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Anandamide prodrugs
1. Water-soluble phosphate esters of arachidonylethanolamide and *R*-methanandamide

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Abstract

Phosphate esters of arachidonylethanolamide (AEA) and *R*-methanandamide were synthesized and evaluated as water-soluble prodrugs. Various physicochemical properties (pK_a, partition coefficient, aqueous solubility) were determined for the synthesized phosphate esters. The chemical stability of phosphate esters was determined at pH 7.4. In vitro enzymatic hydrolysis rates were determined in 10% liver homogenate, and in a pure enzyme-containing (alkaline phosphatase) solution at pH 7.4. The intraocular pressure (IOP) lowering properties of *R*-methanandamide phosphate ester were tested on normotensive rabbits. The phosphate promoiety increased the aqueous solubility of the parent compounds by more than 16 500-fold at pH 7.4. Phosphate esters were stable in buffer solutions, but rapidly hydrolyzed to their parent compounds in alkaline phosphatase solution ($t_{1/2}$<<15 s) and liver homogenate ($t_{1/2}$=8–9 min). The phosphate ester of *R*-methanandamide reduced IOP in rabbits. These results indicate that the phosphate esters of AEA and *R*-methanandamide are useful water-soluble prodrugs.

Keywords: Anandamide; Arachidonylethanolamide; Cannabinoid; Prodrugs; Water-soluble; Phosphate ester

1. Introduction

Arachidonylethanolamide (AEA) was discovered in 1992 by Devane et al. (1992), when they screened for endogenous cannabinoid compounds. They tested the ability of porcine brain extracts to displace a radiolabeled cannabinoid probe ([^{3}H] HU-243) in a ligand binding assay. After purification of promising brain extracts they found a compound which they named anandamide, and subsequently confirmed its structure by synthesis. AEA was later shown to possess such cannabimimetic activities as hypothermia, analgesia, catalepsy and hypomotility (Crawley et al., 1993; Fride and Mechoulam, 1993; Smith et al., 1994). Nowadays, the term anandamides is used to describe structures that are similar to arachidonylethanolamide and its analogs.

In vivo effects of AEA have a short duration due to inactivation by transport into cells (Beltramo et al., 1997), followed by rapid metabolism. AEA is hydrolyzed to arachidonic acid and ethanolamine by fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996). Abadji et al. (1994) synthesized a chiral analog of AEA, *R*-methanandamide, which possessed improved stability and greater affinity at the cannabinoid neuronal receptor (CB_1). Improved stability and affinity of *R*-methanandamide is caused by an *R*-methyl group adjacent to the amide nitrogen. The recent findings have confirmed that the endogenous cannabinoid system and anandamides have a very important role in human physiology and that anandamides may be potential therapeutic agents in treatment of several diseases (Pertwee, 1999, 2000).

As with the classical cannabinoids, anandamides are very lipophilic and poorly water-soluble molecules. The poor aqueous solubility of anandamides causes problems in pharmacological experiments and decreases their pharmaceutical usefulness. Various approaches such as non-aqueous solvents (Wenger et al., 1995; Cabral et al., 1995) and emulsifiers (Adams et al., 1995) have been used to

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overcome this problem. Jarho et al. (1996) used cyclodextrins to significantly improve both the aqueous solubility and chemical stability of AEA.

Introduction of a polar or permanently charged moiety to the anandamide structure opens up new possibilities for use of these molecules as water-soluble prodrugs or analogs. Phosphate esters have been synthesized in the past to increase the water solubility of sparingly soluble molecules (Cho et al., 1982; Varia et al., 1984; Jadhav et al., 1996). Sheskin et al. (1997) synthesized AEA *O*-phosphate and they speculated that it might be a precursor of AEA in the body. They also measured binding affinities of various arachidonyl amides, and AEA *O*-phosphate was five times less active than AEA in their binding assay. Hopper et al. (1996) introduced new phosphorylation methods for mono- and polyunsaturated fatty acid chains, including AEA. In the present study, we have synthesized phosphate esters of AEA and *R*-methanandamide to evaluate their use as water-soluble prodrugs for various dosage forms and administration routes.

