Brief Articles

Synthesis, in Vitro Evaluation, and Intraocular Pressure Effects of Water-Soluble Prodrugs of Endocannabinoid Noladin Ether

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The poor aqueous solubility of 2-arachidonyl glyceryl ether (noladin ether) **2** hinders both pharmacological studies and pharmaceutical development. The synthesized mono- and diphosphate esters of noladin ether (**4** and **6**) considerably increased the aqueous solubility of noladin ether (>40000-fold), showed high stability against chemical hydrolysis in buffer solutions, and were rapidly converted to the parent drug via enzymatic hydrolysis. The monophosphate ester of noladin ether reduced intraocular pressure in normotensive rabbits.

Introduction

Noladin ether (2-arachidonyl glyceryl ether, HU-310) is a newly discovered endogenous cannabinoid CB1 receptor agonist.[1] Previously identified endocannabinoids are amides or esters, of which the best known are arachidonylethanolamide (AEA)[2] or 2-arachidonyl glycerol (2-AG),[3] respectively. AEA and 2-AG have a short duration of action in vivo due to rapid metabolism, and noladin ether would be advantageous because of its higher metabolic stability.

There has been a growing interest in the search for potential therapeutic applications of endocannabinoids, and there are some excellent reviews covering this topic.[4−6] Noladin ether has been shown to reduce intraocular pressure (IOP), and the effect is mediated through the ocular CB1 receptor.[7] Although a lot of research has been on endocannabinoids, not much attention has been paid to their unfavorable physicochemical properties. Endocannabinoids are very lipophilic and poorly water-soluble compounds, which hinders their pharmaceutical development and clinical use. Various approaches such as nonaqueous solvents,[8] emulsifiers,[9] and cyclodextrins[10] have been used to overcome this problem. Another approach is to increase aqueous solubility of endocannabinoids by synthesizing water-soluble prodrugs with ionizable or permanent charge-containing groups. In this article, we describe the synthesis of mono- and diphosphate esters of 2-arachidonyl glyceryl ether and their in vitro and in vivo evaluations as water-soluble endocannabinoid prodrugs.

Results and Discussion

Chemistry. 2-Arachidonyl glyceryl ether was synthesized from arachidonyl alcohol using the method of Hanuš et al.[1] Mono- and diphosphate esters of 2-arachidonyl glyceryl ether were synthesized using dimethyl chlorophosphate as described in Scheme 1.

Chemical Stability. Both prodrugs **4** and **6** showed good chemical stability in tris and phosphate buffers at pH 7.4 at 37 °C (Table 1). No degradation of diphosphate **6** was observed after 10 days. The structures of the prodrugs **4** and **6** resemble surface-active agents, bearing a highly lipophilic (straight hydrocarbon chain) and also a highly hydrophilic portion (phosphate group). This amphiphilic nature allows the formation of micelles in aqueous solutions, which may protect the hydrolytically labile prodrug linkage and unstable double bonds within the micelle interior.[11]

Physicochemical Properties. The aqueous solubility of the phosphate esters was over 5 mg/mL, which is over 40 000 times the solubility of noladin ether (<0.1 μg/mL). The exact prodrug solubility was not determined because of the small amount of phosphate esters available.

Partition or distribution coefficient has been the most frequently used physicochemical parameter to estimate the permeability of drugs or druglike molecules through biological membranes. Optimal log distribution coefficients for membrane permeability are in the range 2−3.[12] The calculated log distribution coefficient for noladin ether at pH 7.4 was 7.05 (calculated using ACDLabs log *D* suite), which shows that the molecule is too lipophilic for optimal membrane penetration. Phosphate esters are in their ionized form at pH 7.4, and prodrugs **4** and **6** have significantly lower log *D* values (Table 1). However, the phosphate prodrug strategy does not necessarily involve membrane permeation of the prodrug. In many cases, it is actually feasible that prior to absorption the water-soluble prodrug undergoes enzymatic degradation to the highly permeable parent drug to ensure high absorption fluxes.[13]

Enzymatic Hydrolysis. Enzymatic hydrolysis of **4** and **6** was studied in alkaline phosphatase solution, liver homogenate, and cornea homogenate to confirm the formation of the parent active compound (Table 2).

