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Synthesis and CB1 receptor activities of dimethylheptyl derivatives of 2-arachidonoyl glycerol (2-AG) and 2-arachidonyl glyceryl ether (2-AGE)

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Abstract—Results from a factor analysis and activity studies of commercially available endocannabinoid-type compounds set the starting point for the current study where dimethylheptyl (DMH) analogues of two endocannabinoids, 2-arachidonoyl glycerol (2-AG) and 2-arachidonyl glyceryl ether (2-AGE), were synthesized and their ability to activate the CB1 receptors was determined by the [^{35}S]GTP$_\gamma$S binding assay using rat cerebellar membranes. The main goal of the study was to examine how the DMH end tail affects the activity properties of 2-AG (**1**) and its stable ether (**2**) and urea analogues (**5**). The importance of the chain length was also explored by synthesizing 2-AG and 2-AGE derivatives (**3** and **4**) possessing the chain length C_{21} instead of C_{22}. Replacement of the pentyl end chain with the DMH resulted in distinct potency decrease as compared to the reference compounds. The modification did not have such a strong impact on the efficacy values. In fact, the efficacy of the derivatives of 2-AGE (**2** and **4**) was comparable or even improved. Introducing a more stable and hydrophilic urea bond led to a dramatic decrease in biological activity.

1. Introduction

In 1995, two independent research groups published significant findings about a new endogenous compound which possessed clear cannabimimetic activity but had an ester linkage and a glycerol head, distinguishing it from the well-known endogenous cannabinoid, *N*-arachidonoyl ethanol amide (AEA).[1,2] It was noteworthy that the concentration of this novel cannabinoid receptor ligand, 2-arachidonoyl glycerol (2-AG), in the rat brain was almost 800 times higher than that of AEA. However, at that time, this new endocannabinoid did not receive the attention it would have deserved, probably because of its poor receptor binding affinity properties as compared to those of AEA (K_i (CB1) for 2-AG 2.4 μM, K_i (CB1) for AEA 99 nM).[1] Possibly, 2-AG would have been found earlier if its susceptibility for enzymatic degradation and acyl migration were recognized earlier. After modifying experimental conditions with serine esterase inhibitors, first with diisopropyl fluorophosphate (DFP) and later with the more potent phenylmethylsulfonyl fluoride (PMSF) and methyl arachidonoyl fluorophosphonate (MAFP),[3] more reliable and promising results of 2-AG and its role in the endocannabinoid system have been gained. Although the first CB1 receptor binding studies gave a reason to postulate that the role of 2-AG as an endocannabinoid is not very significant, it has been later shown that 2-AG is a potent, full efficacy agonist and most probably the main endogenous ligand for both the CB1 and CB2 receptors.[4–7]

HU-310 (2-arachidonyl glyceryl ether, 2-AGE), the ether analogue of 2-AG, was first reported in 1998 by Mechoulam et al. as a stable chemical tool for various cannabinoid related studies both in vitro and in vivo.[8] At the same time, this metabolically stable compound was synthesized and tested by Sugiura et al.[5,9] Later on, 2-AGE was postulated as an endocannabinoid,[10] even though its status as the third endocannabinoid has been under debate since its discovery because it has taken a while for other research groups to repeat this

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finding. However, Di Marzo's and co-workers showed in 2003 that the compound possessing the same mass and similar chemical properties (i.e., chromatographic properties) as 2-AGE can be found in the brain.[11] Nevertheless, 2-AGE's occurrence in the brain did not fully overlap the regional distribution of the CB1 receptors. In the same study, it was also postulated that 2-AGE can be taken up into the cells by the same transporter protein as the other endocannabinoids. Quite opposite findings were reported by Oka et al.[12] They were not able to detect appreciable amounts of 2-AGE from the distinct mammalian brain tissues using GC–MS and fluorometric HPLC. Since the contradictory findings and unclear biosynthetic route of 2-AGE, perhaps it is too early to consider 2-AGE as the third endocannabinoid.

