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FULL PAPER

Synthesis and Biological Evaluation of Endocannabinoid Uptake Inhibitors Derived from WOBE437

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Abstract: WOBE437 ((2*E*,4*E*)-N-(3,4-dimethoxyphenethyl)dodeca-2,4-dienamide, **1**) is a natural product-derived, highly potent inhibitor of endocannabinoid reuptake. In this study, we have synthesized almost 80 analogs of **1** with different types of modifications in the dodecadienoyl domain as well as the dimethoxyphenylethyl head group and we have investigated their effects on anandamide uptake into U937 cells. Intriguingly, none of these analogs was a more potent inhibitor of anandamide uptake than WOBE437 (**1**). At the same time, a number of WOBE437 variants exhibited potencies in the sub-100 nM range with high selectivity over inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase; two compounds were virtually equipotent with **1**. Interestingly, profound activity differences were observed between analogs where either of the two methoxy substituents in the head group had been replaced by the same bulkier alkoxy group. Some of the compounds described here could be interesting departure points for the development of potent endocannabinoid uptake inhibitors with more drug-like properties.

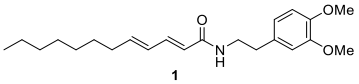
Introduction

The endocannabinoid system (ECS) is a ubiquitous lipid signaling system that comprises two G protein-coupled receptors (GPCRs), the cannabinoid receptors 1 and 2 (CB_1 and CB_2, respectively), their endogenous ligands, 2-arachidonoyl glycerol (2-AG) and *N*-arachidonoyl ethanolamine (anandamide, AEA), and five major enzymes that are responsible for ligand biosynthesis and degradation.[1] While CB_1 is primarily found in the central nervous system[2] (and has been suggested to be the most abundant GPCR in the mammalian brain),[1c] CB_2 is mostly found on immune cells;[3] however, the latter has also been detected in microglial cells, in astrocytes and in certain neurons.[4] The ECS plays a key role in the regulation of cell and tissue homeostasis in virtually all organ systems and its dysregulation, consequently, is associated with a variety pathological conditions, including neurological disorders,[1a] inflammatory conditions, and metabolic diseases.[1c]

Given its involvement in many pathophysiological processes, it is not surprising that the ECS has emerged as an important target system for drug development in different indication areas.[1a-c] In particular, a number of CB_1 or CB_2 agonists and antagonists have been evaluated in clinical trials in humans, although the success of these efforts so far has been limited.[1c] The most advanced example of a synthetic CB receptor ligand is the CB_1 *antagonist* rimonabant, which was approved in 2006 in the European Union for the treatment of obese or overweight patients with additional risk factors such as diabetes or dyslipidemia as adjunct to diet and exercise.[5] However, the compound was never approved in the US[6] and it was withdrawn from the European market in 2008 due to frequent psychiatric side effects.[5] Likewise, CB_1 receptor agonists are fraught with significant central side effects.[1c]

Compared to direct CB activation, promoting EC signalling through inhibition of the major EC-catabolic enzymes fatty acid amide hydrolase (FAAH) or monoacylglycerol lipase (MAGL) may offer a safer alternative towards ECS-directed therapeutics.[1a][6] Based on this premise, a number of FAAH inhibitors have been advanced into clinical trials, mostly against neurological disorders.[1a][1c] Unfortunately, this approach suffered at least a temporary setback in 2016 due to very serious adverse events in a Phase I clinical trial with BIA 10-2474.[7] However, subsequent work has shown that these events were most likely caused by off-targets effects in the brain, rather than inhibition of FAAH.[8]

Apart from inhibition of FAAH or MAGL, EC signaling can also be enhanced through inhibition of AEA or 2-AG uptake,[1a][9] as this prolongs the availability of these transmitters for interaction with CB receptors. In this context, we have recently reported the discovery of the dodeca-2,4-dienamide WOBE437 (**1**) as the first selective, potent AEA uptake inhibitor.[10]



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WOBE437 (**1**) blocks uptake of AEA into U937 cells with an IC_{50} of 10 nM, while neither affecting the uptake of 2-AG nor the hydrolysis of AEA by FAAH (selectivity ratios of 28 and 977, respectively). The compound has been demonstrated to exert cannabimimetic effects in vivo and, more recently, has also been shown to be orally active.[11] At a more general level, the specific effect of WOBE437 (**1**) on AEA uptake, together with other, indirect evidence points to the existence of a dedicated AEA membrane transport system.[9][12] However, the exact component(s) of this system (protein(s), lipids) and its working mechanism still need to be elucidated.

Here, we describe the synthesis of a series of more than 70 analogs of WOBE437 (**1**) with modifications either in the C12 acyl chain or in the (dimethoxyphenyl)ethyl head group and the assessment of their effects on AEA uptake in U937 cells. In addition, we have also determined the effect of most compounds on FAAH activity, in order to ensure that potent AEA uptake inhibition did not result from inhibition of AEA degradation; the latter would reduce the inward-directed concentration gradient that provides the major driving force for AEA uptake into cells.[13] While some of these compounds were already included in the SI of our previous WOBE437 study,[10] their synthesis was not described there and the corresponding biological data were not commented on in detail. Thus, the current study represents the first comprehensive SAR analysis of WOBE437-derived structures. An SAR study around the natural product guineensine, which one of our groups had disclosed as a potent inhibitor of AEA uptake prior to publication of our work on WOBE437 (**1**),[14] has recently appeared.[15] While our investigations of WOBE437 analogs so far have not yielded any compounds with an improved inhibitory profile, our SAR data provide important insights into the relevance of individual structural features of WOBE437 (**1**) for potent and selective inhibition of AEA uptake.

Results and Discussion

Acyl chain modifications. The initial questions to be addressed in our SAR work were related to the significance of the length of the acyl chain in WOBE437 (**1**), the number and positions of double bonds, and the tolerance to modifications in the saturated C7 segment following the dienoyl moiety. The corresponding target structures **2** - **21** are depicted in Fig. 1.

As shown in Scheme 1, analogs **2** - **11** and **17** - **21** were obtained by HWE olefination of phosphonate **82** with the appropriate aldehydes. The latter were either commercially available or prepared according to standard procedures (see the SI). Phosphonate **82** was synthesized from *O*,*O*-dimethyl dopamine (**81**) by acylation with chloroacetyl chloride and reaction of the ensuing chloroacetamide with triethyl phosphite at 150 °C.

