0.2 g, 0.6 mmol) and Et_3N (0.12 mL, 0.87 mmol) were stirred in dry benzene (3 mL) at RT for 10 minutes. DPPA (0.2 g, 0.9 mmol) was added slowly and the mixture was stirred for 2 hours. The solvent was evaporated under vacuum and the product was purified by flash chromatography eluting with 5% EtOAc in PE to yield a yellowish oil (0.11 g, 51%). 15,15-Dimethyl-henicoso-all-*cis*-4,7,10,13-tetraenyl azide (0.1 g, 0.3 mmol) was dissolved in dry toluene (2 mL) and stirred at 65 °C for 2 hours. 2-Phenyl-1,3-dioxan-5-

amine **16** (60 mg, 0.3 mmol, *cis*-isomer) in dry toluene (2 mL) was added to the reaction mixture and the stirring was continued at 65 °C for another 2 hours. The reaction was stopped by evaporating the solvent under reduced pressure to obtain an impure whitish waxy urea (190 mg). ¹H NMR: δ 0.88 (t, 3H, *J*=6.8), 1.09 (s, 6H), 1.25-1.38 (m, 10H), 2.10-2.40 (m, 4H), 2.74-2.95 (m, 6H), 3.23 (qn, 1H, *J*=6.1), 3.89 (d, 1H, *J*=9.1), 4.03 (d, 1H, *J*=11.5), 4.11-4.18 (m, 4H), 5.12-5.51 (m, 8H), 5.53 (s, 1H), 7.34-7.51 (m, 5H).

1-(14,14-Dimethyl-icosa-all-*cis*-3,6,9,12-tetraenyl)-3-(2-hydroxy-1-hydroxymethylethyl)-urea (5). 1-(14,14-Dimethyl-icosa-all-*cis*-4,7,10,13-tetraenyl)-3-(2-phenyl-1,3-dioxan-5-yl)-urea **13** (190 mg, 0.4 mmol) was dissolved in a solution of concentrated HCl/MeOH (11 mL/25 mL) and stirred at room temperature for 4 hours. MeOH was evaporated under reduced pressure and the residue was diluted with EtOAc. The separated organic phase was washed with brine and dried over MgSO₄. The solvent was evaporated and the product was purified by flash chromatography eluting with 1% MeOH in CH₂Cl₂ to yield a whitish waxy product (38 mg, 46%). ¹H NMR: δ 0.88 (t, 3H, *J*=6.9 Hz), 1.09 (s, 6H), 1.22-1.35 (m, 10H), 2.05-2.29 (m, 4H), 2.75-2.94 (m, 6H), 3.18 (qn, 1H, *J*=6.9 Hz), 3.65-3.78 (m, 5H), 4.04 (br s, 1H), 5.12-5.55 (m, 10H); ¹³C NMR: δ 14.1, 22.7, 24.8, 25.7, 26.8 (2C), 27.9, 29.0 (2C), 30.2, 31.9, 36.4, 40.2, 44.4, 53.4, 63.4 (2C), 126.5, 126.9, 127.7, 128.0, 128.5, 129.2, 130.5, 139.3, 159.3; ESI-MS 435.3 [M+H]; elemental analysis: calculated for C₂₆H₄₆N₂O₃·1/3 H₂O: C 70.87%, H 10.67%, N 6.36%; found: C 70.91%, H 10.52%, N 6.00%.

6.6.2 Factor analysis

All computations were carried out on SGI O2 R5000 or R12000 workstations. Ligand structures were constructed using the molecular modeling package SYBYL 6.7. The applied force field was Tripos force field (Clark et al. 1989), and the atomic partial charges for the ligands were calculated by the Gasteiger-Hückel method (Gasteiger et al. 1980). The crystallographic template chosen for building the ligands was taken from the Protein Data Bank: (Berman et al. 2000) arachidonic acid complexed with adipocyte lipid-binding protein (PDB 1adl) (LaLonde et al. 1994). After a light geometry optimization, all the constructed ligands were aligned according to the template and the

factor analysis of the electrostatic and steric fields of the compounds was performed as implemented in SYBYL 6.7.

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7 α -METHYLATED DERIVATIVES OF 2-ARACHIDONOYL GLYCEROL: SYNTHESIS, CB1 RECEPTOR ACTIVITY AND ENZYMATIC STABILITY*

7.1 Introduction

2-AG has been reported to be a full efficacy agonist and probably the main endogenous ligand for both the CB1 and CB2 receptors (Sugiura et al. 1999; Gonsiorek et al. 2000; Sugiura et al. 2000; Savinainen et al. 2001, Savinainen et al. 2003). In spite of the good cannabinergic properties of 2-AG, its poor low enzymatic stability places restrictions on its utilization. This fact represented the starting point for this study, where 2-AG was methylated at its α -position in order to reduce its metabolism to arachidonic acid (AA), and thus, prolong its duration of action. Finally, it was determined if the activity properties of 2-AG could be improved by replacing the head hydroxyl group(s) by fluorine as is the case with AEA (Adams et al. 1995; Lin et al. 1998). The synthesized compounds were evaluated for their CB1 activity using the [35 S]GTP $_{\gamma}$ S binding assay, and their stability was tested in rat cerebellar membranes and in membrane-free buffer solution.