2. Materials and methods

2.1. General procedures

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance (Bruker, Rheinstetter, Germany) operating at 500.1 MHz and 125.6 MHz, respectively. Chemical shifts are reported as parts per million (δ) using TMS as an internal standard. The following abbreviations are used for describing ^{1}H NMR data: s=singlet, d=doublet, t=triplet, q=quartet, qui=quintet, m=multiplet and br=broad. Mass spectra were recorded using a LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). The spray needle was set at 4.5 kV in the positive ion mode and to −4.5 kV in the negative ion mode. The spray was stabilized by a nitrogen sheath flow, the value set to 100. The inlet capillary temperature was 225 °C. The samples were dissolved in 50% methanol–water or in 100% methanol (100 μg/ml) and 5 μl samples were injected. The mobile phase consisted of acetonitrile–water mixture (50:50) and the flow was set to 200 μl/min. Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer. High-performance liquid chromatography (HPLC) determinations were performed with a Merck LaChrom HPLC system consisting of a model L-7250 programmable autosampler, a model L-7100 intelligent pump, a model D-7000 interface, a model L-7455 diode array detector and model D-7000 HPLC system manager software (Hitachi, Tokyo, Japan). A Purospher RP-18e (125 mm×4.0 mm, 5 μm) (Merck, Darmstadt, Germany) reversed-phase column was used for all HPLC separations. pH-metric pK_a determinations were carried out using a Sirius PCA200 automatic titrator (Sirius, Forest Row, UK) and data was analyzed using Refine200 software (Sirius, Forest Row, UK).

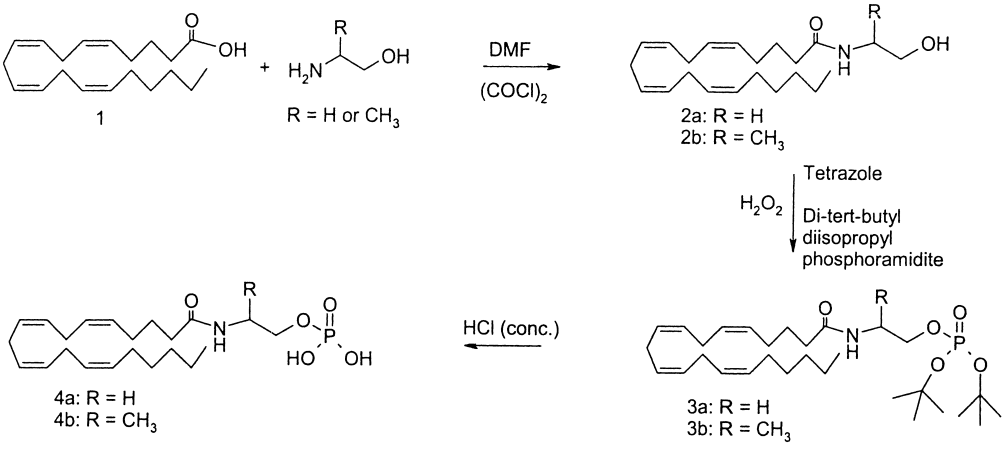
2.2. Chemistry

2.2.1. General

Arachidonic acid (**1**) and AEA (**2a**) were purchased from Deva Biotech (Hatboro, PA, USA). 2-Amino-1-propanol was obtained from Fluka (Buchs, Switzerland) and di-*tert*.-butyl diisopropylphosphoramidite from Aldrich (Milwaukee, WI, USA). Tetrazole was purchased from Acros Organics (Geel, Belgium). *R*-methanandamide (**2b**) was synthesized (Scheme 1) using a slightly modified method of Abadji et al. (1994). Phosphorylation of anandamides was carried out by a modified method of Hopper et al. (1996).