An alternative approach for acyl chain assembly was followed in the synthesis of analogs **13** and **14**, which was based on cross-metathesis between terminally unsaturated amide **83** and non-1-ene in the presence of Grubbs II catalyst[16] (Scheme 1). The reaction gave a 5:1 mixture of **13** and **14** in a total yield of 29%; **13** and **14** could be separated by preparative RP-HPLC to yield stereochemically pure samples of both isomers. Amide **83** was obtained by EDC/HOBt-mediated coupling[17] of 4-pentenoic acid with amine **81**. Likewise, analogs **12** and **16** were prepared from amine **81** and the requisite carboxylic acids by EDC/HOBt-mediated amide bond formation, while **15** was obtained through catalytic hydrogenation of **1**. (See the SI for details).

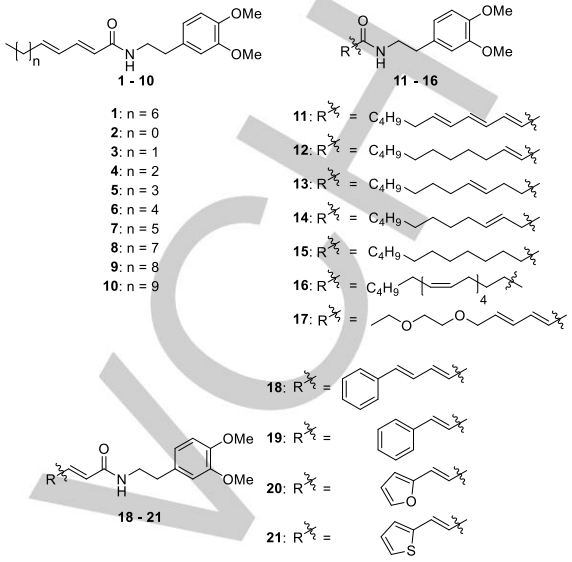
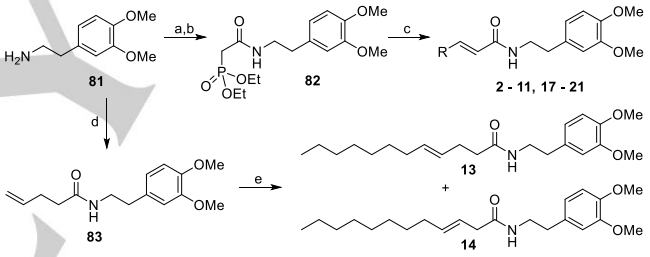


Figure 1. WOBE437 analogs with modified acyl chains I.



Scheme 1. Reagents and conditions: a) Chloroacetyl chloride, Et_3N, CH_2Cl_2, 0 °C to rt, 15 min, 93%; b) $P(OEt)_3$, 150 °C, 2 h, 99%; c) R-CHO, KO*t*Bu, THF, rt, 15 min, 52-88%; d) pent-4-enoic acid, $(COCl)_2$, CH_2Cl_2, rt, 20 min, then **81**, Et_3N, CH_2Cl_2, rt, 10 min, 44%; e) non-1-ene, Grubbs II catalyst, CH_2Cl_2, 40 °C, 6 h, 29% (**13**/**14** = 5:1).

As for the variations in acyl chain length, changes in the number of double bonds led to a clear loss in potency relative to **1**, with the incorporation of an additional double bond between C6 and C7 being better tolerated than the removal of either of the double bonds present in WOBE437 (**1**). Surprisingly, the fully reduced analog **15** was more potent than any of the analogs containing a single double bond. As we have shown previously, the replacement of the dodecadienoyl moiety by an arachidonoyl residue is reasonably well tolerated[10] and the corresponding analog **16** is only 6-fold less potent than **1**, without affecting FAAH activity (selectivity index (SI) = 106; for definition of SI see Table 1).

Overall, the SAR around WOBE437 (**1**) with regard to acyl chain length as well as the number and position of double bonds is intriguingly steep. Thus, with the exception of arachidonoyl derivative **16**, which incorporates the acyl chain of the natural substrate, all modifications investigated here led to a decrease in potency of at least 10-fold. This finding re-enforces our previous conclusion that WOBE437 (**1**) is a highly specific inhibitor of AEA uptake, whose activity is not based on simple, non-specific

lipophilic interactions. We assume that the specificity of **1** arises from both defined interactions with a hitherto uncharacterized protein target but also from specific interactions with membrane components. Perhaps unsurprisingly then, the incorporation of heteroatoms into the saturated segment of the acyl chain (analog **17**) proved to be detrimental for AEA uptake inhibitory activity.

Table 1. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with modified acyl chains I.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
2	>100 (ND)	43	>10 (79)[f]	ND
3	87 (ND)	54	>10 (83)[f]	ND
4	35 (ND)	67	>10 (89)[f]	ND
5	10 (ND)	73	>10 (72)[f]	ND
6	2.1 (1.332-3.366)	69	>10 (74)[f]	ND
7	0.6 (0.3-1)	73	>10 (67)[f]	ND
1	0.01 (0.007-0.015)	79	9.8	980
8	0.14 (0.11-0.19)	74	16.5 (10.3-21.9)	117
9	0.17 (0.12-0.25)	73	9.8 (5.6-15.1)	101
10	0.097 (0.062-0.218)	69	7.7 (4.9-11.0)	79
11	0.11 (0.06-0.19)	96	12.3 (7.5-16.2)	112
12	0.55 (0.44-0.70)	74	5.1 (3.4-6.3)	9
13	1.1 (1.0-1.3)	74	>1	ND
14	2.0 (1.6-2.7)	86	>1	ND
15	0.22 (0.140-0.360)	74	>10	>45
16	0.064 (0.041-0.099)	73	6.8 (4.9-8.2)	106
17	> 10	ND	>10	ND
18	3.02 (1.411-6.455)	65	>1 (69)[f]	ND
19	49.0 (ND)	54	>1 (67)[f]	ND
20	61.7 (ND)	61	>1 (74)[f]	ND
21	69.0 (ND)	65	>1 (78)[f]	ND

[a] For structures, *cf.* Fig. 1. The data for cpds. **2** - **16** are included in the SI for ref[10], but they are not discussed in this reference in any detail. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 95% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay data varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [f] Number in parentheses indicates the remaining FAAH activity at 1 μM cpd. concentration. [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

With the impact of acyl chain length variations on the activity of WOBE437 analogs established, we next wanted to investigate the effects of modifications leading to additional rigidization of the acyl chain at the site of the diene moiety. To this end, we prepared analogs **22** - **27** that incorporate a *para*- or *meta*-substituted phenylene moiety either directly attached to the carbonyl carbon (analogs **22** and **23**) or separated from the carbonyl carbon by one CH_2 group (analogs **24** - **27**) (Fig. 2). For the *meta*-substituted analogs the number of consecutive carbons in the acyl chain (12) corresponds with that in WOBE437 (**1**) (for the shorter path through the aromatic ring).