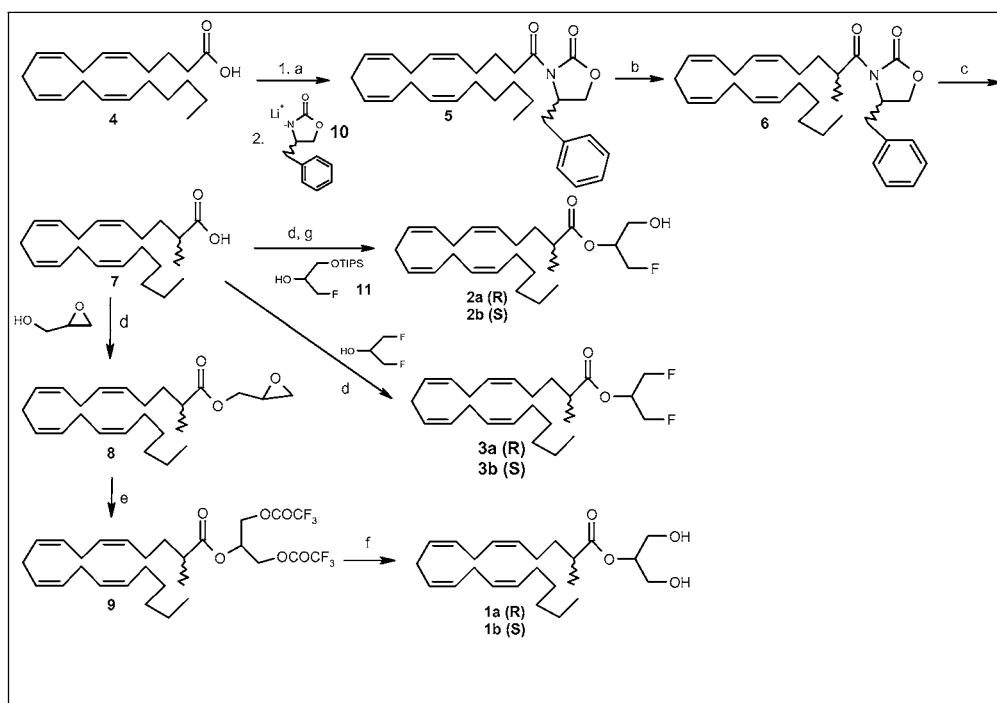
*Adapted from: Teija Parkkari, Mikko Myllymäki, Juha R. Savinainen, Susanna M. Saario, Joel A. Castillo-Meléndez, Jarmo T. Laitinen, Tapio Nevalainen, Ari M.P. Koskinen, and Tomi Järvinen. α -Methylated derivatives of 2-arachidonoyl glycerol: Synthesis, CB1 receptor activity and enzymatic stability.

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7.2 Synthesis

A stereoselective synthesis method was utilized to obtain final products **1-3**, and Evans's chiral auxiliary was used for the purpose (Scheme 7.1). The key synthon **7** was prepared as previously described (Goutopoulos et al. 2001). The final products **1a-b** were finally produced by the method of Stamatov and Stawinski (2002) described in the chapter 6.2. The α -methylated monofluoro derivatives of 2-AG, **2a** (*R*) and **2b** (*S*), and the difluoro derivatives **3a** (*R*) and **3b** (*S*) were prepared by coupling (*S*)- or (*R*)-2-methylarachidonic acid **7** with (*rac*)-1-fluoro-3-(triisopropyl)silyloxy-2-propanol and 1,3-difluoro-2-propanol using EDCI/DMAP. Finally, the TIPS protective groups were removed by adding an excess of TBAF.



Scheme 7.1. (a) pivaloyl chloride, Et₃N, THF; (b) 1. NaHMDS, THF, -78 °C, 2. MeI; (c) LiOOH, THF; (d) EDCI or DCC, DMAP, CH₂Cl₂; (e) TFAA, CH₂Cl₂; (f) pyridine, MeOH, CH₂Cl₂/ hexane; (g) TBAF, THF.

7.3 Results and discussion

As the data presented in the Table 7.1 indicates, only the compounds **1a-b** exhibited appreciable dose-dependent CB1 receptor activity. The (*R*) enantiomer (**1a**) gave significantly better CB1 activity than the (*S*) enantiomer (**1b**) which is a rather interesting finding since it has been reported that stereochemistry at the α -position in anandamide is not very critical when affinity for the CB1 receptor is being measured (Goutopoulos et al. 2001). Weak potency and efficacy values were observed for the monofluoro derivative **2a**, however, with the (*S*) enantiomer no detectable CB1 activation at 10^{-4} M concentration was observed. The replacement of both hydroxyl groups with fluorine (**3a**) led to a loss of CB1 activity. Since the (*R*) enantiomer was proven to be inactive, the activity of the (*S*) form (**3b**) was not determined.

Table 7.1. Comparison of efficacy (E_{\max}) and potency ($-\log EC_{50}$) values of 2-AG and compounds **1a-b**, **2-a-b** and **3a** ($n=3$).

| Compounds | CB1 activation | | |
|-------------|-------------------------------|-------------------------|----------------------|
| | E_{\max} (%basal \pm SEM) | $-\log EC_{50} \pm$ SEM | EC_{50} (μ M) |
| 2-AG | 620 \pm 17 | 6.0 \pm 0.1 | 1.0 |
| 1a | 407 \pm 20 | 5.0 \pm 0.1 | 10 |
| 1b | 227 \pm 19 | 4.8 \pm 0.0 | 16 |
| 2a | 168 \pm 16 | 4.8 \pm 0.4 | 16 |
| 2b | NA | NA | NA |
| 3a | NA | NA | NA |

NA = no detectable CB1 activation at 10^{-4} M concentration

Table 7.2 summarizes the results of the stability studies conducted in rat cerebellar membranes. The ester bond of 2-AG remained intact in membrane free buffer solution, even though acyl migration from 2-AG into 1(3)-AG can be observed. However, in the incubation with rat cerebellar membrane about 77 % of 2-AG was degraded into AA due to the enzymatic activity present in the studied tissue. The acyl migration is not likely to have an impact on the metabolism since it has been reported that the rates of the enzymatic degradation for 2-AG and 1-AG are approximately the same (Saario et al. 2004).

Table 7.2. Relative ($\% \pm s.e.m.$) concentrations of 2-AG (1(3)-AG) and **1a-b** (α -Me-1-AG) and their degradation products (AA or α -Me-AA) in the rat cerebellar membranes after 90 minutes' incubation.

| Compound | 2-AG | | 1a | | 1b | |
|--------------------------|------------|------------|------------|------------|------------|------------|
| | 0 | 90 | 0 | 90 | 0 | 90 |
| 2-AG / α -Me-2-AG | 86 \pm 3 | 5 \pm 1 | 84 \pm 1 | 23 \pm 1 | 82 \pm 2 | 24 \pm 1 |
| 1-AG / α -Me-1-AG | 14 \pm 3 | 18 \pm 4 | 14 \pm 1 | 46 \pm 1 | 17 \pm 2 | 50 \pm 2 |
| AA / α -Me-AA | 0 \pm 0 | 77 \pm 4 | 3 \pm 0 | 31 \pm 2 | 1 \pm 0 | 27 \pm 3 |

Compounds **1a** and **1b** behaved similarly in the membrane-free buffer solution as 2-AG; the α -methylation did not prevent the acyl migration. Nevertheless, the hypothesis that α -methylation of 2-AG could reduce the metabolism was proven to be correct since only about 30 % of **1a** and **1b** were degraded into the corresponding α -methyl-arachidonic acids (α -Me-AA) in the rat cerebellum membranes after 90 minutes' incubation. The stereochemistry of the compound does not play a significant role in its metabolism.

