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Research Article

Synthesis of tritium labelled arachidonic acid amide and ester derivatives with dopamine, serotonin, vanillylamine, and ethyleneglycol moieties

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Summary

Tritium labelled arachidonic acid amides with dopamine, serotonin, vanillylamine and the ethyleneglycol ester moieties with high specific activity (120 Ci/mmol) and yield (70–90%) were prepared from tritiated arachidonic acid by condensation with the corresponding amines and alcohol via mixed anhydrides or acyl fluorides. The labelled compounds were used for studying their uptake by mouse spleen lymphocytes. From the data obtained it was suggested that arachidonic acid amides permeate the membrane by means of passive diffusion, while transmembrane transport of arachidonoylethyleneglycol seems to be driven by the concentration gradient, maintained by hydrolytic enzymes. The compounds synthesized by the reported methods can also be used in receptor binding studies and in the oxidative metabolism of fatty acids amides and esters. Copyright © 2002 John Wiley & Sons, Ltd.

Key Words: tritium; labelled compounds; fatty acid amides; endocannabinoids; transport

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Introduction

Studies over the last decade have demonstrated that arachidonic and other polyenoic fatty acid amides and esters are potent lipid bioregulators involved in the regulation of memory, emotions, nociception, sleep, motor activity, immune and neuroendocrine status (for comprehensive reviews on the subject see[1–3]). Recently, we reported that polyenoic fatty acid derivatives with biogenic amines (dopamine, serotonin, tyramine and others) and alcohols (ethyleneglycol and some nitroderivatives) also have a wide spectrum of biological activity. Thus, arachidonic and eicosapentaenoic acid amides with dopamine and serotonin possess certain anti-aggregatory and cytoprotective properties, being able to inhibit arachidonic acid- and ADP-induced platelet aggregation and to protect early sea urchin embryos from the cytotoxic action of dopamine and serotonin antagonists.[4] Several polyenoic fatty acid amides with dopamine[5] as well as arachidonoylethyleneglycol and its nitroester[6] are potent cannabimimetic compounds, and arachidonoylserotonin proved to be a selective, non-covalent inhibitor of fatty acid amide hydrolase[7] – a key enzyme in endocannabinoid' metabolism – and interfere with lipoxygenases of plant and animal origin[4,6,8]. Besides, a series of vanillylamine *N*-acyl derivatives, analogues of the pungent constituent of hot pepper *Capsicum* sp., capsaicin, were recently shown to bind vanilloid (VR1) and cannabinoid (CB1) receptors and interfere with anandamide facilitated uptake.[9,10]

However, most of the data on the biochemical effects of fatty acid amides and esters were obtained from the competitive binding studies using the Cheng–Prusoff equation and have the drawback of indirect determination of the receptor affinity and effective dose (influence of the ligand depletion, cooperativity, dissimilar affinity to ligands, irreversible binding, etc.). Furthermore, due to the lack of individual isotopically labelled compounds, little is known about their own transport through the plasma membrane. Here, we report the synthesis and application of tritium labelled arachidonoyl dopamine **(1),** serotonin **(2),** vanillylamine **(3)** and ethyleneglycol **(4)** derivatives (Figure 1) and studies of their uptake by mouse spleen lymphocytes.

Results and discussion

The starting compound for the synthesis—[5,6,8,9,11,12,14,15-$^{3}H_{8}$] arachidonic acid – was prepared by selective hydrogenation of

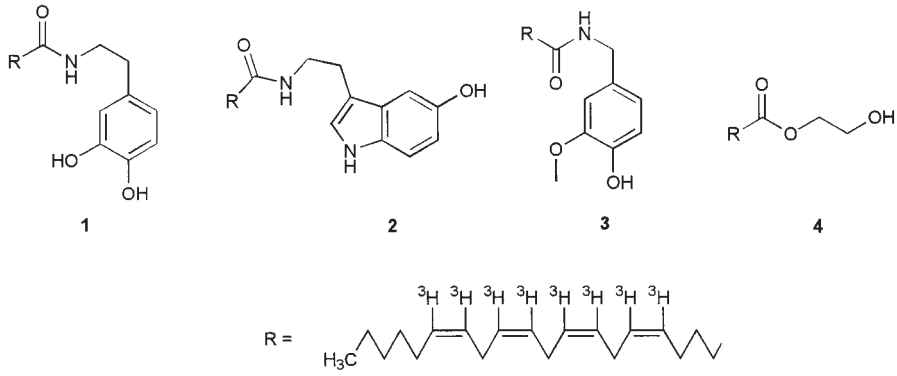

Figure 1. Chemical structures of labelled amides and ester of arachidonic acid. 1 – [^{3}H]arachidonoyl-3-hydroxytyramine, 2 – [^{3}H]arachidonoyl-5-hydroxytryptamine, 3 – [^{3}H]arachidonoyl-4-hydroxy-3-methoxybenzylamine, 4 – [^{3}H]arachidonoylethyleneglycol