2-AG is known as the most efficacious CB1 receptor ligand so far. In the [^{35}S]GTP$_\gamma$S binding assays with the rat cerebellar membranes, E_{max} value for 2-AG was reported to be 620 ± 5 (% basal ± SEM), while the respective figures for AEA, 2-AGE and CP55,940 were 484 ± 7, 415 ± 3 and 510 ± 4.[3] The classical cannabinoid HU-210 has the potency value higher than any other cannabinoid found so far. In our [^{35}S]GTP$_\gamma$S binding assays, *p*EC50 for HU-210 was found to be as high as 8.3 ± 0.1 (unpublished data), whereas the corresponding value for 2-AG is 6.0 ± 0.0.[3] The high potency of HU-210 results most probably from the optimal positioning of the hydroxyl groups in relation to the aliphatic dimethylheptyl (DMH) side chain, which already in 1949 was shown to be favoured in the ligand–receptor recognition of tetrahydrocannabinol-type compounds.[13,14]

The importance of the end pentyl chain on the activity properties of AEA and its derivatives has also been extensively examined by branching and changing the length of the chain.[15,16] Concurrent study of Ryan et al. showed similar results of a DHM derivative of AEA; the activity was enhanced both in vitro and in vivo.[16] In the same study, methyl groups attached to the adjacent carbon produced only minor activity differences. The corresponding monomethyl derivative had also a very similar pharmacological profile. The study also provided further information on an optimal end chain length of AEA. The addition of one to four methylenes into AEA backbone resulted in higher affinity properties; however, such analogues did not show significant in vivo pharmacological effects at doses up to 30 mg/kg.

While the activity properties for the DMH derivatives of AEA have been well studied, the effect of DMH modifications on the other endocannabinoids, 2-AG and 2-AGE, has not yet been reported. In the present study, we explored the CB1 activating properties of endocannabinoid derivatives in the rat cerebellar membranes using the [^{35}S]GTPγS binding assay. As a starting point, a representative group of molecules was tested for the CB1 receptor activation. Based on the 2-AG's activity properties, and the knowledge of the SARs compounds with DMH modification, we decided to explore more closely the DMH derivatives of endocannabinoids. A DMH derivative of 2-AG (**1**) was synthesized in order to study whether the potency and efficacy can be increased. In addition, we synthesized a DMH analogue of 2-AGE (**2**), aimed at an enzymatically more stable, and potent, high efficacy cannabinoid for in vitro studies. Finally, we studied whether the shortening of the chain length has any effect on the activity properties (**3** and **4**), and if the activity properties of 2-AG can be retained by substituting the unstable ester linkage with a more stable and more polar urea linkage (**5**).

2. Results and discussion

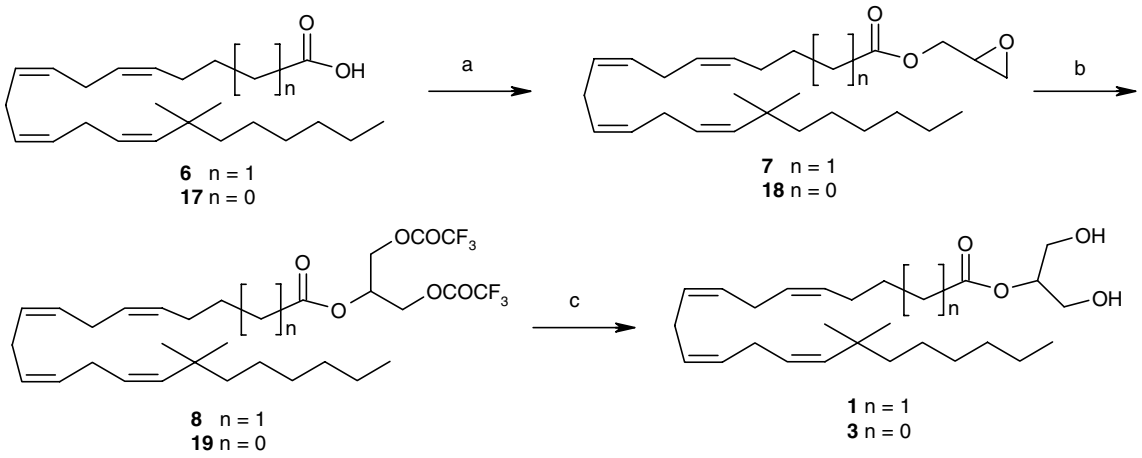
2.1. Selection of compounds for activity tests

The structures of 59 commercially available endocannabinnoid-type compounds were examined. To select a representative group of molecules, 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 (Fig. 2, see Supporting information). The molecular structures and the CB1 receptor-mediated G-protein activation data are presented in Table 2 (see Supporting information). During this study, the [^{35}S]GTPγS binding assay protocol was optimized, and the activity data of these compounds have been produced with a slightly different method than those of the synthesized compounds.