7.4 Conclusions

In order to protect the ester bond of 2-AG against enzymatic degradation, α -methylated derivatives of 2-AG were synthesized using stereoselective synthesis. In addition, the CB1 receptor activities of mono- and difluoro derivatives of 2-AG were studied. The results indicate that even though the stereochemistry of the α -position of 2-AG does not play a role in its ligand affinity for the CB1 receptor, it has a significant role in a G-protein activation. The potency and efficacy values of the α -methylated 2-AG derivatives are slightly weaker compared to 2-AG. However, it is noteworthy, that derivatives are clearly more stable than 2-AG, and therefore, it can be anticipated that their duration of action in the target tissue would be longer. Finally, the results showed that the replacement of the hydroxyl group(s) of 2-AG by fluorine did not improve the activity of 2-AG as is the case with AEA.

7.5 Experimental

7.5.1 Synthesis

(S)-4-Benzyl-oxazolidinone (10b). To the stirred solution of lithium aluminium hydride (1.8 g, 45 mmol) in dry THF (30 mL), L-phenylalanine (1.7 g, 10 mmol) was added through the reflux condenser. The condenser was flushed with dry THF (10 mL) and refluxed for 6 h. The reaction mixture was cooled on an ice-bath. 10% NaOH (2.7 mL) was added dropwise followed by water (3.4 mL) and mixture was stirred for 5 min. 6 M NaOH (7.5 mL) was added slowly followed by CH₂Cl₂ (25 mL). The reaction mixture was cooled to -5 °C and triphosgene (1.4 g, 4.8 mmol) in CH₂Cl₂ (8.5 mL) was added through dropping funnel for 45 minutes. The stirring was continued at RT for 1 hour. The organic phase was separated and the water phase was extracted with CH₂Cl₂ (3 x 40 mL). The combined organic phases were washed with water (90 mL + 40 mL) and brine (20 mL) and dried over anhydrous MgSO₄. The solvents were evaporated and the solid crude product was recrystallized from hexane/EtOAc (8 mL/3 mL). After filtering and drying in vacuo, the title compound yielded 1.3 g (71%) of white needles. R_f=0.2 (10% diethyl ether in CH₂Cl₂); mp 85.5 °C; ¹H NMR (CDCl₃) δ 2.88 (d, *J*= 7.0 Hz, 2H), 4.07-4.19 (m, 2H), 4.46 (t, *J*= 8.2 Hz, 1H), 5.86 (s, 1H), 7.19-7.36 (m, 5H). [α]²⁰ -65 deg cm² g⁻¹ (c= 1.0; CHCl₃). HPLC enantiomer analysis afforded 100% *ee*; (Rt 30.4; Daicel OD chiral column; 10 %(v/v) 2-propanol in hexane; flow 1 mL/min; 254 nm).

(R)-4-Benzyl-oxazolidinone (10a). Compound **10a** was synthesized as **10b** but purified with the flash chromatography (5% diethyl ether in CH₂Cl₂ as an eluent). Yield 22% as crystals. R_f=0.2 (10% diethyl ether in CH₂Cl₂); mp 86 °C. ¹H NMR (CDCl₃): δ 2.88 (d, *J*=7.0, 2H), 4.06-4.17 (m, 2H), 4.46 (t, *J*=8.2, 1H), 5.43 (s, 1H), 7.19-7.36 (m, 5H). [α]²⁰ +68 deg cm² g⁻¹ (c= 1.0; CHCl₃). HPLC enantiomer analysis afforded 100% *ee*; (Rt 38.4; Daicel OD chiral column; 10 %(v/v) 2-propanol in hexane; flow 1 mL/min; 254 nm).

(S)-N-2-Arachidonoyl-4-benzyl-oxazolidinone (5b). The solution of **10b** (710 mg, 4.0 mmol) in dry THF (14 mL) was cooled to -78 °C. *n*-BuLi (1.15 M in hexane, 3.5 mL) was added dropwise and the reaction mixture was stirred at -78 °C for 40 min. To

the stirred solution of arachidonic acid (1.0 g, 3.3 mmol) and Et₃N (0.56 mL, 4.0 mmol) in dry THF (19 mL), distilled pivaloyl chloride (0.58 mL, 4.0 mmol) was added at -78 °C. A white suspension was formed and stirring was continued at -78 °C for 12 minutes and then at 0 °C for 45 minutes. The solution was cooled to -78 °C and lithiated oxazolidinone was cannulated slowly to the reaction mixture. Stirring was continued for 4 h and the reaction mixture was allowed to warm to 0 °C during that time. Stirring was continued for another 3 h and the reaction mixture was quenched with 21 mL of saturated NH₄Cl (aq). The crude product was extracted with 3 x 60 mL of diethyl ether, the organic phase was dried over MgSO₄ and evaporated. Purification with silica gel flash chromatography (CH₂Cl₂:PE) yielded 1.50 g (97%) of the title compound as a light yellow oil. R_f=0.5 (CH₂Cl₂); ¹H NMR (CDCl₃): δ 0.88 (t, *J*= 6.8, 3H), 1.26-1.39 (m, 6H), 1.74-1.82 (m, 2H), 2.05 (q, *J*=7.0, 2H), 2.18 (q, *J*=6.0, 2H), 2.73-2.86 (m, 7H), 2.92-3.03 (m, 2H), 3.28-3.32 (dd, *J*=3.2, 13.3), 4.12-4.22 (m, 2H), 4.64-4.70 (m, 1H), 5.28-5.42 (m, 8H), 7.20-7.35 (m, 5H).