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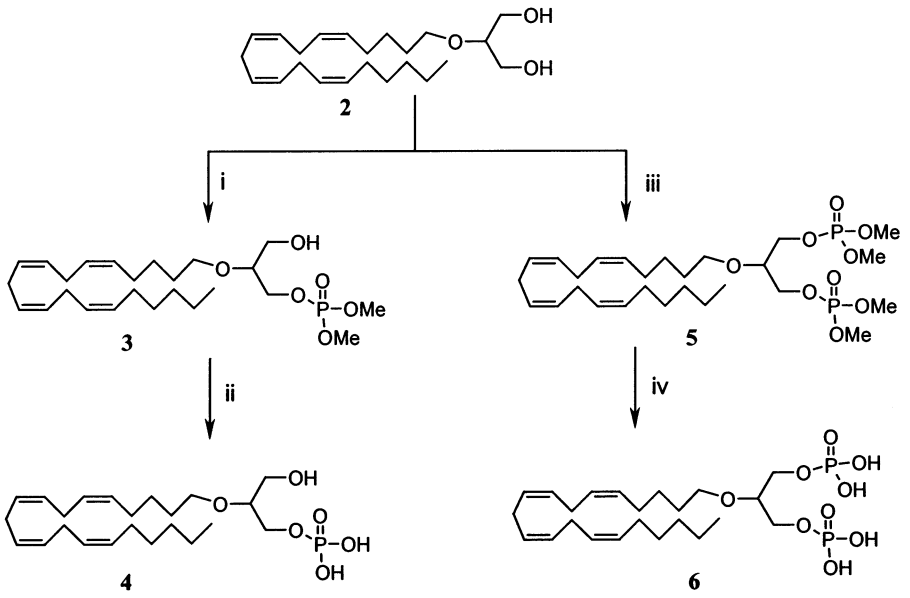
Scheme 1[a]



[a] (i) Pyridine, Cl-P(O)(OMe)$_2$ (1.5 equiv), 0 °C, 2 h, 20 °C overnight; (ii) NaI (2.2 equiv), Me_3SiCl (2.2 equiv), 40 °C, 1 h, MeOH, 20 °C 1 h; (iii) pyridine, Cl-P(O)(OMe)$_2$ (3 equiv), 0 °C, 2 h, 20 °C overnight; (iv) NaI (4.4 equiv), Me_3SiCl (4.4 equiv), 40 °C, 1 h.

Table 1. Chemical Stability, Aqueous Solubility, and Distribution Coefficients of Noladin Ether **2** and Its Phosphate Esters **4** and **6**

	chemical stability			
compd	phosphate buffer, pH 7.4 ($t_{1/2}$, days)	tris buffer, pH 7.4 ($t_{1/2}$, days)	solubility, tris buffer, pH 7.4	log $D_{7.4}$, pH 7.4 (mean ± SD, $n = 3$)
2	nd[a]	nd[a]	<0.1 μg/mL	7.05[b]
4	17.9	9.0	>5 mg/mL	1.98 ± 0.06
6	stable[c]	stable[c]	>5 mg/mL	0.65 ± 0.07

[a] nd = not determined. [b] Calculated using ACDLabs log *D* suite. [c] No degradation was observed after 10 days.

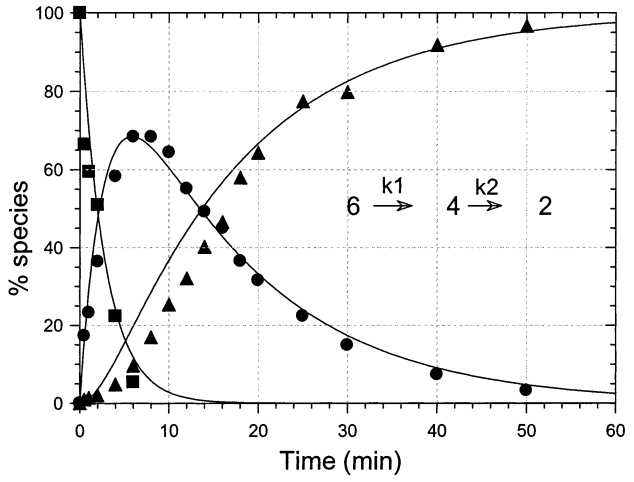


Figure 1. Time courses for noladin ether diphosphate ester **6** (■), noladin ether monophosphate ester **4** (●), and noladin ether **2** (▲) during hydrolysis of **6** in alkaline phosphatase solution. The solid lines are constructed by the proposed reaction scheme shown and calculated first-order rate constants.