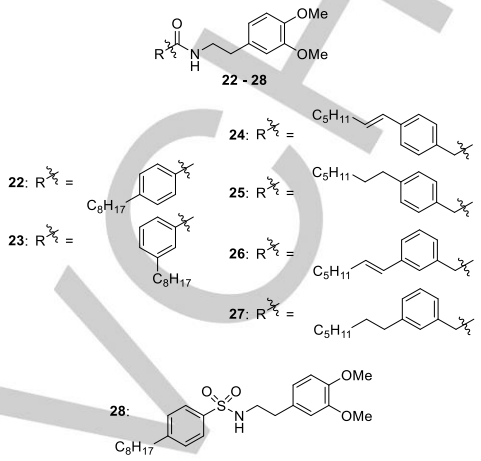
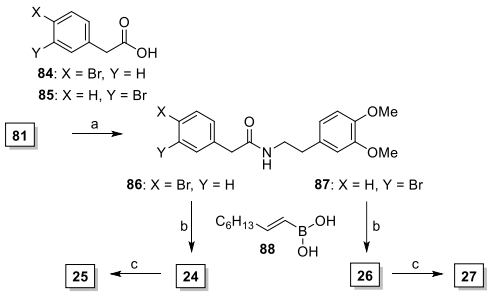


Figure 2. WOBE437 analogs with modified acyl chains II.

Analogs **22** and **23** were prepared by EDC/HOBt-mediated amide bond formation[17][18] from amine **81** (*cf.* Scheme 1) and *p*- or *m*-octylbenzoic acid, respectively. (See the SI for details). The synthesis of WOBE analogs **24** - **27** is summarized in Scheme 2 and in the key step involved the attachment of the distal part of the acyl side chain to aromatic bromides **86** and **87** by Suzuki coupling;[19] the latter, in turn, had been obtained by EDC/HOBt-mediated couplings[17][18] between amine **81** and *p*- and *m*-bromo phenylacetic acids **84** and **85**, respectively. The Suzuki couplings proceeded in good yields (59% and 76%) and the ensuing unsaturated analogs **24** and **26** were then transformed into saturated derivatives **25** and **27**, respectively, by Pd/C-catalyzed reduction with Et_3SiH.[20] Sulfonamide-based analog **28** was obtained by the reaction of *p*-octylphenylsulfonyl chloride with amine **81** (see the SI for details).



Scheme 2. Reagents and conditions: a) EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 8-16 h, 60-90%; b) $Pd(OAc)_2$, K_2CO_3, DME/H_2O 2:3, 90 °C, 10-14 h, **24**: 59%, **26**: 76%; c) Et_3SiH, Pd/C, EtOH, 1-2 h, **25**: 83%, **27**: 96%.

All analogs **22** - **27** were less potent inhibitors of AEA uptake into U937 cells than WOBE437 (**1**), albeit to different extents (Table 2). While **22** was only ca. 10-fold less active than **1**, the IC_{50} for **24** was increased by more than 100-fold. *Para*-substituted analog

22 appeared to be slightly more potent than the *meta*-substituted variant **23**, although the *meta*-substitution pattern provides the better match for a dodeca-2,4-dienoyl chain in a fully extended conformation; however, the difference between **22** and **23** is very small and may not be significant. For analog pairs **24/26** and **25/27**, there is no clear preference for one of the substitution patterns; likewise, saturation of the double bond of the styryl moiety of **25** and **26** led to opposite effects on inhibitory activity. The replacement of the amide bond in **22** by a sulfonamide moiety resulted in a *ca.* 20-fold reduction in potency.

Table 2. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with modified acyl chains II.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d]	SI[e]
22	0.11 (0.08-0.15)	65	5.3 (4.5-5.9)	49
23	0.23 (0.17-0.32)	71	12.3 (10.6-14.1)	52
24	1.2 (0.3-4.2)	65	>5	ND
25	0.69 (0.47-1.00)	64	13.6 (12.3-15.4)	20
26	0.33 (0.047-2.33)	68	21.8 (18.5.-22.9)	66
27	0.72 (0.367-1.41)	63	>5	ND
28	2.3 (0.846-6.31)	81	ND	ND
29	0.28 (0.18-0.44)	103	13.7 (11.6-17.0)	49
30	1.0 (0.47-2.4)	128	>10 (85)[f]	ND
31	1.3 (0.74-2.5)	74	14.1 (11.1-16.8)	11
32	0.73 (0.38-1.4)	64	16.7 (13.0-21.1)	23
33	0.96 (0.67-1.36)	56	2.1 (1.4-3.0)	2.2
34	2.8 (1.2-6.6)	63	4.6 (3.3-6.3)	1.6
35	1.3 (0.6-2.9)	58	12.6 (8.6-18.1)	9.5
36	Ca. 20% Inh. @1uM	ND	ND (15)[g]	ND
37	Ca. 41% Inh. @1uM	ND	ND (30)[g]	ND

[a] For structures, *cf*. Figs. 2 and 3. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 95% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay data varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [f] Number in parentheses indicates the remaining FAAH activity at 1 μM cpd. concentration. [g] Number in parentheses indicates the remaining FAAH activity at 5 μM cpd. concentration. [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

Based on the observations with WOBE437 analogs **22** - **27**, we subsequently prepared a series of congeners of **22**, with variations in the nature of the substituent *para* to the amide moiety (Fig. 3). The synthesis of analog **31** involved Suzuki coupling[17] between aromatic bromide **92** and boronic acid **94**, which proceeded in high yield (84%) (Scheme 3); reduction of the double bond in **31** with Et_3SiH in the presence of Pd/C gave **32**. Hetero-substituted analogs **35** and **37** were derived from aromatic fluoride **90** by nucleophilic aromatic substitution with 4-heptylphenol and heptylamine, respectively (33% and 90% yield).