(*R*)-*N*-2-Arachidonoyl-4-benzyl-oxazolidinone (5a). Compound **5a** was synthesized as **5b** by coupling arachidonic acid with (*R*)-4-benzyl-oxazolidinone. The yield of light yellow oil was 84%. ¹H NMR (CDCl₃): δ 0.88 (t, *J*=6.8, 3H), 1.26-1.39 (m, 6H), 1.74-1.82 (m, 2H), 2.05 (q, *J*=7.0, 2H), 2.18 (q, *J*=6.0, 2H), 2.73-2.86 (m, 7H), 2.92-3.03 (m, 2H), 3.28-3.32 (dd, *J*=3.2, 13.3, 1H), 4.12-4.22 (m, 2H), 4.64-4.70 (m, 1H), 5.28-5.42 (m, 8H), 7.20-7.35 (m, 5H).

(*S*)-*N*-((*S*)-2-Methyl-arachidonoyl)-benzyl-2-oxazolidinone (6b). The stirred solution of 1.0 M sodium bis(trimethylsilyl)amide in THF (0.4 mL, 0.4 mmol) was cooled to -78 °C. The 0 °C solution of **5b** (160 mg, 0.35 mmol) in dry THF (5 mL) was added dropwise for 15 minutes. The reaction mixture went yellow and after stirring for 30 minutes methyl iodide (0.11 mL, 1.8 mmol) was added. The reaction mixture was stirred at -78 °C for 4 h and then quenched with 2.6 mL of saturated NH₄Cl (aq). The crude product was extracted with 3 x 10 mL of diethyl ether, washed with water and organic phase was dried with MgSO₄ and solvents were evaporated. Purification with flash chromatography (2.5% EtOAc in PE) yielded 100 mg (60%) of a title compound as a light yellow oil. R_f=0.7 (CH₂Cl₂); ¹H NMR (CDCl₃): δ 0.89 (t, *J*=7.0, 3H), 1.24 (d, *J*=6.9, 3H), 1.27-1.41 (m, 6H), 1.45-1.54 (m, 1H), 1.82-1.91 (m, 1H), 2.03-2.17 (m,

4H), 2.75-2.88 (m, 7H), 3.26 (dd, $J=3.2, 13.3$, 1H), 3.73 (st, $J=7.0$, 1H), 4.15-4.21 (m, 2H), 4.65-4.70 (m, 1H), 4.29-5.41 (m, 8H), 7.21-7.32 (m, 5H).

(R)-N-((R)-2-Methyl-arachidonoyl)-benzyl-2-oxazolidinone (6a). Compound **6a** was synthesized as **6b**, but with **5a** as the starting material. Purification with flash chromatography (2.5% EtOAc in PE) yielded 82% of title compound as a colourless oil. $R_f=0.7$ (CH_2Cl_2); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=7.0$, 3H), 1.24 (d, $J=6.9$, 3H), 1.27-1.41 (m, 6H), 1.45-1.54 (m, 1H), 1.82-1.91 (m, 1H), 2.03-2.17 (m, 4H), 2.75-2.88 (m, 7H), 3.26 (dd, $J=3.2, 13.3$, 1H), 3.73 (st, $J=7.0$, 1H), 4.15-4.21 (m, 2H), 4.65-4.70 (m, 1H), 4.29-5.41 (m, 8H), 7.21-7.32 (m, 5H).

(S)-2-Methylarachidonic acid (7b). The stirred solution of **6b** (200 mg, 0.42 mmol) in THF:H₂O (3:1, 8.4 mL) was cooled to 0 °C. 30% H₂O₂ (aq., 0.34 mL, 3.4 mmol) and LiOH·H₂O (40 mg, 0.95 mmol) were added to the reaction mixture successively. The reaction mixture was allowed to warm to RT, stirred overnight and quenched with 2.5 mL of 1.5 M Na₂SO₃. THF was evaporated and the residue was acidified with 10% HCl to pH 1-2. The product was extracted with 3 x 20 mL of diethyl ether, the organic phase was dried with MgSO₄ and solvents were evaporated. Purification with silica gel chromatography (20% EtOAc in hexane) yielded 110 mg (83%) of title compound as a light yellow oil. $R_f=0.3$ (20% EtOAc in hexane); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=7.0$, 3H), 1.20 (d, $J=7.0$, 3H), 1.20-1.39 (m, 6H), 1.46-1.53 (m, 1H), 1.75-1.83 (m, 1H), 2.05 (q, $J=7.0$, 2H), 2.49-2.53 (st, $J=7.0$, 1H), 2.13 (q, $J=7.0$, 2H), 2.78-2.83 (m, 6H), 5.26-5.34 (m, 8H); $[\alpha]^{20}_D = +18 \text{ deg cm}^2 \text{ g}^{-1}$ ($c=1.0$; CH_2Cl_2).

(R)-2-Methylarachidonic acid (7a). Compound **7a** was synthesized as **7b**, but with **6a** as the starting material. Purification with silica gel chromatography (10% EtOAc and 1% acetic acid in PE) yielded 65% of title compound as a light yellow oil. $R_f=0.3$ (20% EtOAc in hexane); $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=7.0$, 3H), 1.20 (d, $J=7.0$, 3H), 1.20-1.39 (m, 6H), 1.46-1.53 (m, 1H), 1.75-1.83 (m, 1H), 2.05 (q, $J=7.0$, 2H), 2.13 (q, $J=7.0$, 2H), 2.49-2.53 (st, $J=7.0$, 1H), 2.78-2.83 (m, 6H), 5.26-5.34 (m, 8H); $[\alpha]^{20}_D = -15 \text{ deg cm}^2 \text{ g}^{-1}$ ($c=0.38$; CH_2Cl_2).