2.2.2. Preparation of (5z,8z,11z,14z)-eicosa-5,8,11,14-tetraenoic acid (2-hydroxy-1-methyl-ethyl)-amide (*2b*)

Arachidonic acid (**1**) (300 mg, 0.985 mmol) was



Scheme 1. Synthesis of phosphate esters of AEA and *R*-methanandamide.

dissolved in dry methylene chloride (10 ml) and DMF (72 mg, 0.985 mmol) was added. The solution was cooled to 0 °C and oxalyl chloride (250 mg, 1.970 mmol) was added dropwise. The solution was stirred at room temperature for 2 h. The solvent was evaporated to yield arachidonic acid chloride. Arachidonic acid chloride was dissolved in dry methylene chloride and the solution was cooled to 0 °C. *R*(−)-2-amino-1-propanol (740 mg, 9.85 mmol) in 5 ml of methylene chloride was added dropwise and the reaction mixture was stirred for 1 h under a nitrogen atmosphere. The mixture was washed twice with water (15 ml) and dried with magnesium sulfate. The product was purified with flash chromatography on 40 μm silica gel (J.T. Baker, Deventer, Holland) using 4% methanol–96% methylene chloride as eluent to give 279 mg (78.3%) of *R*-methanandamide (**2b)** as a slightly yellow oil. ^{1}H NMR ($CDCl_3$) δ: 0.84 (t, ^{3}J=6.8 Hz, 3H), 1.16 (d, ^{3}J=6.8 Hz, 3H), 1.23–1.39 (m, 6H), 1.63–1.76 (m, 2H), 2.05 (q, ^{3}J=6.9 Hz, 2H), 2.12 (q, ^{3}J=6.8, 2H), 2.19 (t, ^{3}J=7.4 Hz, 2H), 2.76–2.88 (m, 6H), 3.48–3.56 (m, 1 H), 3.60–3.69 (m, 1H), 4.00–4.11 (m, 1H), 5.28–5.43 (m, 8H), 5.62 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.04, 17.04, 22.55, 25.45, 25.62, 25.63, 26.61, 27.20, 29.30, 31.50, 36.07, 47.80, 67.38, 127.51, 127.83, 128.14, 128.26, 128.61, 128.83, 129.03, 130.52, 173.64. ESI–MS: 362.3 (M+1).

*2.2.3. Preparation of phosphoric acid di-tert.-butyl ester 2-((5z,8z,11z,14z)-eicosa-5,8,11,14-tetraenoylamino)-ethyl ester (**3a**)*

AEA (**2a**) (222 mg, 0.638 mmol) and tetrazole (134 mg, 1.92 mmol) were dissolved in a 50 ml mixture of dry tetrahydrofuran and methylene chloride (1:1). Di-*tert.*-butyl diisopropyl-phosphoramidite (531 mg, 1.92 mmol) was added and the reaction mixture was stirred overnight under a nitrogen atmosphere. After addition of 0.2 ml 30% hydrogen peroxide, the reaction mixture was stirred for 2 h. The mixture was cooled to 0 °C and saturated sodium metabisulfite solution was added to hydrolyze excess hydrogen peroxide. The mixture was diluted with 50 ml of ethyl acetate and washed twice with saturated sodium metabisulfite solution (2×50 ml). The organic phase was dried with sodium sulfate and the solvent was evaporated in vacuo. The product was purified by flash chromatography on silica gel with ethyl acetate to give 190 mg (55%) of **3a** as a clear oil. The purification was done as fast as possible to avoid breakdown of the protecting *tert.*-butyl groups. ^{1}H NMR ($CDCl_3$) δ: 0.90 (t, ^{3}J=7.3 Hz, 3H), 1.20–1.40 (m, 6H), 1.49 (s, 18H), 1.72 (qui, ^{3}J=7.6 Hz, 2H), 2.01–2.14 (m, 4H), 2.22 (t, ^{3}J=7.6 Hz, 2H), 2.74–2.87 (m, 6H), 3.53 (q, ^{3}J=5.1 Hz, 2H), 4.05 (qui, ^{3}J=4.3 Hz, 2H), 5.29–5.43 (m, 8H), 6.52 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.06, 22.58, 25.54, 25.66, 25.68, 26.76, 27.25, 29.34, 29.87, 29.90, 31.54, 36.07, 40.00, 40.04, 65.97, 66.02, 127.59, 127.92, 128.25, 128.63, 128.76, 129.14, 130.52, 173.23.