Molecule **34** (Table 2, see Supporting information) 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 tested for the CB1 receptor activity as well (Table 2, see Supporting information). Another molecule with a similar DMH side chain to that of **34** was found to have a comparable potency and efficacy with **34** and 2-AG. Consequently, we focussed on the design and synthesis of new endocannabinoid derivatives with DMH side chain in order to evaluate structure–activity relationships and to discover novel active CB1 lead structures.

2.2. Chemistry

A synthesis route for the final products **1** and **3** is illustrated in Scheme 1. The key synthons **6** and **17** were prepared as described in the literature.[17] The synthesis of endogenous 2-AG and its analogues is very challenging. First, 2-AG is very easily isomerized due to the acid, base and heat promoted migration of the acyl group, and second, the double bond system in fatty acids is extremely sensitive to auto-oxidation limiting the number of suitable synthesis methods. Stamatov and Stawinski[17,18] developed an efficient synthetic strategy to 2-AG, which was utilized in the synthesis of final products **1** and **3**. The corresponding carboxylic acids **6** and **17** were esterified with (±)-glycidol in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 4-dimethylaminopyridine (DMAP) to give the glycidyl derivatives **7** and **18**, which were treated



Scheme 1. Reagents: (a) EDC, DMAP, CH_2Cl_2; (b) TFAA, CH_2Cl_2; (c) pyridine, MeOH, CH_2Cl_2/hexane.

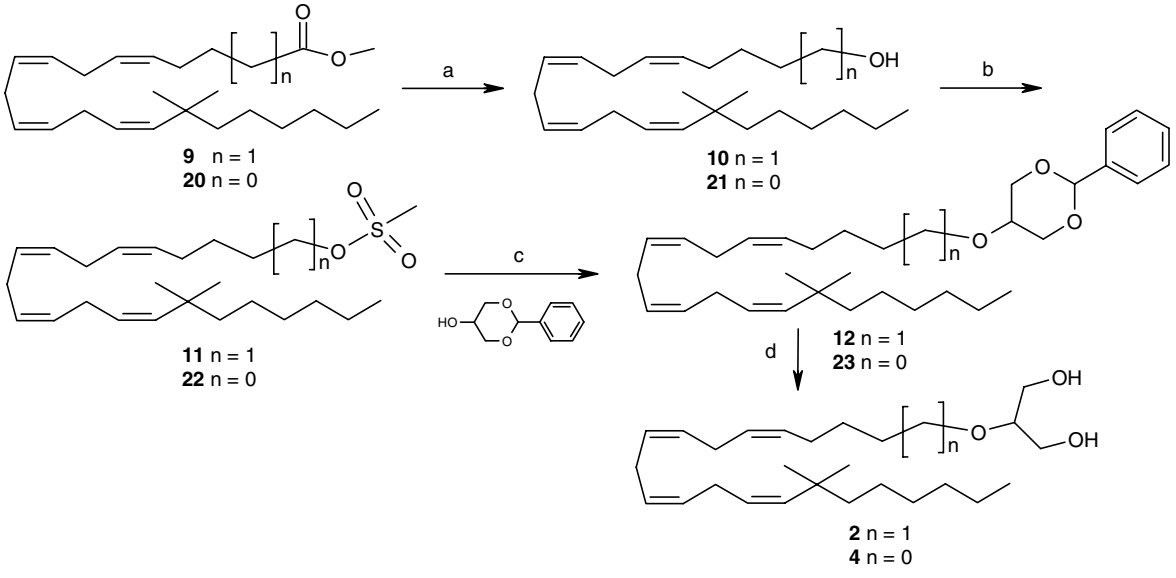
with TFAA giving trifluoroacetate esters **8** and **19**. These were further converted into the desired products by transesterification using pyridine and methanol in dichloromethane/hexane.