1,3-Difluoro-2-propyl (S)-2-methylarachidonate (3b). To a stirred solution of **7b** (100 mg, 0.31 mmol) and 1,3-difluoro-2-propanol (80 mg, 0.83 mmol) in toluene (27

mL) DCC (130 mg, 0.62 mmol) and DMAP (12 mg, 0.1 mmol) were added. The reaction mixture was stirred at RT overnight and then diluted to EtOAc (46 mL). The organic phase was washed with water (46 mL) and brine (46 mL), dried over MgSO₄ and evaporated. Purification with flash chromatography (2% methyl-*t*-butyl ether in hexane) yielded 66 mg (54%) of title compound as a colourless oil. R_f = 0.4 (10% EtOAc in hexane); ¹H NMR (CDCl₃): δ 0.89 (t, J =6.9, 3H), 1.20 (d, J =7.0, 3H), 1.26-1.39 (m, 6H), 1.47-1.54 (m, 1H), 1.75-1.82 (m, 1H), 2.03-2.13 (m, 4H), 2.55 (st, J =7.0, 1H), 2.80-2.85 (m, 6H), 4.58 (dd, J =4.8, J_{H-F} = 46.9, 4H), 5.23 (tq, J =4.6, J_{H-F} =19.7, 1H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.0, 17.0, 22.5, 24.8, 25.6 (3C), 27.2, 29.3, 31.5, 33.4, 38.9, 70.1 (t, J_{C-F} = 20.8), 80.3 (ddd, J_{C-F} = 1.4, 6.9 and 173), 127.5, 127.8, 128.1, 128.3, 128.6, 128.8, 128.9, 130.5, 175.6; GC-MS(EI): M = 396.

1,3-Difluoro-2-propyl (*R*)-2-methylarachidonate (3a). Compound **3a** was synthesized as **3b**, by coupling **7a** with 1,3-difluoro-2-propanol using EDCI and DMAP as coupling reagents. Purification with flash chromatography (2% PE in EtOAc) yielded 93% of a title compound as colourless oil. R_f =0.4 (10 % EtOAc in hexane); ¹H NMR (CDCl₃): δ 0.89 (t, J =6.9, 3H), 1.20 (d, J =7.0, 3H), 1.26-1.39 (m, 6 H), 1.47-1.54 (m, 1H), 1.75-1.82 (m, 1H), 2.03-2.13 (m, 4H), 2.55 (st, J =7.0, 1H), 2.80-2.85 (m, 6H), 4.58 (dd, J =4.6, J_{H-F} =46.9, 4H), 5.23 (tq, J =4.6, J_{H-F} =19.8, 1H), 5.31-5.43 (m, 8H); ¹³C NMR (CDCl₃): δ 14.0, 16.9, 22.6, 24.8, 25.6 (3C), 27.2, 29.3, 33.4, 39.0, 70.1 (t, J_{C-F} =20.8), 80.3 (ddd, J_{C-F} =1.4, 6.9 and 173), 127.5, 127.9, 128.1, 128.3, 128.6, 128.8, 128.9, 130.5, 175.7.

(*S*)-2-Methylarachidonic acid oxiranylmethyl ester (8b). **7b** (150 mg, 0.47 mmol), glycidol (42 mg, 0.56 mmol), EDCI (180 mg, 0.94 mmol) and DMAP (17 mg, 0.14 mmol) in dry CH₂Cl₂ (7 mL) were stirred at RT overnight. CH₂Cl₂ was added and the solution was washed with brine. The organic phase was dried over Na₂SO₄, filtered and evaporated. The crude product was purified by flash chromatography eluting with 5% EtOAc in PE yielding 114 mg (59%) of oily product. ¹H NMR: δ 0.89 (t, J =6.9, 3H), 1.19 (dd, ² J =1.3, J =7.0, 3H), 1.26-1.39 (m, 6H), 1.46-1.53 (m, 1H), 1.74-1.82 (m, 1H), 2.03-2.12 (m, 4H), 2.52 (st, J =7.0, 1H), 2.63-2.65 (m, 1H), 2.80-2.85 (m, 7H), 3.18-3.21 (m, 1H), 3.91-3.95 (m, 1H), 4.39-4.43 (m, 1H), 5.31-5.43 (m, 8H).

(R)-2-Methylarachidonic acid oxiranylmethyl ester (8a). Yield 75%. ^1H NMR: δ 0.89 (t, $J=6.9$, 3H), 1.19 (dd, $^2J=1.3$, $J=7.0$, 3H), 1.26-1.39 (m, 6H), 1.46-1.53 (m, 1H), 1.74-1.82 (m, 1H), 2.03-2.12 (m, 4H), 2.52 (st, $J=7.0$, 1H), 2.63-2.65 (m, 1H), 2.80-2.85 (m, 7H), 3.18-3.21 (m, 1H), 3.91-3.95 (m, 1H), 4.39-4.43 (m, 1H), 5.31-5.43 (m, 8H).

(S)-2-methylarachidonic acid 2'-(1',3'-bistrifluoroacetyl propyl) ester (9b). Corresponding oxiranylmethyl ester (93 mg, 0.25 mmol) in dry CH_2Cl_2 (2 mL) was cooled to -20°C and TFAA (0.21 mL, 1.5 mmol) in dry CH_2Cl_2 (3 mL) was added. The cooling bath was removed, and the mixture was allowed to warm to room temperature. After 2 hours, the reaction was concluded by evaporating the solvent under vacuum. The residue was purified by flash chromatography eluting with toluene yielding 133 mg (92%) of an oily product. ^1H NMR: δ 0.89 (t, $J=6.9$, 3H), 1.17 (d, $J=7.0$, 3H), 1.26-1.39 (m, 6H), 1.44-1.51 (m, 1H), 1.71-1.79 (m, 1H), 2.03-2.10 (m, 4H), 2.50 (st, $J=7.0$, 1H), 2.79-2.85 (m, 6H), 4.46 (dd, $^2J=5.6$, $J=11.9$, 2H), 4.62-4.66 (m, 2H), 5.31-5.44 (m, 9H).

(R)-2-methylarachidonic acid 2'-(1',3'-bistrifluoroacetyl propyl) ester (9a). Yield 96%; ^1H NMR: δ 0.89 (t, $J=6.9$, 3H), 1.17 (d, $J=7.0$, 3H), 1.26-1.39 (m, 6H), 1.44-1.51 (m, 1H), 1.71-1.79 (m, 1H), 2.03-2.10 (m, 4H), 2.50 (st, $J=7.0$, 1H), 2.79-2.85 (m, 6H), 4.46 (dd, $^2J=5.6$, $J=11.9$, 2H), 4.62-4.66 (m, 2H), 5.31-5.44 (m, 9H).