*2.2.4. Preparation of phosphoric acid di-tert.-butyl ester 2-((5z,8z,11z,14z)-eicosa-5,8,11,14-tetraenoylamino)-propyl ester (**3b**)*

Compound **3b** was synthesized from *R*-methanandamide (**2b**) (210 mg, 0.580 mmol), tetrazole (483 mg, 1.74 mmol) and di-*tert.*-butyl diisopropyl phosphoramidite (122 mg, 1.74 mmol) as described for **3a**. The product was purified by flash chromatography on silica gel with ethyl acetate to give 150 mg (47%) of **3b** as a clear oil. The purification was done as fast as possible to avoid breakdown of the protecting *tert.*-butyl groups. ^{1}H NMR ($CDCl_3$) δ: 0.89 (t, ^{3}J=6.8 Hz, 3H), 1.21 (d, ^{3}J=6.8 Hz, 3H), 1.23–1.40 (m, 6H), 1.48 (s, 9H), 1.49 (s, 9H), 1.62–1.77 (m, 2H), 2.00–2.14 (m, 4H), 2.19 (t, ^{3}J=7.6 Hz, 2H), 2.73–2.87 (m, 6H), 3.84–3.93 (m, 1H), 3.95–4.04 (m, 1H), 4.15–4.26 (m, 1H), 5.26–5.45 (m, 8H), 6.43 (s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.08, 17.15, 22.58, 25.56, 25.61, 25.62, 26.64, 26.70, 27.22, 29.33, 29.81, 29.84, 29.88, 31.52, 36.13, 45.15, 45.20, 69.66, 69.71, 127.56, 127.89, 128.21, 128.22, 128.59, 128.69, 129.13, 130.51, 172.79.

*2.2.5. Preparation of phosphoric acid mono-[2-((5z,8z,11z,14z)-eicosa-5,8,11,14-tetraenoyl amino)-ethyl] ester (**4a**)*

To a solution of **3a** (95 mg, 0.175 mmol) in ethyl acetate (2 ml) three drops of concentrated hydrochloric acid were added. The mixture was stirred at room temperature for 6 h and evaporated in vacuo to give 75 mg (100%) of **4a**. ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, ^{3}J=6.8 Hz, 3H), 1.23–1.42 (m, 6H), 1.64–1.76 (m, 2H), 2.00–2.12 (m, 4H), 2.33 (t, ^{3}J=7.5 Hz, 2H), 2.70–2.86 (m, 6H), 3.51 (br s, 2H), 4.07 (br s, 2H), 5.25–5.43 (m, 8H), 8.43 (br s, 2H). ^{13}C NMR ($CDCl_3$) δ: 14.07, 22.58, 25.64, 25.65, 25.66, 26.62, 27.24, 29.34, 31.53, 35.49, 127.56, 127.86, 128.13, 128.40, 128.70, 128.83, 128.98, 130.55, 176.32. ESI–MS: 426.5 (M-1). Anal. ($C_{22}H_{38}NO_5P \cdot 0.33H_2O$) C, H, N.

*2.2.6. Preparation of phosphoric acid mono-[2-((5z,8z,11z,14z)-eicosa-5,8,11,14-tetraenoyl amino)-propyl] ester (**4b**)*

Compound **4b** was synthesized from **3b** (110 mg, 0.199 mmol) as described for **4a** to give 85 mg (97%) of **4b**. ^{1}H NMR ($CDCl_3$) δ: 0.88 (t, ^{3}J=6.7 Hz, 3H), 1.20 (d, ^{3}J=6.0 Hz, 3H), 1.23–1.42 (m, 6H), 1.62–1.76 (m, 2H), 1.99–2.14 (m, 4H), 2.22–2.38 (m, 2H), 2.73–2.88 (m, 6H), 3.91 (br s, 1H), 4.04 (br s, 1H), 4.21 (br s, 1H), 5.26–5.45 (m, 8H), 8.25 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ: 14.09, 16.46, 22.58, 25.61, 25.64, 25.70, 26.55, 27.22, 29.33, 31.51, 35.56, 127.53, 127.85, 128.14, 128.33, 128.64, 128.84, 128.92, 130.52, 175.44. ESI–MS: 440.5 (M-1). Anal. ($C_{23}H_{40}NO_5P$) C, H, N.