Scheme 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.[17] In the first step, corresponding ester (**9** or **20**) was reduced to alcohol with lithium aluminium hydride (LAH), and alcohol was activated by methane sulfonyl chloride (mesyl chloride, MsCl) prior to the addition of 2-phenyl-[1,3]-dioxan-5-ol. Finally, the benzylidene protective group was cleaved by concentrated HCl in methanol.[19]

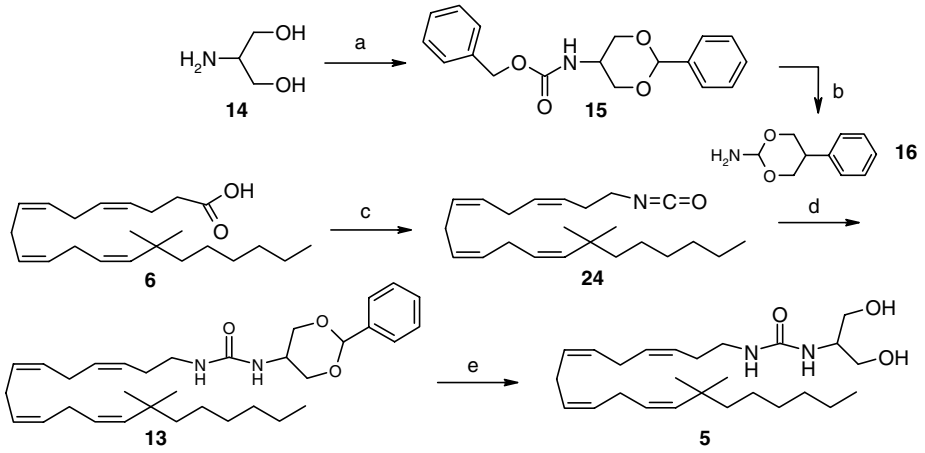
The urea **5** was synthesized by converting the acid **6** to a corresponding azide with diphenylphosphorylazide (DPPA), which was further rearranged by heating to an isocyanate **24**[20] (Scheme 3). 2-Phenyl-[1,3]-dioxan-5-yl-amine **16** was produced by protecting an amino group of 2-amino-1,3-propanediol with benzyl chloroformate, which enabled a selective protection of hydroxyl groups with benzaldehyde.[21] The benzyl carbamate **15** was deprotected by catalytic hydrogenation at elevated pressure and then treated with isocyanate **24**.[20] Finally, the protecting group of the 1,3-diol was removed with concentrated HCl in methanol.

2.3. [^{35}S]GTPγS membrane binding studies

Ability of the synthesized compounds to activate the CB1 receptors was determined by an optimized [^{35}S]GTPγS binding assay protocol where noise due to tonic adenosine A_1 receptor activity and enzymatic degradation of endocannabinoids have been eliminated (Method G in Experimental).[3] The efficacy and potency values are presented in Table 1, and dose–response curves for compounds **1**–**4** are shown in Figure 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).



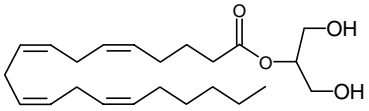
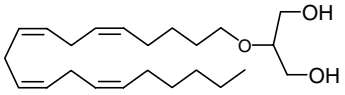
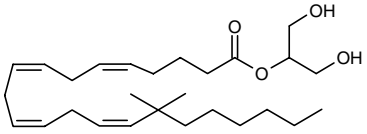
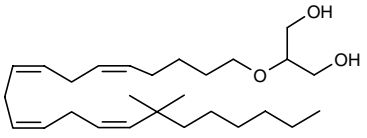
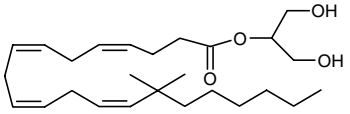
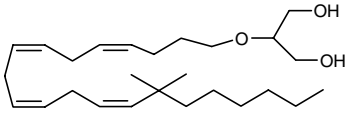
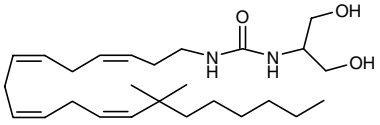
Scheme 2. Reagents: (a) LAH, Et_2O; (b) MsCl, Et_3N, CH_2Cl_2; (c) KOH, benzene; (d) HCl/MeOH.