5,8,11,14-eicosatetraynoic acid with gaseous tritium over Lindlar catalyst according to a procedure described earlier for the corresponding methyl ester.[11] Although direct tritiation of free eicosatetraynoic acid to labelled arachidonic acid proceeds with a lower yield than in the case of its methyl ester, the approach utilizes an acetylenic precursor stored in the carboxylic form, thus avoiding two additional steps: methylation of the starting compound and subsequent saponification of the hydrogenation product.

The synthesis of the amides and ester of labelled arachidonic acid was performed using the mixed anhydride and carbonyl fluorides techniques, respectively. Unlabelled compounds are usually prepared from milligram and above quantities of arachidonic acid with equivalents of other reagents. The reaction conditions allow the straightforward separation and chromatographic isolation of the final product. In the case of labelled arachidonic acid, a huge excess of the unlabelled reagents was applied to ensure its full conversion into target compounds. To avoid loss of labelled compounds during water workup, which is usually applied to eliminate most of the reagents,[12] the reaction mixture was directly separated by HPLC followed by evaporation to dryness and dissolution of the residue in methanol. Due to a considerable difference between the retention time of the labelled amides or ester and other reagents, and since the column load did not exceed 1 mg, the target compounds were isolated with a radiochemical purity of more than 98% within a single run.

The compounds thus prepared were used to study their transport into mouse splenic cells. Amides (1–3) undergo rapid ($t_{1/2} < 1$ min) uptake by mouse splenocytes (Figure. 2A); the amount of the label in the lipid extracts of cells after 15 min incubation averages 8–9% of the total radioactivity of the samples and does not depend on temperature for all amides (data not shown).

The ester derivative **(4),** which was previously demonstrated to be exhaustively hydrolysed in mouse splenocytes by a PMSF- and pHMB-insensitive lipase-like activity, also accumulates in the chloroform:-methanol extracts (10% of total activity after 15 min). The time course curve does not reach the plateau during the 15 min incubation period (Figure. 2B).

The results suggest that arachidonic acid amides **(1–3)** permeate the membrane by means of passive diffusion. Moderate permeability of the membrane for these compounds might be due to the presence of polar hydrophilic functionalities in their structure. Transmembrane transport of arachidonoylethyleneglycol **(4)** seems to be driven by the concentration gradient, maintained by hydrolytic enzymes and subsequent drain of arachidonic acid by remodelling into other lipid species.

Experimental

Materials

Catalysts, solvents and other reagents were purchased from Fluka (Buchs, Switzerland). Preparative purification of the labelled compounds was performed using a Gilson high-performance liquid

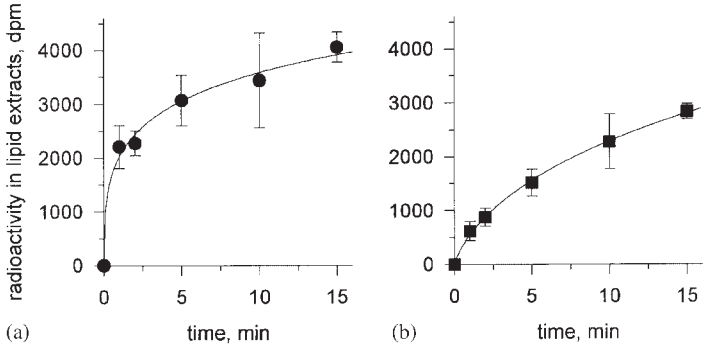

Figure 2. Time course of arachidonoylserotonin (2) – a and arachidonoylethyleneglycol (4) – b uptake by mouse spleen lymphocytes

chromatography system (France) equipped with a radioactivity detector and a variable wavelength UV detector.

Synthesis of labelled arachidonic acid: This was performed as described,[11] with minor modifications. The solution of 5,8,11,14-eicosatetraynoic acid in dioxane was exposed to gaseous tritium in the presence of Lindlar catalyst (acetylenic acid-catalyst-quinoline ratio 1:1:2, mg/mg/μ1) for 2 h at room temperature, the pressure of gaseous tritium was 400 hPa. The final product was isolated by HPLC[11] with a specific radioactivity of 170–180Ci/mmol, yield 20–25%.