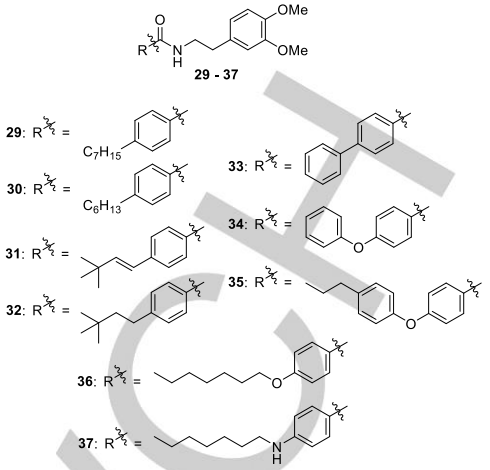
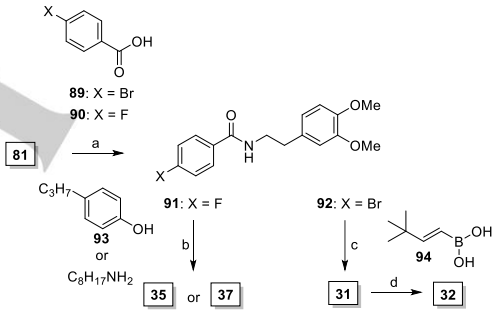


Figure 3. WOBE437 analogs with modified acyl chains III.

Analogs **33**, **34**, and **36** were obtained through EDC/HOBt-mediated coupling[17][18] of the corresponding (commercially available) carboxylic acids and amine **81** (see the SI for details).



Scheme 3. Reagents and conditions: a) EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 8-16 h, **91**: 90%, **92**: 98%; b) **35**: **93**, K_2CO_3, DMF, rt, 20 h, 90%; **37**: $C_7H_{15}NH_2$, K_2CO_3, DMSO, 120 °C, 12 h, 33%; c) $Pd(OAc)_2$, K_2CO_3, DME/H_2O 2:3, 90 °C, 14 h, 84%; d) Et_3SiH, Pd/C, EtOH, 1 h, 99%.

As illustrated by the data in the lower part of Table 2, all modifications of the substituent in the *para*-position of the phenylacetamide moiety in **22** led to a reduction in potency by ca. 3- (**29**) to ca. 30-fold (**34** and perhaps **36**). Thus, shortening of the octyl substituent in **22** is not tolerated, even if the nominal length of the acyl chain in **29** corresponds with that of WOBE437 (**1**) (12 carbons), while it is one carbon longer in **22**. Of note, the simple replacement of the CH_2 group directly attached to the aromatic ring by a heteroatom led to less potent analogs (**36** and **37**), compared to the parent compound **29**.

Head group modifications. In a first set of head group modifications (i. e. modifications of the *N*-phenylethyl moiety), we investigated the importance of each of the methoxy groups on the phenyl ring (analogs **38** - **47**) and of the length of the linker between the phenyl group and the amide nitrogen (analogs **48** and **49**) (Fig. 4).

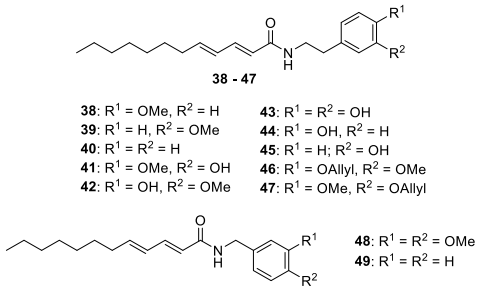
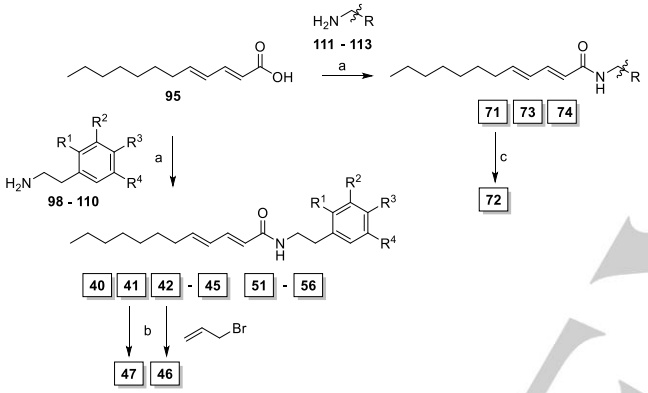


Figure 4. WOBE437 analogs with modified head groups I.

The majority of these analogs were prepared by direct EDC/HOBt-mediated coupling[17][18] of acid **95** with the respective (commercially available) amines; **46** and **47** were obtained by alkylation of **42** and **41**, respectively with allyl bromide in excellent yields (Scheme 4).



Scheme 4. Reagents and conditions: a) For all compounds, except for **51** and **56**: EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 8-16 h, 27-91%. For **51**: $(COCl)_2$, CH_2Cl_2, rt, 20 min, then 2-(2-fluoro-4,5-dimethoxyphenyl)ethylamine (**105**), CH_2Cl_2, rt, 10 min, 27%; for **56**: $(COCl)_2$, CH_2Cl_2, rt, 15 min, then 2-(3,4,5-trimethoxyphenyl)ethylamine (**110**), CH_2Cl_2, rt, 15 min, 27%; b) allyl bromide, K_2CO_3, DMF, 50 °C, 5.5 h, **46**: 84%, **47**: 78%; c) 4M HCl in 1,4-dioxane, rt, 1 h, 87%.

We have previously reported that the removal of each or both of the methoxy groups in WOBE437 (**1**) (analogs **38** - **40**) leads to a significant loss in AEA uptake inhibitory activity;[10] these results have been confirmed in the current study (within the bounds of the reproducibility of our transport assay) (Table 3).

A similar loss in potency was incurred by demethylation of the methoxy group in position *meta* to the ethyl linker to the amide nitrogen (analog **41**), while demethylation of the *p*-methoxy group appeared to be less critical (analog **42**). Thus, **42** was only 7-fold less potent than WOBE437 (**1**) (Table 3). Removal of both methyl groups produced an analog (**43**) with an activity that was >100-fold lower than that of WOBE437 (**1**). Interestingly, the demethylation products of mono-methoxy derivatives **38** and **39**, i. e. **44** and **45**, respectively, both exhibited comparable activity as the corresponding parent compound. Thus, once one of the methoxy substituents in WOBE437 (**1**) is removed, the demethylation of the remaining methoxy group is of no further consequence, in contrast to the effects observed upon demethylation of **1**, **41**, and **42**. In fact, mono-hydroxylated analogs **44** and **45** were more potent than bis-phenol **43**.