Scheme 3. Reagents: (a) 1—CbzCl, EtOH, Et_3N, 2—benzaldehyde, toluene, *p*-TSA; (b) Pd/C, H_2, 300 psi, 60 °C; (c) 1—DPPA, Et_3N, benzene, 2—toluene, Δ; (d) toluene; (e) HCl/MeOH.

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**

Table 1. Comparison of efficacy (E_{max}) and potency (pEC_{50}) values of 2-AG, 2-AGE and **1**–**5**

Compound	Structure	CB1 activation ($n = 3$)	
		E_{max} (% basal ± SEM)	$pEC50$ ± SEM
2-AG		620 ± 5	6.0 ± 0.0
2-AGE		484 ± 7	5.2 ± 0.0
1		521 ± 35	4.6 ± 0.1
2		475 ± 2	4.6 ± 0.0
3		590 ± 76	4.3 ± 0.1
4		548 ± 27	4.5 ± 0.1
5		309 ± 8[a]	4.0 ± 0.1[a]

The activation data have been produced using the method G described in Section 4.
[a] $n = 2$.

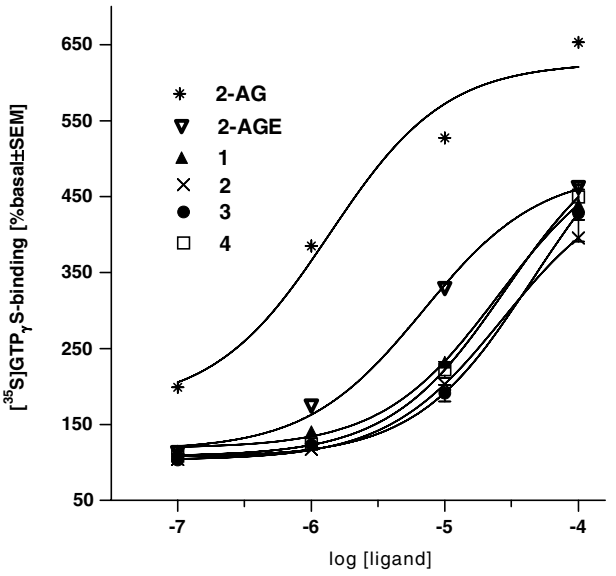


Figure 1. Dose–response curves for 2-AG, 2-AGE and **1**–**4** for the G-protein binding in rat cerebellar membranes (mean ± SEM, $n = 3$–4).

having the structure closer to that of 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; shorter chain length gives higher efficacy values. It seems that the ligand–receptor interactions of ester derivatives of endocannabinoids are substantially more sensitive to structural changes than amide derivatives. This is in good agreement with the previous findings of Suhara et al. 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.[22] 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. 2-AG analogues with different degree of double bonds have also been investigated and arachidonic backbone with four double bonds seemed to be favoured, although the effect on the activity was not very drastic.[5]

Razdan and co-workers published results on carbamate and urea analogues of endocannabinoids aimed at better enzymatic stability.[20] In this 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. 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 and immobility) it was more potent than AEA or other urea derivatives synthesized. The authors were not able to explain this observation but it resembles the data published on 2-AG.[1,4,5,23] 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 flash chromatography, the compound requires clearly more polar solvents for elution, and the state of the product is more like wax than oil. In addition, the weak activation properties may also be a consequence of the strong intramolecular hydrogen bonds which can be observed in the ^{1}H NMR spectrum.

3. Conclusions

In the present study, dimethylheptyl derivatives of the endocannabinoids, 2-AG and 2-AGE, have been synthesized and their ability to activate the CB1 receptor have been determined by the [^{35}S]GTP$_\gamma$S binding assay using the rat cerebellar membranes. Modification of a pentyl tail of 2-AG with dimethylheptyl led to a dramatic potency decrease, while the impact on efficacy was much weaker. Replacement of the pentyl chain of 2-AGE with DMH resulted in similar loss of potency, whereas the efficacy remained comparable to that of 2-AGE. Shortening of the chain length from C_{22} to C_{21} 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, we conclude that unlike AEA-type compounds and classical cannabinoids, the activity properties of 2-AG and 2-AGE cannot be improved by replacement of the end pentyl chain with the DMH structure.