2-((S)-2-Methylarachidonoyl)glycerol (1b). The corresponding trifluoroacetyl propyl ester (130 mg, 0.23 mmol) was dissolved in dry hexane/ CH_2Cl_2 (2 mL/1 mL) solution and the mixture was cooled to -20°C . Dry pyridine (0.2 mL) and methanol (0.15 mL) in dry hexane/ CH_2Cl_2 (2 mL/1 mL) were added slowly into the reaction mixture. The cooling bath was removed and the solution was stirred at room temperature for 2 h. The reaction was concluded by evaporating solvents under reduced pressure without a heating bath. The remaining solvents were removed under high vacuum yielding 88 mg (97 %) of an oily product. $R_f=0.3$ (50% EtOAc in PE); ^1H NMR (CDCl_3): δ 0.89 (t, $J=6.9$, 3H), 1.19 (d, $J=7.0$, 3H), 1.26-1.39 (m, 6H), 1.47-1.54 (m, 1H), 1.75-1.82 (m, 1H), 2.03-2.13 (m, 4H), 2.23 (s, 2H), 2.53 (st, $J=7.0$, 1H), 2.80-2.85 (m, 6H), 3.82-3.83 (m, 4H), 4.92 (qn, $J=4.8$, 1H), 5.31-5.43 (m, 8H); ^{13}C NMR (CDCl_3): δ 14.0, 17.0, 22.5, 24.8, 25.6 (3C), 27.2, 29.3, 31.5, 33.5, 39.1, 62.4, 75.0, 127.5, 127.8, 128.1, 128.3, 128.6, 128.7, 129.0, 130.5, 176.9.

2-((R)-2-Methylarachidonoyl)glycerol (1a). Quantitative yield. ^1H NMR (CDCl_3): δ 0.89 (t, $J=6.9$, 3H), 1.19 (d, $J=7.0$, 3 H), 1.26-1.39 (m, 6 H), 1.47-1.54 (m, 1H), 1.75-1.82 (m, 1H), 2.03-2.13 (m, 4H), 2.23 (s, 2H), 2.53 (st, $J=7.0$, 1H), 2.80-2.85 (m, 6H), 3.82-3.83 (m, 4H), 4.92 (qn, $J=4.8$, 1H), 5.31-5.43 (m, 8H); ^{13}C NMR (CDCl_3) δ : 14.0, 17.0, 22.5, 24.8, 25.6 (3C), 27.2, 29.3, 31.5, 33.5, 39.1, 62.4, 75.0, 127.5, 127.8, 128.1, 128.3, 128.6, 128.7, 129.0, 130.5, 176.9.

(rac)-2-Fluoro-1-(hydroxymethyl)ethyl (S)-2-methylarachidonate (2b). The mixture of **7b** (100 mg, 0.31 mmol) and **11** (86 mg, 0.35 mmol) in CH_2Cl_2 (5 mL) was stirred at RT. EDCI (170 mg, 0.89 mmol) and DMAP (14 mg, 0.11 mmol) were added and the reaction mixture was stirred at RT overnight (15 hours) and evaporated. The purification with flash chromatography (0-3% EtOAc in PE) yielded 120 mg (70%) of title compound as a colourless oil. $R_f=0.8$ (10% EtOAc and 0.1% AcOH in PE); ^1H NMR (CDCl_3): δ 0.89 (t, $J=6.9$, 3H), 1.10-1.15 (m, 21H), 1.15-1.19 (m, 3H), 1.24-1.39 (m, 6H), 1.42-1.52 (m, 1H), 1.72-1.82 (m, 1H), 2.02-2.12 (m, 4H), 2.44-2.54 (m, 1H), 2.74-2.88 (m, 6H), 3.85 (d, $J=6.0$, 2H), 4.52-4.68 (m, 2H), 5.00-5.10 (m, 1H), 5.28-5.44 (m, 8H). Solution of (rac)-1-fluoromethyl-2-(triisopropylsilyloxy)ethyl (S)-2-methylarachidonate (120 mg, 0.22 mmol) in THF (2 mL) was stirred at 0 °C. TBAF (730 mg, 2.3 mmol) was dissolved to THF (3 mL), acidified to pH 6 with glacial acetic acid and added to the reaction mixture. Stirring was continued at 0 °C for 3 hours, at 5 °C for overnight (15 hours) and at RT for 9 hours. Altogether 1500 mg (22 eq) of TBAF (200-300 mg portions) was added as pH 6 -solution of THF and AcOH during the reaction time. The reaction was stopped by evaporating the THF in a rotavapor. Purification with flash chromatography (5% EtOAc in PE) yielded 56 mg (89%) of title compound as a yellowish oil. ^1H NMR (CDCl_3): δ 0.89 (t, $J=6.9$, 3H), 1.19 (d, $J=7.0$, 3H), 1.25-1.39 (m, 6H), 1.47-1.54 (m, 1H), 1.74-1.82 (m, 1H), 2.06 (q, $J=6.9$, 2H), 2.11 (q, $J=7.0$, 2H), 2.54 (st, $J=7.0$, 1H), 2.80-2.85 (m, 6H), 3.82 (br s, 2H), 4.58 (dd, $J=4.5$ and $J_{\text{H-F}}=47.1$, 2H), 5.08 (dq, $J=4.6$ and $J_{\text{H-F}}=20.9$, 1H), 5.31-5.43 (m, 8H); ^{13}C NMR (CDCl_3): δ 14.0, 17.0, 22.6, 24.8, 25.6 (3 C), 27.2, 29.3, 31.5, 33.5, 39.0, 61.0 (d, $J_{\text{C-F}}=6.9$), 72.8 (d, $J_{\text{C-F}}=19.8$), 81.4 (d, $J_{\text{C-F}}=172.4$), 127.5, 127.9, 128.1, 128.3, 128.6,

128.8, 128.9, 130.5, 176.3; $[\alpha]^{20} +15 \text{ deg cm}^2 \text{ g}^{-1}$ ($c= 0.13$; CH_2Cl_2); GC-MS(EI): $M= 394$.