Replacement of either of the methoxy groups in **1** by a larger allyloxy group (analogs **46** and **47**) was reasonably well tolerated, with the corresponding analogs both exhibiting IC_{50} values for AEA transport inhibition below 100 nM.

Table 3. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with modified head groups I.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
38	0.22 (0.16-0.35)	64	2.61 (0.8-3.0)	12
39	0.27 (0.10-0.72)	56	12.4 (10.4-14.1)	46
40	0.36 (0.18-0.73)	69	16.5 (12.2-19.3)	46
41	0.31 (0.21-0.46)	77	18.2 (15.2-23.4)	59
42	0.067 (0.045-0.098)	67	5.9 (2.6-7.4)	88
43	1.1 (0.4-2.9)	98	>10 (100)[f]	ND
44	0.29 (0.20-0.44)	72	2.2 (1.0-2.6)	7.6
45	0.22 (0.11-0.44)	79	1.0 (0.7-1.6)	4.5
46	0.08 (0.016-0.480)	66	12.9 (9.7-15.2)	161
47	0.057 (0.038-0.085)	78	20.5 (18.3-22.7)	44
48	0.41 (0.29-0.58)	51	>100	ND
49	0.32 (0.063-1.6)	73	>100	ND

[a] For structures, *cf.* Fig. 4. Data for cpds. **38** - **40, 43,** and **49** were already reported in the SI of ref[10]. Data for **38**, **39**, and **43** are directly taken from ref[10], those for **40** and **49** were (re)determined in this study. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 95% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [f] Value in parentheses indicates the remaining FAAH activity at 1 μM cpd. concentration. [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

Shortening the *N*-ethyl linker in WOBE437 (**1**) to a CH_2 group produced an analog (**48**) with ca. 40-fold reduced potency; interestingly, and in contrast to what is observed for the parent compound **1**, the activity of **48** was not reduced upon removal of the methoxy groups (Table 3).

As an extension of the above studies on demethoxylated and demethylated derivatives of WOBE437 (**1**), we have also investigated head group-modified analogs incorporating an additional substituent at position 2 or 3 of the dimethoxyphenyl moiety (Fig. 5).

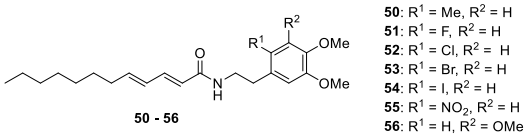
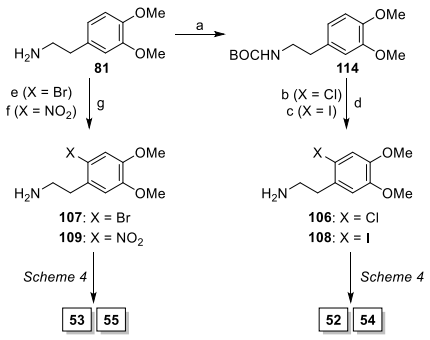


Figure 5. WOBE437 analogs with modified head groups II.

Analogs **52** - **55** were prepared by EDC/HOBt-mediated coupling[17][18] of acid **95** with the requisite amines; **51** and **56** were obtained via the acid chloride (Scheme 4). For the synthesis of **50** see the SI.

As depicted in Scheme 5, chlorinated and iodinated amine precursors **106** and **108**, respectively, were obtained by halogenation of *N*-BOC-protected 2-(4,5-dimethoxyphenyl)ethyl amine (**114**) with *N*-chlorosuccinimide or elementary iodine followed by BOC-removal with HCl/dioxane in good overall yields. Brominated amine **107** and nitro-substituted derivative **109** were prepared by direct bromination and nitration, respectively, of unprotected amine **81**. The syntheses of 2-(2-methyl-4,5-dimethoxyphenyl)ethyl amine, 2-(2-fluoro-4,5-dimethoxyphenyl)-ethyl amine, and 2-(3,4,5-trimethoxyphenyl)ethyl amine are described in the SI.



Scheme 5. Reagents and conditions: a) Boc_2O, THF, 0 °C to rt, 12 h, 99%; b) NCS, $Si(CH_3)_3Cl$, MeCN, rt, 2 h, 81%; c) I_2, CF_3COOAg, $CHCl_3$, rt, 2 h, 97%; d) 4M HCl in 1,4-dioxane, 1,4-dioxane, rt, 2 h, **106**: quant, **108**: 99%; e) Br_2, AcOH, rt, 16 h, 98%; f) HNO_3, H_2O, rt, 3 h, 77%.

Substitution of WOBE437 (**1**) at the 2-position of the aromatic ring was associated with a decrease in potency for all substituent groups investigated, although IC_{50} values for AEA uptake inhibition still remained below 200 nM or even 100 nM (**53** and **54**) (Table 4). No difference was observed between the effect of a slightly electropositive methyl group and (strongly) electron-withdrawing substituents, such as fluoro, chloro, or nitro. For analogs **51** - **54** there was a trend of increasing potency with decreasing electronegativity/increasing size of the halogen substituent. It may thus be speculated that the potent activity of iodo derivative **56** is related to halogen bonding[21] with the hitherto elusive protein target(s) of WOBE437-type AEA uptake inhibitors.

Compared to all other analogs in Fig. 5, **56**, which incorporates an additional methoxy group at the 3-position of the phenyl ring, showed substantially lower activity. The IC_{50} value for **56** was >3 μM, which makes the compound ca. 300-fold less potent than WOBE437 (**1**). Whether this effect reflects a general incompatibility of transport inhibition with the presence of substituents at the 3-position of WOBE437 (**1**) or if it may be caused by the increased electron density in the aromatic ring remains to be determined.

Based on the results obtained with allyloxy analogs **46** and **47**, which had indicated that alkoxy substituents larger than a simple methoxy group are not necessarily detrimental for AEA uptake inhibitory activity, we have subsequently investigated WOBE437 analogs with significantly extended alkoxy substituents and bulky end groups (Fig. 6).