4. Materials and experimental procedures

4.1. Factor analysis

All computations were carried out on SGI O2 R5000 or R12000 workstations. Ligand structures were constructed using the molecular modelling package SYBYL 6.7.[24] The applied force field was Tripos force field,[25] and the atomic partial charges for the ligands were calculated by the Gasteiger–Hückel[26] method. A crystallographic template chosen for building the ligands was taken from the Protein Data Bank:[27] arachidonic acid complexed with adipocyte lipid–binding protein (PDB 1adl[28]). 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.

4.2. Chemistry

^{1}H NMR and ^{13}C NMR were recorded on a Bruker Avance 500 spectrometer operating on 500.1 and 125.8 MHz, respectively. $CDCl_3$ 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, Departemento Quìmica Orgànica, Universidade de Santiago de Compostela, Spain). Chemical shifts (δ) are reported in parts per million (ppm) downfield from

TMS. Following abbreviations are used: s = singlet, d = doublet, t = triplet, dd = doublet of doublets, dq = doublet of quartets, br s = broad singlet, qn = quintet and m = multiplet. Coupling constants are reported in hertz and the letter J indicates ^3J 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 groups. Elemental analyses for C, H and N were performed on a ThermoQuest CE Instruments EA1110-CHNS-O elemental analysator (ThermoQuest, Italy.) TLC was performed using silica gel (60 F_{254}) coated aluminium sheets (Merck, Germany).

4.3. Method A. EDC coupling

Corresponding acid (**6** or **17**) (1 equiv), glycidol (1.2 equiv), EDC (1.8 equiv) and DMAP (0.3 equiv) in dry CH_2Cl_2 (15 ml/mmol) were stirred at room temperature for 20 h. The solvent was evaporated under reduced pressure.

4.3.1. 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 petroleum ether/ethyl acetate (8/1). ^{1}H 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).

4.3.2. 15,15-Dimethyl-henicosa-all-*cis*-4,7,10,13-tetraenoic acid oxiranylmethyl ester (18). The crude product was purified by flash chromatography eluting with petroleum ether/ethyl acetate (10/1) yielding a colourless oil (52%). ^{1}H 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).

4.4. Method B. TFAA promoted stereoselective transformation of glycidyl ester

Corresponding oxiranylmethyl ester (**7** or **18**) (1 equiv) in dry CH_2Cl_2 (5 ml/mmol) was cooled to −20 °C and TFAA (6 equiv) 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 h, the reaction was concluded by evaporating the solvent under vacuum. The residue was purified by flash chromatography eluting with toluene.

4.4.1. 1,3-Bis-(trifluoroacetyl)-2-(16,16-dimethyl-docosa-all-*cis*-5,8,11,14-tetraenoic acid) propyl ester (8). Yield 83%. ^{1}H 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).

4.4.2. 1,3-Bis-(trifluoroacetyl)-2-(15,15-dimethyl-henicosa-all-*cis*-4,7,10,13-tetraenoic acid) propyl ester (19). Light yellow oil; yield 81%. ^{1}H 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).

4.5. Method C. Cleavage of trifluoroacetyl groups

Corresponding trifluoroacetyl propyl ester (**8** or **19**) (1 equiv) 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 equiv) and methanol (18.5 equiv) 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 concluded by evaporating solvents under reduced pressure without a heating bath.

4.5.1. 1,3-Bis-(hydroxy)-2-(16,16-dimethyl-docosa-all-*cis*-5,8,11,14-tetraenoic acid) propyl ester (1). ^{1}H 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 $C_{27}H_{46}O_4 \cdot 1/4H_2O$: C, 73.84; H, 10.62. Found: C, 73.50; H, 10.80.

4.5.2. 1,3-Bis-(hydroxy)-2-(15,15-dimethyl-henicosa-all-*cis*-4,7,10,13-tetraenoic acid) propyl ester (3). Yellowish oil; yield 95%. ^{1}H 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 $C_{26}H_{44}O_4 \cdot 1/7H_2O$: C, 73.79; H, 10.55. Found: C, 73.77; H, 10.82.