Alkaline phosphatase quantitatively degraded **4** and **6** to noladin ether as presented in Figure 1 showing the hydrolysis of **6**, where release of noladin ether is preceded by the formation of monophosphate ester **4**. The hydrolysis reactions can be described by pseudo-first-order rate constants k_1 and k_2, which were calculated to be 0.36 and 0.065 min^{-1}, respectively. The solid lines in Figure 1 were constructed by calculated constants according to the proposed reaction scheme and show good agreement with experimental data. The somewhat higher k_2 (0.11 min^{-1}) in the hydrolysis experiment of **4** is probably due to the inhibition of alkaline phosphatase by the higher amount of released phosphate in the hydrolysis experiment of **6**.

Table 2. Half-Lives (Mean ± SD) of Mono- (**4**) and Diphosphate Esters (**6**) of Noladin Ether **2** in Alkaline Phosphatase Solution, Liver Homogenate, and Cornea Homogenate at 37 °C

compd	$t_{1/2}$ (min), $n = 2$, alkaline phosphatase	$t_{1/2}$ (min), $n = 3$, 10% liver homogenate	$t_{1/2}$ (min), $n = 3$, 4% cornea homogenate
4	4.6 ± 0.7	7.1 ± 0.1	18.1 ± 1.4
6	1.6 ± 0.0	6.4 ± 0.7	17.2 ± 1.7

Hydrolysis rates in 4% bovine cornea homogenate were studied in order to clarify whether these phosphate esters can hydrolyze during permeation of the cornea. Both **4** and **6** hydrolyzed to noladin ether in cornea homogenate (Table 2). These results suggest that phosphate esters of noladin ether are at least partly hydrolyzed during permeation of the cornea.

Intraocular Pressure Study in Normotensive Rabbits. The IOP study was performed to confirm that these phosphate ester prodrugs also work in vivo. In vitro studies already showed that prodrug **6** is rapidly converted to **4**; therefore, only **4** was tested. Both noladin ether (dissolved in HP-β-CD solution) and its phosphate ester **4** caused a statistically significant ($p < 0.05$) fall in IOP of the treated eye when compared to a buffer solution (Figure 2). The maximal observed decrease in IOP was 8.5 ± 4.5% at 2 h after a topical administration of 172 nmol of noladin ether and was 9.7 ± 4.4% at 3 h after a topical administration of 172 nmol of phosphate ester **4**. No statistically significant difference between noladin ether in HP-β-CD formulation and **4** could be observed. The reduction of IOP was

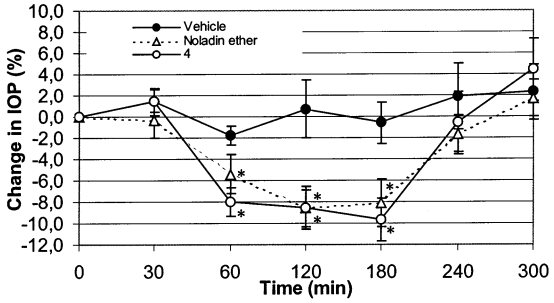


Figure 2. IOP changes (mean ± SE, $n = 5$) in treated eyes of normotensive rabbits after unilateral ocular administration of isotonic citrate buffer (●), 172 nmol of noladin ether **2** (△), or 172 nmol of phosphate ester **4** (○). The asterisk (∗) indicates data significantly different from values for the citrate buffer.

most probably a local effect in the eye because no statistically significant decrease of IOP was observed in the corresponding untreated eyes when compared to the buffer solution (data not shown).

Conclusions

The physicochemical properties of noladin ether **2** were successfully improved by introducing a phosphate moiety to the structure. High water solubility and chemical stability, together with a rapid and quantitative enzymatic hydrolysis in vitro and the ability to reduce IOP in vivo, prove that these phosphate esters are promising prodrug candidates of endocannabinoid noladin ether.

Experimental Section

General Procedures. ^{1}H, ^{31}P, and ^{13}C NMR, spectra were recorded on a Bruker Avance (Bruker, Rheinstetter, Germany) spectrometer operating at 500.13, 202.45, and 125.76 MHz, respectively. TMS was used as an internal standard for ^{1}H and ^{13}C, and 85% H_3PO_4 was used as an external standard for ^{31}P. Mass spectra were recorded using an LCQ quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA). Elemental analyses were carried out on a ThermoQuest CE Instruments EA 1110-CHNS-O elemental analyzer. HPLC determinations were performed with a Merck LaChrom HPLC system equipped with a diode array detector (Hitachi, Tokyo, Japan). A Purospher RP-8e (125 mm × 4.0 mm, 5 μm) (Merck, Darmstadt, Germany) reversed-phase column was used for all analytical HPLC determinations.