Table 4. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with modified head groups II.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
50	0.14 (0.078-0.27)	72	1.4 (1.0-2.0)	10
51	0.146 (0.123-0.173)	81	>1 (70)[f]	ND
52	0.185 (0.078-0.435)	63	15.0 (11.1-17.4)	81
53	0.081 (0.049-0.134)	62	14.7 (12.4-16.9)	181
54	0.043 (0.017-0.111)	62	13.9 (10.5-15.1)	323
55	0.12 (0.017-0.18)	74	0.8 (0.5-1.2)	6.8
56	3.4 (1.1-9.9)	71	>10	ND

[a] For structures, *cf.* Fig. 5. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 95% CI, 95% confidence interval. [c] Maximal inhibition of uptake of [^{3}H]-AEA into U937 cells. Maximum inhibition data in our uptake assay varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [f] The value in parentheses indicates the remaining FAAH activity at 1 μM cpd. concentration. [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

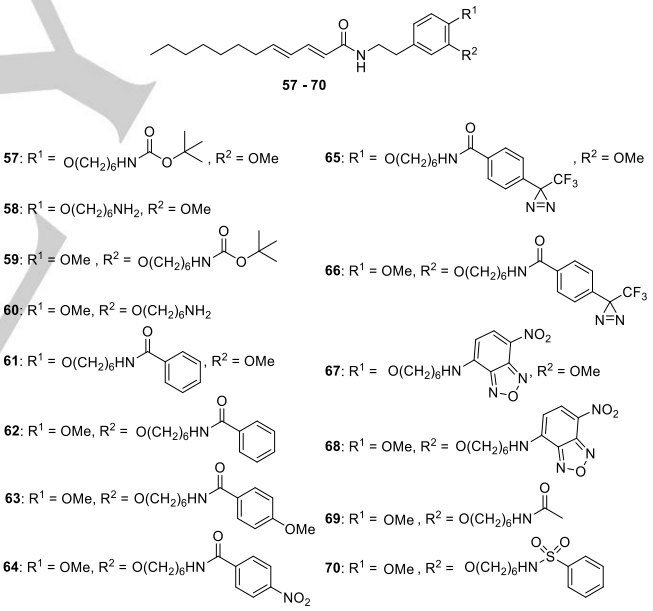
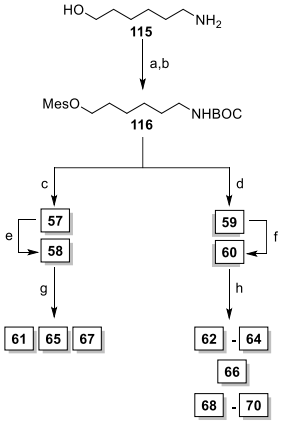


Figure 6. WOBE437 analogs with modified head groups III.

These studies were carried out in the context of our work on the identification of fluorescently labelled and/or reactive probe molecules for the identification of proteins involved in EC transport. This work has led to the discovery of the previously reported, highly versatile probe RX-55 (**66** in Fig. 6),[10] but it has also revealed some intriguing features of the WOBE437 SAR (*vide infra*).

The synthesis of analogs **57** - **70** proceeded through analogs **41** and **42** as intermediates, which were alkylated with mesylate **116** in yields of 66% and 84%, respectively (Scheme 6). Subsequent removal of the BOC-protecting group with TFA gave the

corresponding free amines, which were further elaborated into the desired target structures by acylation (**61** - **66**, **69**), alkylation (**67**, **68**), or sulfonylation (**70**) in very good to excellent yields (Scheme 6). (The synthesis of **65** and **66** has also been described in ref[10]).



Scheme 6. Reagents and conditions: a) Boc_2O, CH_2Cl_2, rt, 16 h, quant.; b) MesCl, Et_3N, CH_2Cl_2, 0 °C, 20 min, quant.; c) **42**, K_2CO_3, KI, DMF, 90 °C, 12 h, 84%; d) **41**, K_2CO_3, KI, DMF, 90 °C, 12 h, 66%; e) TFA, CH_2Cl_2, rt, 2 h, 87%; f) TFA, CH_2Cl_2, rt, 2 h, 86%; g) **61**: PhCOCl, Et_3N, CH_2Cl_2, rt, 5 min, 85%; **65**: RCOOH, EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 12 h, 88%; **67**: 4-chloro-7-nitrobenzoxadiazole, $NaHCO_3$, $CHCl_3$/ MeOH 1:1, rt, 18 h, 98%; h) **62**: PhCOCl, Et_3N, CH_2Cl_2, rt, 5 min, 96%; **63**, **64**, and **66**: RCOOH, EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 8-16 h, 85-87%; **68**: 4-chloro-7-nitrobenzoxadiazole, $NaHCO_3$, $CHCl_3$/ MeOH 1:1, rt, 18 h, 78%; **69**: Ac_2O, Et_3N, DMAP, CH_2Cl_2, rt, 30 min, 87%; **70**: $PhSO_2Cl$, Et_3N, CH_2Cl_2, rt, 30 min, 84%.

As illustrated by the data summarized in Table 5, several of the analogs **57** - **70** were highly potent inhibitors of AEA uptake, with **63** and **66** closely approaching the activity of WOBE437 (**1**). It is noteworthy that these analogs retained the outstanding selectivity over FAAH, thus clearly indicating that inhibition of AEA uptake was not caused by compromised FAAH activity; as the only example, selectivity was somewhat reduced for analog **59**, although the compound was still selective. Compared to their BOC-protected precursors **57** and **59**, amines **58** and **60**, respectively, were clearly more potent; with no differences in activity being observed between the respective regioisomers. In contrast, and very intriguingly, a profound dependence of AEA uptake inhibitory potency on the position of the extension on the aromatic ring is observed for **65** and **66** and, albeit to a lesser extent, for the pairs of regioisomers **61**/**62** and **67**/**68**. In all three cases the isomer with the extension attached to the *meta* oxygen is significantly more potent. It is somewhat puzzling, why such a difference is not observed for amines **58** and **60** or, in particular, their BOC-derivatives **57** and **59**, as the latter also carry a bulky *N*-substituent at the chain terminus. Comparison of **62**, **63**, and **64** reveals that both electron-donating as well as electron-withdrawing substituents are reasonably well tolerated at the *para* position of the benzamide moiety. Likewise, sulfonamide-based analog **70** almost retains the activity of the amide-based parent compound **61**. Our previous report on WOBE437 (**1**)[10] also included analogs with an *N*-*iso*-butyl or an *N*-(2-hydroxyethyl) amide head group, which are part of the natural product (2*E*,4*E*)-*N*-*iso*-butyl dodeca-2,4-dienamide from different *Echinacea* species[22] and of the natural transport substrate AEA (anandamide), respectively. Both compounds were found to be poor inhibitors of AEA uptake into U937 cells.