2.3. HPLC analysis

Gradient elution with a 20 mM phosphate buffer (pH

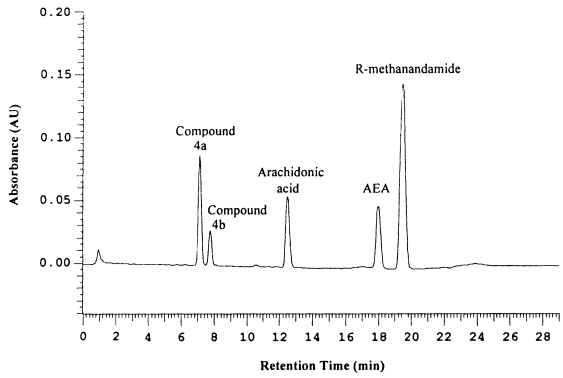


Fig. 1. HPLC separation of arachidonic acid, anandamides and their phosphate esters.

7.4)–90% acetonitrile solvent system achieved a good chromatographic separation of arachidonic acid, anandamides and their phosphate esters (Fig. 1). A flow-rate of 0.9 ml/min was used and compounds were detected at 211 nm.

2.4. Hydrolysis in aqueous solution

The rates of chemical hydrolysis for phosphate esters at 37 °C were determined in 50 mM phosphate buffer (pH 7.4, ionic strength 0.5) and in 185 mM borate buffer (pH 7.4). The solutions were prepared by dissolving an appropriate amount of phosphate esters in buffer solution (initial concentration 198–246 μM). The filtered solutions (Millipore 0.45 μm) were placed in a water bath at 37 °C and samples were withdrawn at appropriate intervals. Samples were analyzed for remaining phosphate ester by HPLC.

2.5. Aqueous solubility

The aqueous solubilities of phosphate esters were determined at room temperature in 185 mM borate buffer (pH 7.4). Compounds **4a** (8.43 mg) and **4b** (3.81 mg) were added to 1 ml of buffer solution and the solution was vigorously shaken for 4 h, filtered (0.45 μm Millipore), diluted and analyzed for phosphate ester by HPLC. The pH of the mixtures was held constant throughout the study.

2.6. Distribution coefficient

The distribution coefficients (log D) of phosphate esters **4a** and **4b** were determined at 25 °C in a 1-octanol-borate buffer (pH 7.4 and pH 5.0) system. Before use, the 1-octanol was saturated with borate buffer by vigorously shaking for 24 h. A known amount of phosphate ester was dissolved in the borate buffer and the pH was checked and adjusted (if necessary). The solution was filtered using a 0.45 μm membrane filter and 2 ml of the filtered solution was shaken with 0.4 ml 1-octanol for 4 h. The phases were separated by centrifuging the tubes 10 min at 1700 rpm. The concentration of phosphate esters in the buffer, before and after the shaking, was determined by HPLC.

2.7. Determination of pK_a

pK_a was determined by the pH-metric technique (Avdeef, 1993) using Sirius PCA200 computerized titration system. Measurements were performed at 25±1 °C under an argon stream to minimize CO_2 absorption. Sample concentrations ranged from 0.40 mM to 0.65 mM. Two independent measurements were made for each compound and the results were combined into a multiset. HCl and KOH solutions used in titrations were made using standardized ampoules (Merck, Darmstadt, Germany). Average ionic strength during the titrations was 0.151 M (adjusted with 0.15 M KCl). Titrations were performed from high to low pH (pH 9.88–1.66).