Synthesis of labelled amides: Unlabelled arachidonic acid amides **(1–3)** were synthesized as described earlier.[5] Corresponding labelled compounds were prepared as follows. A solution of [^{3}H]arachidonic acid in acetonitrile (3 mCi, 120 Ci/mmol) was treated with a 100-fold excess of isobutylchloroformate and NEt_3. The reaction was carried out at 23°C in an argon atmosphere; reaction time is indicated in Table 1. The reaction mixture was evaporated under an argon stream and treated with 0.1 ml of the corresponding amine in DMF (100-fold excess of unlabelled over labelled compounds; 23°C, argon atmosphere). The reaction mixture was further evaporated under an argon stream, diluted in the eluent, and loaded onto the Kromasil C_{18} column (7 μm, 4 × 150 mm).

Synthesis of labelled ester: The ester derivative, arachidonoylethyleneglycol **(4),** was synthesized by the treatment of [^{3}H]arachidonic acid solution in acetonitrile (3 mCi, 120 Ci/mmol) with a solution of cyanuric fluoride and pyridine in acetonitrile (100-fold excess of non-labelled over labelled compounds; 23°C, argon atmosphere; reaction time indicated in Table 1). Then 0.1 ml of ethyleneglycol and a pyridine

Table 1. Reaction conditions for the synthesis of labelled amides (1–3) and ester (4) and corresponding HPLC retention times

Compound	Reaction time (h)		Retention time[a] (min)	Yield, (%)
	Stage 1 reagents: isobutylchloroformate (**1–3**) or cyanuric fluoride (**4**)	Stage 2 reagents: corresponding amines (**1–3**) or ethyleneglycol (**4**)		
1	2	20	9.21	91
2	2	20	8.49	83
3	2	20	9.86	90
4	4	48	12.67	73

[a] HPLC, see Experimental.

solution in acetonitrile was added to the reaction mixture; the solvent was evaporated under an argon stream, and the residue dissolved in the eluent and loaded onto the column. The corresponding unlabelled ester was prepared according to the instruction given in Reference [6].

The compounds were purified by HPLC in MeOH:H_2O:AcOH:CF_3-COOH (90:10:0.1:0.01, v:v:v:v); elution rate 1 ml/min. The peaks were assigned by comparison of the retention times of the respective standards, prepared from 'cold' arachidonic acid (see Table 1).

Isolation of mouse spleen lymphocytes: Female CBA mice (18–25 g), maintained at 22°C with free access to food and water were sacrificed by means of cervical dislocation. Spleens were isolated and dissected in a Petri dish in a small amount of Hanks' balanced salt solution (HBSS). The cell suspension was filtered through a fine mesh (6.25×10^{-4} mm^2) nylon sieve, and erythrocytes were lysed by addition of 0.83% NH_4Cl solution to the suspension (6:1, v:v), followed by 15 min centrifugation at $300 \times g$ at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of 0.25 M sucrose solution containing 3 mM $MgCl_2$. Centrifugation was repeated, and the resulting pellet was washed in HBSS twice. Cells were counted in a cytometer; their viability was assessed using a Trypan blue dye exclusion test and exceeded 95–97%.

Incubation: Mouse spleen lymphocytes (2×10^7 cells/ml) were incubated in HBSS for 1–15 min at 4° or 37°C with the compounds under study (~2 nM; 0.11–0.22 μCi per sample). The reaction was terminated by rapid (10–15 s) centrifugation at $8000 \times g$, the pellet washed with 0.2% BSA solution in HBSS and extracted with an acidified mixture of chloroform:methanol (2:1, v:v). The radioactivity of lipid extracts was measured by liquid scintillation counting in 'Universol' scintillation cocktail (ICN Biomedicals) (counting efficiency ~40%) using Beckman LS9800 (Beckman) or Packard 2100TR (Packard BioSystems) counters.

Statistical analysis and data presentation: Statistical analysis of the temperature dependence of the uptake was performed using student's paired two-tailed *t*-test ($p < 0.05$). Points on the charts represent mean ± S.D. of the respective samples ($n = 3$).

Conclusions

Tritium labelled compounds of high specific activity closely related to endocannabinoids were synthesized by means of a one pot method.

These compounds can be used as tracers in studies of transport, receptor interaction and metabolism of the corresponding amides and ester of arachidonic acid.

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