Table 5. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with modified head groups II.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
57	0.16 (0.056-0.50)	43	2.1 (1.2-2.9)	13
58	0.04 (0.03-0.06)	85	2.24 (1.0-4.3)	56
59	0.16 (0.07-0.37)	69	1.0 (0.63-1.7)	6
60	0.036 (0.026-0.051)	88	0.62 (0.35-0.90)	17
61	0.19 (0.108-0.34)	78	>10	>50
62	0.013 (0.010-0.018)	72	13.2 (10.1-17.9)	1015
63	0.028 (0.018-0.043)	76	16.8 (10.1-20.0)	600
64	0.069 (0.058-0.828)	80	>10	>145
65	2.0 (0.60-6.5)	65	ND	ND
66	0.016 (0.011-0.023)	82	14.8 (12.4-18.0)	925
67	1.9 (0.55-6.4)	94	ND	ND
68	0.21 (0.14-0.31)	86	6.0 (2.3-14.7)	28
69	0.12 (0.079-0.18)	82	9.2 (6.7-11.2)	77
70	0.063 (0.054-0.071)	85	12.9 (5.6-15.5)	204

[a] For structures, *cf.* Fig. 6. Data for cpds. **65** and **66** have already been reported in ref[10]. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 950% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay data varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

Notwithstanding these negative findings, we have also investigated a very small set of WOBE437 analogs with other non-aromatic head groups; in addition, we have prepared one analog incorporating an alternative (non-phenylethyl/benzyl) aromatic amide substituent (Fig. 7).

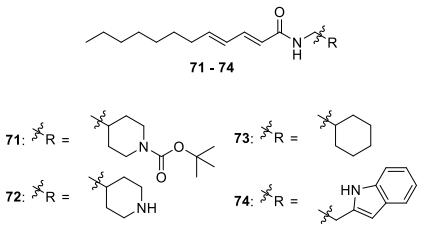


Figure 7. WOBE437 analogs with non-phenylethyl/benzyl amide head groups

Compounds **71**, **73**, and **74** were prepared by EDC/HOBt-mediated coupling[17][18] of acid **95** with the (commercially available) amines **111**-**113**, respectively, in good yields (Scheme 4; for structures of **111**-**113** see the SI); **72** was obtained by BOC-removal from **71** with HCl/dioxane.

Analogs **71** and **72** were >10-fold and >30-fold less active, respectively, than the corresponding parent compound **49** (and, thus, several hundred-fold less active than WOBE437 (**1**);

similarly, indole derivative **74** was >100-fold less potent than WOBE437 (**1**) (Table 6).

Table 6. Inhibition of AEA uptake into U937 cells by WOBE437 analogs with non-phenylethyl/benzyl amide head groups.[a]

Cpd.[a]		EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
71	9.5	(3.5-15.6)	49	>10	ND
72	4.19	(1.37-12.5)	79	>10	ND
73	0.18	(0.069-0.49)	49	ND	ND
74	1.11	(0.68-1.83)	68	ND	ND

[a] For structures, *cf*. Fig. 7. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 950% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay data varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

In contrast, cyclohexyl derivative **73**, even if being ca. 20-fold less active than WOBE437 (**1**), retained substantial potency; in fact, the compound was somewhat more potent than the corresponding phenyl derivative **49** (Fig. 4, Table 3) and it is orders of magnitude more potent than its *iso*-butyl amide congener.

In order to assess the effects of substitution of either the acyl chain or the ethyl linker on AEA uptake inhibition, we finally investigated the chiral WOBE437 analogs depicted in Fig. 8.

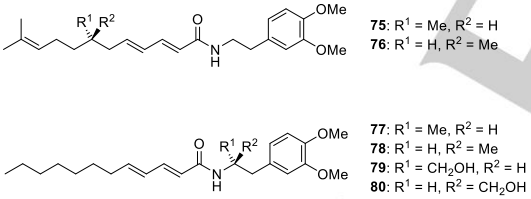
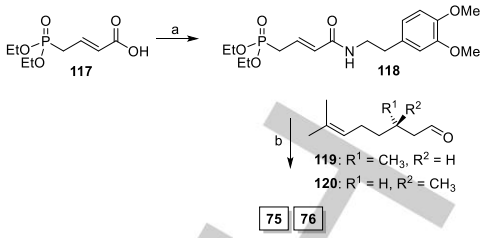


Figure 8. Chiral WOBE437 analogs.

We were well aware that analogs **75** and **76** incorporate multiple modifications, which would prevent an unambiguous structural interpretation of any activity differences to WOBE437 (**1**); however, these compounds were attractive, due to their ready accessibility from citronellal, which is commercially available in both enantiomeric forms. Thus, HWE reaction of either *S*-citronellal (**119**) or *R*-citronellal (**120**) with phosphonate **118** directly led to **75** and **76**, respectively (Scheme 7). (For the synthesis of **77** - **80** see the SI).



Scheme 7. Reagents and conditions: a) **81** (*cf*. Scheme 1), EDC•HCl, HOBt, Et_3N, CH_2Cl_2, rt, 12 h, 100%; b) For **75**: LDA, THF, -78 °C, 30 min, then **119**, -78 °C to -20 °C, 20 min, 32%; for **76**: LDA, THF, -78 °C, 30 min, then **120**, -78 °C to -20 °C, 20 min, 47%.