2.8. Hydrolysis in enzyme solution

The rate of hydrolysis in alkaline phosphatase solution was determined at 37 °C. Alkaline phosphatase (Type VII-S: from bovine intestinal mucosa, 2.745 u/mg protein) was purchased from Sigma (St Louis, MO, USA). **4a** (0.5 μmol) or **4b** (0.6 μmol) were each dissolved in an 185 mM borate buffer or 50 mM phosphate buffer (pH 7.4) and each solution was filtered through a 0.45 μm membrane filter. A tube containing each filtered solution (2 ml) was placed in a 37 °C water bath and 4 μl (20.9 units) of alkaline phosphatase was added. Samples (120 μl) were removed from each tube and 120 μl of acetonitrile was added to stop enzymatic hydrolysis. After centrifugation (14 000 rpm, 10 min), samples were analyzed for the remaining phosphate ester and for released anandamide by HPLC.

2.9. Hydrolysis in liver homogenate

The hydrolysis of **4a** and **4b** was studied in 10% rabbit liver homogenate at 37 °C. The 20% liver homogenate was prepared by homogenizing rabbit liver with four equivalents of isotonic phosphate buffer (pH 7.4). The homogenate was centrifuged for 90 min at 9000×g at 4 °C and the supernatant was stored at −80 °C until used. One volume of 1.15 mM phosphate ester solution in borate buffer (pH 7.4) was added to one volume of stirred, pre-incubated (37 °C) 20% liver homogenate to give 10% liver homogenate. The solution was kept at 37 °C and 200 μl samples were withdrawn and added to 300 μl of cold acetonitrile. After mixing and centrifugation (14 000 rpm, 10 min), the samples were analyzed for the remaining phosphate ester, and for released anandamide, by HPLC.

2.10. IOP studies

2.10.1. Formulation of eyedrops

R-methanandamide was dissolved in aqueous 15% HP-β-CD solution. Earlier studies (Pate et al., 1995, 1998) have demonstrated that topical administration of HP-β-CD (up to 30% solution) does not affect the IOP of rabbits. The pH of *R*-methanandamide solution was adjusted to 7.4 with sodium hydroxide and the solution was made isotonic with sodium chloride. Isotonicity of the solution was confirmed by using an Osmostat OM-6020 Auto-Osmometer (Kyoto Daiichi Kagaku, Kyoto). The phosphate ester of *R*-methanandamide (**4b**) was dissolved to an isotonic 50 mM phosphate buffer at pH 7.4. Final drug concentration of *R*-methanandamide and of the phosphate ester of *R*-methanandamide in the eyedrop solutions was 6.91 μmol/ml. The isotonic 50 mM phosphate buffer was used as a negative control treatment. Drug concentrations were analyzed by HPLC.

2.10.2. Intraocular pressure (IOP) measurements

The method for IOP measurements has been described earlier (Pate et al., 1998; Laine et al., 2002). The experimental animals used were normotensive Dutch Belted rabbits of either gender (2.4–3.5 kg, $n=5$). The rabbits were housed singly in cages under standard laboratory conditions: 12 h dark/12 h light cycle, 20.0±0.5 °C and 55–75% relative humidity. Rabbits were given water and food ad libitum except during the tests. Animals were treated in accordance with the ARVO Statement for the use of animals in ophthalmic and vision research.

During the tests, rabbits were placed in plastic restraining boxes located in a quiet room. A drop (25 μl) of the test solution was instilled unilaterally into each left eye of the rabbits. During installation, the upper eyelid was pulled away slightly from the globe. IOP was measured using a BioRad (Cambridge, MA) Digilab Modular One Pneumatonometer. Before each measurement, one or two drops of 0.06% oxybuprocaine were applied to the cornea to eliminate discomfort. For each determination, at least two readings were taken from the treated and untreated eye, and the mean of these readings was used. IOP of the rabbits were measured 1 and 0 h before, and at 0.5, 1, 2, 3, 4 and 5 h after the administration of the eyedrop. IOP at the time of eyedrop administration (0 h) was used as a baseline value. Baseline IOPs ranged between 25.5–16.8 mmHg. All studies were set up using a randomized crossover design. At least 72 h of wash-out time was allowed for the rabbits between dosings.

2.10.3. Statistical analysis of IOP data

Results are given as a change in IOP (mmHg) mean±S.E.M. A one-factor analysis of the variance (ANOVA) for repeated measurements was used to test the statistical differences between the *R*-methanandamide, the phosphate ester **4b** and control treated groups. Significance in differences of the means was tested using the Fisher's Protected Least Significant Difference (PLSD) method at the 95% confidence level.