(rac)-2-Fluoro-1-(hydroxymethyl)ethyl (R)-2-methylarachidonate (2a). Compound **2a** was synthesized as **2b** by coupling **7a** with **11**. $^1\text{H NMR}$ (CDCl_3): δ 0.89 (t, $J=7.0$, 3H), 1.19 (dd, $J=1.3, 7.0$, 3H), 1.25-1.39 (m, 6H), 1.47-1.54 (m, 1H), 1.74-1.82 (m, 1H), 2.06 (q, $J=6.9$, 2H), 2.11 (q, $J=7.0$, 2H), 2.54 (st, $J=7.0$, 1H), 2.80-2.85 (m, 6H), 3.82 (br s, 2H), 4.58 (dd, $J=4.5, J_{\text{H-F}}=47.1$, 2H), 5.08 (dq, $J= 4.6, J_{\text{H-F}}= 20.9$, 1H), 5.31-5.43 (m, 8H); $^{13}\text{C NMR}$ (CDCl_3) δ : 14.0, 17.0, 22.5, 24.8, 25.6 (3 C), 27.2, 29.3, 31.5, 33.5, 39.0, 60.9 (d, $J_{\text{C-F}}= 7.0$), 72.8 (d, $J_{\text{C-F}}= 19.7$), 81.4 (d, $J_{\text{C-F}}=172.3$), 127.5, 127.8, 128.1, 128.3, 128.6, 128.8, 128.9, 130.5, 176.3; $[\alpha]^{20} -15 \text{ deg cm}^2 \text{ g}^{-1}$ ($c= 0.36$; CH_2Cl_2); GC-MS(EI) $M= 394$.

7.5.2 Enzymatic stability

The enzymatic stability studies were carried out in rat cerebellar membranes prepared as previously described (Savinainen et al. 2001). Preincubations (80 μL , 30 min at 25 $^\circ\text{C}$) contained 10 μg membrane protein, 44 mM Tris-HCl (pH 7.4), 0.9 mM EDTA, 0.5% BSA and 1.25% (v/v) DMSO as a solvent for drugs. The incubations (90 min at 25 $^\circ\text{C}$) were initiated by adding 40 μL of preincubated membrane cocktail, giving a final volume of 400 μL . The final volume contained 5 μg membrane protein, 52 mM Tris-HCl (pH 7.4), 1.0 mM EDTA, 95 mM NaCl, 4.8 mM MgCl_2 , 0.5% BSA and 50 μM of the drug (2-AG, **1a-b**). At time-points of 0 and 90 min, 100 μL samples were removed from the incubation, acetonitrile (200 μL) was added to stop the enzymatic reaction, and pH of the samples was simultaneously decreased with phosphoric acid (added to acetonitrile) to 3.0, in order to stabilize the drugs against any possible post-incubation chemical acyl migration reaction. The samples were centrifuged at 23,700 g for 4 min at RT prior to HPLC analysis of the supernatant. Retention times were 5.6 min for 2-AG, 6.1 min for 1-AG, 7.4 min for α -methyl-2-AG, 7.9 min for α -methyl-1-AG, 10.0 for AA, and 13.2 min for α -methyl-AA. The susceptibilities of 2-AG and **1a-b** towards enzymatic degradation were finally determined by the formation of AA or α -methyl-AA during the 90 min on the basis of the corresponding peak areas.

7.6 References

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8 SUMMARY AND CONCLUSIONS

8.1. Summary for novel CB1 receptor ligands

8.1.1 Arachidonyl alcohol derivatives

A series of hydroxypropionyl ester derivatives (Figure 8.1; **1a-b**) as well as carbamate (Figure 8.1; **2**) and carbonate (Figure 8.1; **3**) derivatives of arachidonyl alcohol were developed in order to study their CB1 activity. Arachidonyl alcohol was used as a starting material in order to prevent formation of an unwanted metabolite, arachidonic acid.

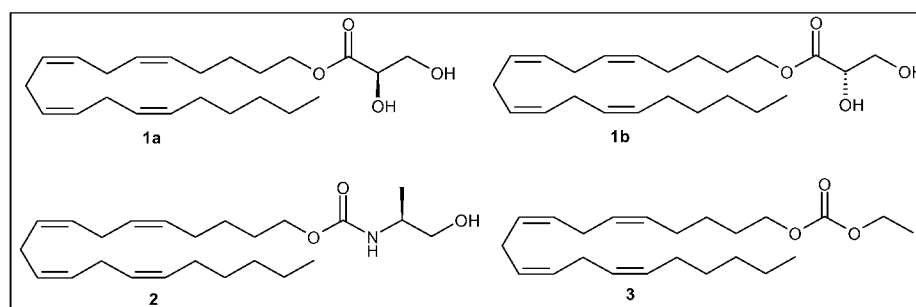


Figure 8.1. Representative structures of synthesized arachidonyl alcohol derivatives.

From the fourteen compounds synthesized, only **1a** and **1b** exhibited dose-dependent CB1 activity. Carbamates were able to produce some activity, however, the activity achieved was not abolished by the CB1 receptor antagonist, indicating that the response was not CB1 receptor mediated. The reason for the inactivity of carbonates remained partly unclear but most probably these compounds are too unstable and lipophilic for the conditions used in *in vitro* studies. In conclusion, synthesized arachidonyl alcohol derivatives cannot serve as potential CB1 receptor ligands.

8.1.2 Reversed amide derivatives of AEA

Based on previously made observation of the enzymatic stability of the AEA analogue, retroanandamide (Figure 8.2; **4**), we designed a series of reversed amides to study their cannabinoid receptor activation, and stability in rat brain homogenate and

membrane free buffer. In the [35 S]GTP γ S binding assay in rat cerebellar membranes, the reversed amides, as well as AEA, acted as partial agonists. Within the series, compound **5** (Figure 8.2) which has a cyclopropyl head group had superior CB1 activity. Other compounds, however, were not able to show improved CB1 activity compared to AEA.

All the studied compounds behaved as partial agonists and had comparable efficacy and potency values to AEA at hCB2-CHO lysates. The most interesting finding in the both C₁₉ and C₂₀ series was that these compounds with a butanoyl tail (Figure 8.2; **6a-b**) possessed potency that equal to that of 2-AG (7.4 ± 0.1). In addition, compound **6b** was relatively efficacious. To conclude, in contrast to claims in previous publications, reversed amides were able to activate both cannabinoid receptors.