Analogs **75** and **76** both were less potent than WOBE (**1**) by ca. 25- to 30-fold, with no significant difference being detectable between the two enantiomers (Table 7). Likewise, the incorporation of a methyl or a hydroxymethyl substituent at C1 of the ethylene linker between the amide nitrogen and the phenyl ring resulted in a reduction in AEA uptake inhibitory activity; however, measurable differences were now observed between enantiomers. Thus, methyl-substituted analog **78**, which is only 7-fold less active than WOBE437 (**1**), is ca. 3-fold more potent than its *R*-enantiomer **77**. The stereochemical preference is reversed for hydroxymethyl substitution, with **79** now being ca. 4.5-fold more potent than **80** (note that due to a change in priority for a hydroxymethyl *vs*. a methyl substituent, the more potent isomer **79** is still *S*-configured). Overall, there is an almost 8-fold preference for a C1-methyl over a C1-hydroxymethyl group for one spatial orientation of the substituent (**78** vs. **80**), while the difference is only 2-fold for the other (**77** vs. **90**). However, it needs to be kept in mind that all differences between analogs **75** - **80** are relatively small and should not be overinterpreted. Nevertheless, the fact that chirality can be incorporated into WOBE437 (**1**) with tolerable effects on potency could be important for future optimization efforts on the WOBE437 scaffold, as chiral molecules have been suggested to be less prone to promiscuous effects than those without chiral centers.[23][24]

Table 7. Inhibition of AEA uptake into U937 cells by chiral WOBE437 analogs.[a]

Cpd.[a]	EC_{50} [μM] (95% CI)[b]	Max. Inh. (%)[c]	FAAH IC_{50} [μM][d] (95% CI)[b]	SI[e]
75	0.279 (0.18-0.44)	103	>1	ND
76	0.247 (0.18-0.34)	121	>1	ND
77	0.23 (0.16-0.33)	60	2.2 (1.4-3.5)	9.6
78	0.071 (0.040-0.12)	61	ND	ND
79	0.12 (0.05-0.2)	71	4.4 (2.9-5.7)	37
80	0.54 (0.38-0.77)	72	7.9 (4.5-13.0)	15

[a] For structures, *cf*. Fig. 7. ND = Not determined. [b] Concentration required for half-maximal inhibition of uptake of AEA into U937 cells. EC_{50} values represent the mean of at least three independent experiments. 95% CI, 95% confidence interval. [c] Maximal inhibition of uptake of AEA into U937 cells. Maximum inhibition data in our uptake assay data varied significantly; thus, at this point, we consider numbers >60% as essentially indistinguishable. [d] IC_{50} for the inhibition of FAAH in U937 cell homogenates. [e] Selectivity index = EC_{50} (AEA uptake inhibition)/IC_{50} (FAAH inhibition). [^{3}H]-AEA was used to determine uptake inhibition by WOBE437 analogs; FAAH inhibition was assessed in U937 cell homogenates. For experimental details *cf.* ref[14].

Conclusions

We have assessed the inhibition of AEA uptake into U937 cells by almost 80 analogs of the potent AEA uptake inhibitor WOBE437 ((2*E*,4*E*)-*N*-(3,4-dimethoxyphenethyl)dodeca-2,4-dienamide, **1**), incorporating a diverse set of modifications both in the acyl chain as well as the dimethoxyphenylethyl head group. This has included variations in acyl chain length and its degree of unsaturation, the introduction of rigidifying elements, the complete removal, demethylation or extension of methoxy groups, the full replacement of the phenyl moiety by other entities, and the incorporation of chirality in both the fatty acid moiety as well as the head group. As the bottom line finding, none of these compounds was more active than WOBE437 (**1**), with 23 out of 79 analogs exhibiting IC_{50} values for AEA uptake of >1 μM (vs. 10 nM for WOBE (**1**)). At the same time, several analogs exhibited IC_{50} values close to or below 100 nM; importantly, these compounds proved to be highly selective AEA uptake inhibitors (with selectivity indices vs. FAAH inhibition between 17 and >1000), thus attesting to the exquisite selectivity potential associated with the WOBE437 scaffold. The most potent analogs identified were **62** and **66**, whose activity is essentially identical with that of WOBE437 (**1**). Perhaps the most intriguing finding of this SAR study is the >100-fold activity difference between regioisomers **65** and **66**; similarly, **62** is more potent than **61** and **68** is more active than **67**, although the differences are less pronounced (ca. 13-fold and 9-fold, respectively). While these differences cannot be rationalized in the absence of structural information on the interacting protein(s) (which are still elusive at this point), our findings provide important guidance for future optimization studies. Likewise, analog **22**, which does not contain any olefinic double bonds and exhibits an IC_{50} for AEA uptake of 107 nM and chiral analog **78** (IC_{50} of 78 nM) are interesting departure points for the development of potent AEA inhibitors with more drug-like properties. In particular, **22**, with a selectivity index of 49, offers significant room for variations of and around the newly introduced aromatic site and efforts in this direction have been initiated in our laboratories.

At this point in time, the molecular mechanism of AEA uptake has not been elucidated fully. At the same time, the effects that have been observed for WOBE437 (**1**) in vitro and in vivo[10][11] clearly support the notion that the inhibition of EC uptake (in the absence of inhibition of EC-degrading enzymes) is an important and promising therapeutic concept. In this context, the development of new and also more drug-like scaffolds for EC uptake inhibition is highly desirable, even if WOBE437 (**1**) itself has recently been shown to be orally bioavailable in mice.[11] We are pursuing the discovery and pharmacological evaluation of such inhibitors, building on some of the findings revealed in this SAR study, but also based on alternative hit finding strategies. The results of these efforts will be reported in due course.

Experimental Section

Detailed protocols for the synthesis of all final products and intermediates, analytical data for all new compounds, and copies of the relevant ^{1}H- and ^{13}C-NMR spectra can be found in the SI. All compounds tested were at least 95% pure if not stated otherwise in the SI. For biological testing, compounds were dissolved in HPLC-grade DMSO.

Acknowledgements

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Conflict of interest

This authors declare no conflict of interest.

Keywords: endocannabinoid system • endocannabinoid membrane transport • anandamide transport inhibitor • SAR • WOBE437

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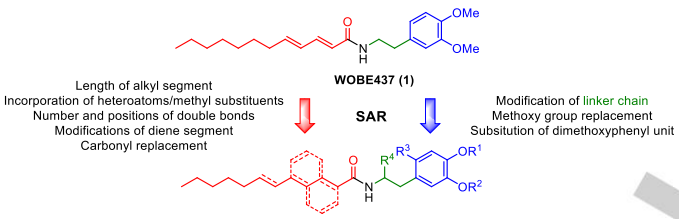

The dodeca-2,4-dienamide WOBE437 (**1**) is a potent inhibitor of endocannabinoid uptake. To elucidate the key structural features underlying the specific inhibitory activity of **1**, we have conducted a comprehensive SAR study on almost 80 WOBE437 analogs with modifications in the dodecadienoyl domain as well as the dimethoxyphenylethyl head group. Several of these variants exhibited potencies in the sub-100 nM range and were highly selective over inhibition of the endocannabinoid-degrading enzyme fatty acid amide hydrolase. However, none of the analogs surpassed the potency of WOBE437 (**1**).