Phosphoric Acid 2-((5*Z*,8*Z*,11*Z*,14*Z*)-Eicosa-5,8,11,14-tetraenyloxy)-3-hydroxypropyl Ester Dimethyl Ester (3). 2-((5*Z*,8*Z*,11*Z*,14*Z*)-Eicosa-5,8,11,14-tetraenyloxy)propane-1,3-diol (**2**) (300 mg, 0.82 mmol) was dissolved in 15 mL of pyridine, and the mixture was cooled to 0 °C. Dimethyl chlorophosphate[14] (**1**) (178 mg, 1.23 mmol) was dissolved in 2 mL of diethyl ether and then added dropwise to the solution, and the mixture was stirred for 2 h at 0 °C. The reaction mixture was stirred at 20 °C overnight, and water (2 mL) was added to dissolve pyridinium chloride. The mixture was extracted with diethyl ether (3 × 30 mL). The combined ether fractions were washed with 0.1 M H_2SO_4 (2 × 20 mL), 5% $NaHCO_3$ (20 mL), and water (3 × 20 mL). The organic phase was dried over Na_2SO_4 and purified by flash chromatography to give 190 mg (49%) of **3**. ^{1}H NMR ($CDCl_3$) δ: 5.45−5.30 (m, 8H), 4.17−4.10 (m, 2H), 3.785 (d, 3H, $J = 11.1$ Hz), 3.782 (d, 3H, $J = 11.1$ Hz), 3.75−3.72 (m, 1H), 3.68−3.50 (m, 4H), 2.88−2.78 (m, 6H), 2.37 (br s, 1H), 2.14−2.00 (m, 4H), 1.64−1.56 (m, 2H), 1.50−1.23 (m, 8H), 0.89 (t, 3H, $J = 6.9$ Hz).

Phosphoric Acid Mono-[2-((5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,-11,14-tetraenyloxy)-3-hydroxypropyl] Ester (4). The protected phosphate ester **3** (190 mg, 0.402 mmol) and NaI (133 mg, 0.884 mmol) were placed in a round-bottom flask, and 5 mL of dry acetonitrile was added. Trimethylsilyl chloride (96 mg, 0.844 mmol) was added, and the mixture was stirred for 1 h at 40 °C. Solvent was evaporated in vacuo, MeOH (5 mL) was added, and the mixture was stirred for 1 h at room temperature. Solvents were evaporated, and the product was purified using reversed-phase preparative HPLC to give 70 mg (39%) of **4** as a sticky semisolid product. ^{1}H NMR ($CDCl_3$) δ: 6.66 (br s, 3H), 5.48−5.27 (m, 8H), 4.11 (br s, 2H), 3.83−3.67 (m, 2H), 3.65−3.48 (m, 3H), 2.87−2.72 (m, 6H), 2.12−2.00 (m, 4H), 1.64−1.53 (m, 2H), 1.45−1.21 (m, 8H), 0.89 (t, 3H, $J = 8.0$ Hz). ^{13}C NMR δ: 130.49, 129.85, 128.59, 128.34, 128.19, 128.15, 127.93, 127.59, 77.90 (d, $J_{CP} = 7.4$ Hz), 70.61, 64.98 (d, $J_{CP} = 5.3$ Hz), 60.83, 31.53, 29.46, 29.34, 27.24, 27.01, 26.01, 25.66, 22.59, 14.08. ^{31}P NMR δ: 1.52. ESI-MS, *m*/*z*: 443.7 (M − 1). Anal. ($C_{23}H_{41}O_6P \cdot 0.33\ H_2O$) C, H.

Phosphoric Acid (Dimethoxyphosphoryloxy)-((5*Z*,8*Z*,-11*Z*,14*Z*)-eicosa-5,8,11,14-tetraenyloxy)propyl Ester Dimethyl Ester (5). Compound **5** was synthesized from **2** (300 mg, 0.82 mmol) and **1** (357 mg, 2.47 mmol) as described for **3** to give 210 mg (44%) of **5**. ^{1}H NMR ($CDCl_3$) δ: 5.44−5.28 (m, 8H), 4.20−4.04 (m, 4H), 3.782 (d, 6H, $J = 11.1$ Hz), 3.779 (d, 6H, $J = 11.1$ Hz), 3.70 (qui, 1H, $J = 5.1$ Hz), 3.59 (t, 2H, $J = 6.5$ Hz), 2.87−2.77 (m, 6H), 2.12−2.02 (m, 4H), 1.64−1.54 (m, 2H), 1.48−1.23 (m, 8H), 0.89 (t, 3H, $J = 6.9$ Hz).