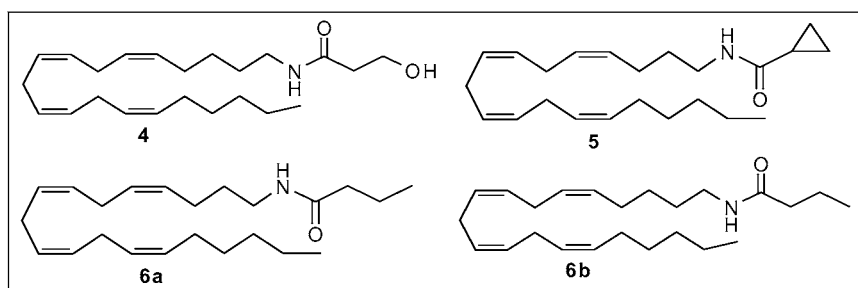


Figure 8.2. Representative structures for reversed amides.

Stability studies with a few representative reversed amide structures in rat brain homogenate showed that significantly improved enzymatic stability could be achieved by reversing the amide bond of AEA.

8.1.3 DMH derivatives of 2-AG and 2-AGE

Classical cannabinoids have distinctly better biological activity when their pentyl chain at position 3 is replaced by 1',1'-dimethylheptyl structure. A similar activity improvement is also observed in the case of AEA. These facts served as a starting point in this study where DMH derivatives of endogenous 2-AG and 2-AGE were synthesized (Figure 8.3; **7-9**), and their biological activity was determined in rat cerebellar membranes by the [35 S]GTP γ S binding assay.

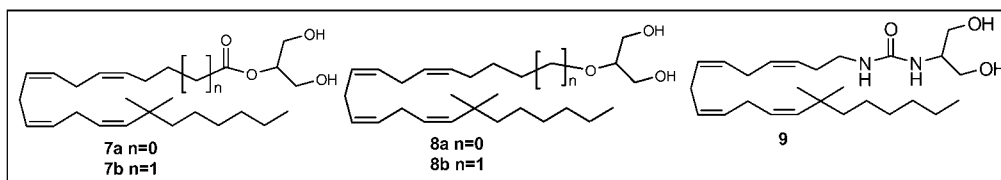


Figure 8.3. DMH derivatives of 2-AG and 2-AGE.

The synthesis of DMH derivatives was very time consuming since they had to be built from smaller synthons. The NMR interpretation proved also to be a major challenge due to extensive peak overlapping, and in addition, extra attention had to be paid to the double bonds since a *cis* form is an absolute requirement for CB1 activity. Finally, 2-AG's susceptibility to undergo isomerization during the synthesis and even during the storage had to be taken into consideration.

The DMH modification for 2-AG and 2-AGE led to a dramatic potency decrease. However, efficacy values were only slightly weaker or remained comparable to those of the reference compounds. Analogues with a shorter chain length (C_{22} vs C_{21}) proved to be more efficacious. Replacement of the ester bond with more hydrophilic and stable urea bond produced only weak agonistic activity. Based on these results, we concluded that unlike AEA-type compounds and classical cannabinoids, the activity profile of 2-AG and 2-AGE, cannot be improved by replacement of the end pentyl chain with the DMH structure.

8.1.4 α -Methylated derivatives of 2-AG

2-AG is a superior ligand for the cannabinoid receptors. However, its low enzymatic stability places restrictions for its utilization. Therefore, the main goal of this study was to introduce a methyl group at the α -position of 2-AG in order to reduce its hydrolysis to arachidonic acid (AA), and thus, prolong its duration of action.

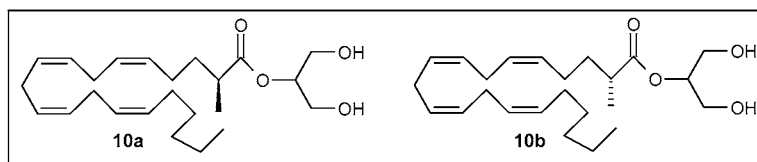


Figure 8.4. α -Methylated derivatives of 2-AG.

Since the introduction of a methyl group to the α -position of 2-AG results in formation of a chiral compound, a stereoselective synthesis method was chosen. Asymmetric induction was achieved by Evans's chiral 4-benzyl-2-oxazolidinone. The final compounds were produced diastereoselectively with methyl iodide.

The CB1 activity of the α -methylated 2-AG derivatives (Figure 8.4; **10a-b**) was slightly weaker compared to 2-AG. Interestingly, we observed that stereochemistry of the α -position has a significant role in the G-protein activation, although it has been reported to be insignificant in receptor affinity studies. Finally, the stability studies conducted in rat cerebellar membranes showed that the α -methylation protected 2-AG from enzymatic degradation, and therefore, this modification may prolong the duration of action.

8.2 Conclusions

In the present study, four series of endocannabinoid derivatives were synthesized and evaluated for their cannabinergic activity in the [35 S]GTP $_{\gamma}$ S binding assay. The enzymatic and chemical stability of a few representative compounds were determined in rat brain or rat cerebellum homogenate and membrane-free buffer. The following main conclusions can be made:

1. Carbamate, carbonate, and hydroxypropionyl ester derivatives of arachidonyl alcohol do not serve as potential ligands for the CB1 receptors.
2. Reversed amide derivatives of AEA, especially those with short hydrophobic alkyl head groups, act as partial agonists for both cannabinoid receptors.

3. Reversed amides of AEA possess significantly higher enzymatic stability in rat brain homogenate compared to AEA.
4. 2-AG and 2-AGE seem to be more sensitive to the structural modifications, since the replacement of the pentyl tail with the DMH structure does not lead to any improvement in the CB1 activity, in contrast to the situation with AEA and classical cannabinoids.
5. The stereochemistry of the α -position of 2-AG does not play a role in a ligand affinity for the CB1 receptor, however, it has a significant role in the G-protein activating properties of these compounds.
6. α -Methylation of 2-AG provides good protection against enzymatic degradation, and therefore, prolonged duration of action.
7. Although synthesis of fatty acid derivatives was found to be occasionally very challenging, effective synthesis and purification methods were developed and they can be further utilized in future ligand development.

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