4.6. Method D. $LiAlH_4$ reduction and alcohol activation with methanesulfonyl chloride

$LiAlH_4$ (1 equiv) was added several portions into dry THF (1.7 ml/mmol), and the solution was cooled to 0 °C. Corresponding ester (**9** or **20**) (1 equiv) 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 °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 ether. The aqueous layer was separated from the filtrate and extracted several times with 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 equiv) and triethylamine (2.7 equiv) were dissolved in dry CH_2Cl_2 (10 ml/mmol). The reaction mixture was cooled to 0 °C and methanesulfonyl chloride (1.3 equiv) was added to the mixture. The cooling bath was removed and the mixture was stirred for 2 h 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.

4.6.1. Methanesulfonic acid 16,16-dimethyl-docosa-all-*cis*-5,8,11,14-tetraenyl ester (11). ^{1}H 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).

4.6.2. Methanesulfonic acid 15,15-dimethyl-henicosa-all-*cis*-4,7,10,13-tetraenyl ester (22). ^{1}H 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).

4.7. Method E. Addition of 5-hydroxy-2-phenyl-[1,3]-dioxane

KOH (12 equiv) and 5-hydroxy-2-phenyl-1,3-dioxane (12 equiv) were crushed together and dissolved in dry benzene (50 ml/mmol). The mixture was stirred at 50 °C for half an hour. Corresponding alcohol activated with methanesulfonyl group (**11** or **22**) (1 equiv) in dry benzene (50 ml/mmol) was added slowly to the reaction mixture and the solution was stirred at 50 °C for three days. The reaction was concluded by adding crushed KOH (6 equiv) and 5-hydroxy-2-phenyl-1,3-dioxane (6 equiv) to the reaction mixture and stirring it 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 ether, and the combined organic layers were washed with brine and dried over $MgSO_4$. The solvent was evaporated under vacuum.

4.7.1. 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 petroleum ether yielding a colourless oil (60%). ^{1}H 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).

4.7.2. 5-(15,15-Dimethyl-henicosa-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 petroleum ether yielding a yellow oil (50%). ^{1}H 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).

4.8. Method F. Deprotection of a diol

Corresponding 2-phenyl-[1,3]-dioxane derivative (**12** or **23**) was dissolved in a solution of concentrated HCl/MeOH (1/2) and stirred at room temperature for 3 h. MeOH was evaporated under reduced pressure, and the residue was diluted with ethyl acetate. The separated organic phase was washed with 5% $NaHCO_3$ followed by brine and finally dried over MgSO4, and the solvent was evaporated under reduced pressure.

4.8.1. 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% ethyl acetate in petroleum ether yielding a yellowish oil (68%). ^{1}H 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 (br s, 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, ^2J = 11.5, J = 5.1), 3.76 (dd, 2H, ^2J = 11.6, J = 4.4), 5.13–5.42 (m, 8H). ^{13}C 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 $C_{27}H_{48}O_3 \cdot 1/10H_2O$: C, 76.76; H, 11.50. Found: C, 76.62; H, 11.80.

4.8.2. 2-(15,15-Dimethyl-henicosa-all-*cis*-4,7,10,13-tetraenyloxy)-propane-1,3-diol (4). The crude product was purified by flash chromatography eluting with ethyl acetate/petroleum ether (1/1) solution yielding a brownish oil (37%). ^{1}H 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, ^2J = 11.6, J = 5.0), 3.77 (dd, 2H, ^2J = 11.6, J = 4.4), 5.12–5.43 (m, 8H). ^{13}C 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 $C_{26}H_{46}O_3 \cdot 1/4H_2O$: C, 75.95; H, 11.40. Found: C, 75.87; H, 11.36.

4.8.3. (2-Phenyl-[1,3]-dioxan-5-yl) carbamic acid benzyl ester (15). 2-Amino-1,3-propanediol **14** (1.0 g, 11.0 mmol) and Et_3N (2.3 ml, 16.6 mmol) were dissolved in dry EtOH (40 ml). The reaction mixture was cooled to 0 °C and benzyl chloroformate (4.7 ml, 32.9 mmol) was added slowly to the mixture. The cooling bath was removed and the mixture was stirred for 1 h at room temperature. The reaction was concluded by evaporating the solvent under vacuum, and the residue was purified