3. Results and discussion

3.1. Hydrolysis in aqueous solution

The effect of a buffer system on the stability of phosphate esters **4a** and **4b** was considerable. Phosphate esters **4a** and **4b** hydrolyzed more slowly in 185 mM borate buffer ($t_{1/2}=11.9$ days and 13.9 days, respectively) compared to 50 mM phosphate buffer ($t_{1/2}=2.4$ days and 3.1 days, respectively), which is most probably due to a catalytic effect of phosphate ions on the hydrolysis of the phosphate ester group (Table 1).

3.2. Aqueous solubility

Jarho et al. (1996) measured the aqueous solubility of AEA to be 0.4 μg/ml (1.15×10^{-6} M) at pH 7.4. Phosphate esters **4a** and **4b** were completely dissolved during the solubility experiment. Because there was no undissolved compound left after the experiment, exact solubilities can not be given. The maximum solubility of **4a** and **4b** could not be measured due to the small amount of each phosphate ester available and their high respective aqueous solubilities. The aqueous solubilities of **4a** and **4b** in a pH 7.4 solution at room temperature were determined to be at least 8.15 mg/ml (19.06×10^{-3} M) and 3.8 mg/ml (8.60×10^{-3} M), respectively (Table 2). In any case, introduction of a phosphate moiety to the anandamide structure significantly increased the aqueous solubility of the parent drug (Table 2).

3.3. Distribution coefficient

Compound **4b** showed a higher distribution coefficient (log *D*) at pH 5.0 and 7.4 compared to **4a** (Table 2) which is probably due to the α-methyl substituent on the former structure. The high partition coefficients suggest ion-pair partitioning. Partition of **4a** or **4b** between octanol and the aqueous phase is strongly dependent on the pH of the aqueous phase due to their respective ionizable phosphate moieties. The lipophilic character of both **4a** and **4b**

Table 1
Half-lives (days) of **4a** and **4b** in phosphate buffer (50 mM; pH 7.4) and in borate buffer (185 mM; pH 7.4) at 37 °C

Compound	$t_{1/2}$ (days) Phosphate buffer (50 mM; pH 7.4)	$T_{1/2}$ (days) Borate buffer (185 mM; pH 7.4)
4a	2.4	11.9
4b	3.1	13.9

Table 2
Aqueous solubility (one determination), distribution coefficient at pH 5.0 and 7.4 (log D, mean±S.D.; $n=3$) and pK_a of AEA, **4a** and **4b**

Compound	Solubility (mM) (pH 7.4)	Solubility compared to AEA	Log $D_{5.0}$ (pH 5.0)	Log $D_{7.4}$ (pH 7.4)	pK_{a1}	pK_{a2}
AEA	1.15×10^{-3} [a]	–	–	–	–	–
4a	>19.06	>16500	1.91±0.02	1.15±0.09	2.68	6.88
4b	>8.6	>7500	2.60±0.30	1.53±0.02	2.65	6.73

[a] Jarho et al., 1996.

decreases when pH is increased. These phosphate esters **4a** and **4b** have high aqueous solubilities (>8.15 mg/ml and >3.8 mg/ml, respectively) and adequate distribution coefficients (log $D_{7.4}=1.15$ and 1.53, respectively) which is a very good combination for further drug development.

3.4. Determination of pk_a

The two pK_as of phosphate ester **4a** and **4b** are shown in Table 2. Goodness-of-fit values (Avdeef, 1993) for the multiset of two determinations were 1.48 and 1.31 for **4a** and **4b**, respectively. The good solubility of the phosphate esters at pH 7.4 can be readily explained by the pK_a-values. At pH 7.4 the hydroxyl group with the lower pK_a (2.65) is completely ionized and more than 80% of the other hydroxyl group is in ionized form.