Phosphoric Acid Mono-[2-((5*Z*,8*Z*,11*Z*,14*Z*)-eicosa-5,8,-11,14-tetraenyloxy)-3-phosphonooxypropyl] Ester (6). Compound **6** was synthesized from **5** (210 mg, 0.36 mmol), NaI (237 mg, 1.58 mmol), trimethylsilyl chloride (172 mg, 1.58 mmol), and MeOH (5 mL) as described for **4** to give 30 mg (16%) of **6**. ^{1}H NMR ($CDCl_3$) δ: 9.28 (br s, 4H), 5.49−5.24 (m, 8H), 4.16 (br s, 4H), 3.82−3.70 (m, 2H), 3.62 (br s, 1H), 2.89−2.71 (m, 6H), 2.13−1.95 (m, 4H), 1.60 (br s, 2H), 1.48−1.20 (m, 8H), 0.88 (t, 3H, $J = 6.9$ Hz). ^{13}C NMR δ: 130.47, 129.88, 128.59, 128.37, 128.19, 128.13, 127.94, 127.60, 76.57, 70.98, 64.46, 31.53, 29.34, 29.25, 27.23, 26.99, 25.89, 25.66, 25.64, 22.59, 14.09. ^{31}P NMR δ: 0.98. ESI-MS, *m*/*z*: 523.8 (M − 1). Anal. ($C_{23}H_{42}O_9P_2 \cdot H_2O$) C, H.

Distribution Coefficient. The distribution coefficients (log *D*) of **4** and **6** were determined at 25 °C in a 1-octanol-tris buffer (50 mM, pH 7.4, ionic strength 0.15) system. A known amount of compound was dissolved in the buffer, and the pH was checked and adjusted (if necessary). A 1 mL aliquot of this solution was shaken with 1 mL of 1-octanol for 1 h. The phases were separated by centrifugation for 10 min at 4000 rpm. The concentration of the compound in the buffer, before and after the shaking, was determined by HPLC.

Eye Drop Formulation. Noladin ether was dissolved in 25% aqueous hydroxypropyl-β-cyclodextrin (HP-β-CD). The pH of the noladin ether solution was adjusted to 7.4 with NaOH solution, and the solution was made isotonic with NaCl. Because of its higher water solubility, the monophosphate ester of noladin ether (**4**) was dissolved in 10 mM citrate buffer at pH 5.0 and the solution was made isotonic with NaCl. Final drug concentrations of noladin ether and its monophosphate prodrug **4** in eye drop solutions were 2.5 mg/mL and 3.05 mg/mL (6.86 mmol/L), respectively. Isotonic 10 mM citrate buffer (pH 5.0) was used as a control vehicle.

Intraocular Pressure Experiments. A single drop (25 μL) of the test solution or vehicle was instilled unilaterally into the left eye in the upper corneoscleral limbus. Before each measurement, one or two drops of topical anesthetic (0.06% oxybuprocaine) were applied to the cornea to reduce possible discomfort. The IOP of the rabbits was measured using a pneumatonometer (Digilab Modular One, Bio-Rad, Cambridge, MA). For each determination, at least two readings were taken from the treated and untreated eye and the mean of these readings was used. The IOP of the rabbits was measured 1 h before administration and then at 0, 0.5, 1, 2, 3, 4, and 5 h after application of the eye drops. The IOP at the time of eye drop administration (0 h) was used as a baseline value. Baseline IOPs ranged between 18 and 23.9 mmHg. All studies were set up using a randomized crossover design. At least a 72 h wash-out time was allowed for each rabbit between doses.

Data Analysis. Results are given as a change in IOP (%) mean ± SE (standard error). A one-factor analysis of the variance (ANOVA) for repeated measurements was used to test for possible statistical differences between noladin ether **2**, prodrug **4**, and vehicle treated groups. Significance in differences of the mean values was tested by Fisher's protected least significant difference (PLSD) method at the 95% confidence level. Experimental data fitting (nonlinear regression analyses) of hydrolysis data was performed by Scientist Software (MicroMath Scientific Software, Salt Lake City, UT).

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Supporting Information Available: Hydrolysis and animal information. This material is available free of charge via the Internet at http://pubs.acs.org.

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