by flash chromatography eluting with 2% MeOH in CH_2Cl_2 to yield *N*-benzyloxycarbonyl-2-amino-1,3-propanediol as a white solid compound (2.0 g, 81%). ^{1}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). *N*-Benzyloxycarbonyl-2-amino-1,3-propanediol (2.0 g, 8.9 mmol), benzaldehyde (1.2 ml, 11.9 mmol) and *p*-TSA (20.0 mg, 0.1 mmol) in dry toluene (40 ml) were refluxed under a Dean–Stark water separator for 2 h. 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 ethyl acetate/petroleum ether (1/5) solution to yield a mixture of the cis- and trans-isomers as a white solid product (2.0 g, 72%). ^{1}H NMR: (trans) δ 3.56–3.60 (m, 2H ax), 4.07 (br s, 1H), 4.34 (dd, 2H eq, $^2J = 10.9$, $J = 4.8$), 4.60 (br s, 1H), 5.13 (s, 2H), 5.43 (s, 1H), 7.32–7.47 (m, 10H). ^{1}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).

4.8.4. 2-Phenyl-[1,3]-dioxan-5-yl-amine (16). *N*-Benzyloxycarbonyl-(2-phenyl-[1,3]-dioxan-5-yl)-amine **15** (2.0 g, 6.4 mmol) and 10% Pd/C (0.1 g) were dissolved in dry EtOH (150 ml). The reaction mixture was placed in a high-pressure hydrogenation reactor (300 psi, 60 °C). After 3 h, 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 (0.95 g, 92%). ^{1}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}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).

4.8.5. 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-heneicosa-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 room temperature for 10 min. DPPA (0.24 g, 0.9 mmol) was added slowly and the mixture was stirred for 2 h. The solvent was evaporated under vacuum and the product was purified by flash chromatography eluting with 5% ethyl acetate in petroleum ether to yield a yellowish oil (0.11 g, 51%). 15,15-Dimethyl-heneicosa-all-*cis*-4,7,10,13-tetraenyl azide (0.11 g, 0.3 mmol) was dissolved in dry toluene (2 ml) and stirred at 65 °C for 2 h. 2-Phenyl-[1,3]-dioxan-5-yl-amine **16** (60.0 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 h. The reaction was concluded by evaporating the solvent under reduced pressure to obtain an impure whitish waxy urea (0.19 g). ^{1}H 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).

4.8.6. 1-(14,14-Dimethyl-icosa-all-*cis*-3,6,9,12-tetraenyl)-3-(2-hydroxy-1-hydroxymethyl-ethyl)-urea (5). 1-(14,14-Dimethyl-eicosa-all-*cis*-4,7,10,13-tetraenyl)-3-(2-phenyl-[1,3]-dioxan-5-yl)-urea **13** (0.19 g, 0.4 mmol) was dissolved in a solution of concentrated HCl/MeOH (11/25 ml) and stirred at room temperature for 4 h. MeOH was evaporated under reduced pressure and the residue was diluted with ethyl acetate. The separated organic phase was washed with brine and dried over $MgSO_4$. The solvent was evaporated and the product was purified by flash chromatography eluting with 1% MeOH in CH_2Cl_2 to yield a whitish waxy product (38.0 mg, 46%). ^{1}H 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). ^{13}C 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: $m/z = 435.3$ $[M+H]^+$. Elemental analysis. Calculated for $C_{26}H_{46}N_2O_3 \cdot 1/3H_2O$: C, 70.87; H, 10.67; N, 6.36. Found: C, 70.91; H, 10.52; N, 6.00.

4.9. CB1 receptor activity based on [^{35}S]GTPγS membrane binding studies

4.9.1. Method G. The [^{35}S]GTPγS membrane binding studies were preformed as previously described.[3] Maximal agonist responses (E_{max}, % basal) and potencies (pEC_{50}) were determined from dose–response curves. The CB1-dependent activity was confirmed by antagonizing half-maximal responses with the CB1 selective antagonist AM251 (10^{-6} M). Results are presented as means ± SEM of at least three independent experiments, performed in duplicate. Data-analysis was calculated as non-linear regression by GraphPad Prism 4.0 for Windows.

4.9.2. Method H. The [^{35}S]GTPγS membrane binding studies were preformed as previously described.[4] For agonist dose–response and antagonist experiments, results are presented as mean ± 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 was calculated as non-linear regression by GraphPad Prism 3.0 for Windows.

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.12.007.

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