3.5. Hydrolysis in enzyme solution

Enzymatic hydrolysis of phosphate esters **4a** and **4b** in borate buffer was very rapid (completely hydrolyzed before first sample) in the presence of alkaline phosphatase (Table 3), releasing AEA and *R*-methanandamide, respectively. The buffer system is important in these enzymatic hydrolyses, as the reaction was much slower in phosphate buffer than in borate buffer. Both phosphate and borate are known to be inhibitors of alkaline phosphatase (Fernandez et al., 1981). Phosphate ion inhibits alkaline phosphate much more effectively than borate ion, which is logical as the inorganic phosphate binds to alkaline phosphatase thereby inhibiting the enzyme. Inorganic phosphate is also produced when alkaline phosphatase hydrolyses phosphate esters. The slow dissociation of inorganic phosphate from the enzyme could be avoided by using buffer like Tris, which is a good phosphoryl acceptor (Simopoulos and Jencks, 1994).

Table 3
Half-lives (min) (mean±S.D., $n=3$) of **4a** and **4b** in alkaline phosphatase solution and liver homogenate at 37 °C

Compound	$t_{1/2}$ (min) Alkaline phosphatase		$t_{1/2}$ (min) 10% Liver homogenate
	Borate buffer	Phosphate buffer	
4a	Rapid hydrolysis[a]	209±9	8±1
4b	Rapid hydrolysis[a]	473±24	9±0

[a] Completely hydrolyzed before the first sample (15 s).

3.6. Hydrolysis in liver homogenate

The breakdown of **4a** and **4b** in liver homogenate released AEA and *R*-methanandamide, respectively. Both **4a** and **4b** were hydrolyzed with half-lives of 8 and 9 min, respectively, (Table 3) and AEA was further hydrolyzed to arachidonic acid. The breakdown of *R*-methanandamide to arachidonic acid was probably prevented by the *R*-α-methyl group.

3.7. Intraocular pressure (IOP) measurements

Both *R*-methanandamide (dissolved in HP-β-CD solution) and phosphate ester **4b** caused a statistically significant ($P<0.05$) fall of IOP in the treated eye when compared to a buffer solution (Fig. 2). The maximal observed decrease of IOP was 3.1±0.7 mmHg and 2.4±0.4 mmHg at 2 h after administration of 173 nmol of *R*-methanandamide or **4b**, respectively. No statistically significant difference between *R*-methanandamide and **4b** could be observed.

Administration of 173 μmol of *R*-methanandamide or **4b** did not cause statistically significant decrease of IOP in the corresponding untreated eyes when compared to a buffer solution (data not shown) which suggests that the reduction of IOP is a local effect.

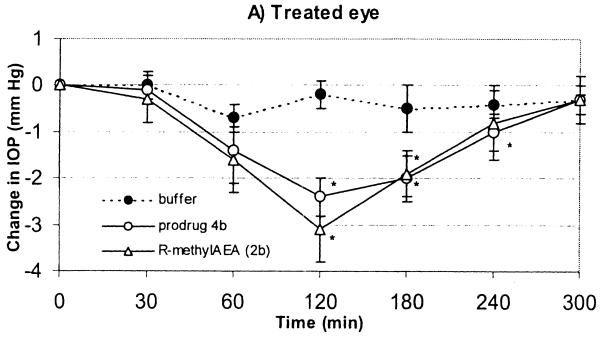


Fig. 2. IOP changes (mean±S.E.M., $n=5$) in treated eyes of normotensive rabbits after unilateral ocular administration of isotonic phosphate buffer (●), 173 μmol *R*-methanandamide (△) or 173 nmol phosphate ester **4b** (○). Symbols (*) indicate data significantly different from values for the phosphate buffer.

4. Conclusions

Phosphate esters of AEA and *R*-methanandamide significantly increased the aqueous solubility of their respective parent compounds, were adequately stable in buffer solutions, and released the parent drugs after enzymatic hydrolysis in alkaline phosphatase-containing solution and in liver homogenate. The phosphate ester of *R*-methanandamide reduced IOP in rabbits, and the effect was comparable to *R*-methanandamide in hydroxypropyl-β-cyclodextrin solution. Thus, phosphate esters of arachidonylethanolamide and *R*-methanandamide can be considered as potential water-soluble prodrugs of anandamides.

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