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The preparation of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol

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2-*O*-Arachidonoyl-1-*O*-stearoyl-*sn*-glycerol is the most abundant molecular species of the 1,2-diacyl-*sn*-glycerol signaling lipids in neural tissue. The facile preparation of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol from 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine at a hexane and phosphate buffer interface with phospholipase C was demonstrated on a 20 μCi scale in 83% radiochemical yield. The specific activity of the product 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol was 57.0 mCi/mmol and the radiochemical purity was determined to be >99% by TLC. The hydrolysis of this lipid biosynthetic intermediate with lipoprotein lipase was shown to produce 2-*O*-[1′-^{14}C]arachidonoylglycerol (2-AG). The ^{14}C-radiolabeled monoacylglycerol 2-AG is an endogenous cannabinoid receptor-signaling molecule (endocannabinoid).

Keywords: 2-*O*-arachidonoyl-1-*O*-stearoyl-*sn*-glycerol; ^{14}C-labeled; diacylglycerol; 2-arachidonoylglycerol; 2-AG

Introduction

1,2-Diacyl-*sn*-glycerols have important physico-chemical and juxtacrine signaling functions.[1–4] Although diacylglycerols are found in extremely low concentration in cell membranes, 2-*O*-arachidonoyl-1-*O*-stearoyl-*sn*-glycerol is the most abundant molecular species in brain[5] and peripheral nerve.[6] The hydrolysis of this lipid by diacylglycerol lipase (DAGL) produces the endocannabinoid 2-*O*-arachidonoylglycerol (2-AG).[7,8] The endocannabinoid system is the focus of our research and includes the two most abundant endocannabinoid lipids, 2-AG and *N*-arachidonoylethanolamine (AEA), each having distinct signaling functions.[9,10] Radiolabeled 2-*O*-arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) (see Scheme 1) was required for our studies of DAGL enzyme activity and the biosynthesis of ^{14}C-labeled endocannabinoid 2-AG (**3**). 2-*O*-[1′-^{14}C]Arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) has recently been used to assay the activity of hDAGL-α preparations,[11,12] including in the presence of inhibitors, but is no longer commercially available.

This report describes the convenient preparation of radiolabeled 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) via a biomimetic route that uses the phosphocholine phosphodiester group for protection. Syntheses of labeled 1,2-diacyl-*sn*-glycerols have previously used the 3-phosphocholine group,[13–18] 3-phosphate group,[19] or 3-phosphoinositol group[20] as protecting groups in chemo-enzymatic syntheses. Other classical protecting groups have also been used in purely chemical syntheses of unlabeled[21–23] and labeled[11] 1,2-diacyl-*sn*-glycerols and labeled *rac*-1,2-diacylglycerols,[24,25] although such routes involve the technical challenge of avoiding acyl group migration during a microscale radiosynthesis.

Our conversion of the commercially available labeled substrate 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine (**1**) utilized the regiospecificity of the enzymatic hydrolysis by phospholipase C.[26] The hydrolysis of the phosphatidylcholine **1** with phospholipase C from *Bacillus cereus* was performed at neutral pH. The mild reaction conditions are critical for 1,2-diacyl-*sn*-glycerols, where variation of pH from 7 or increased temperature results in rearrangement of 1,2-diacyl-*sn*-glycerol to 1,3-diacyl-*sn*-glycerol.[27–29] 1,2-Diacylglycerols are reportedly more stable in hydrocarbon solution.[30] The 1,2-diacylglycerol and 1,3-diacylglycerol isomers are readily separable by analytical TLC[28,31,32] and HPLC.[24,25] However, our 1,2-diacyl-*sn*-glycerol isolation following the enzymatic protecting group cleavage required only flash chromatographic filtration to remove trace amounts of unreacted starting material as well as the phosphocholine salt byproduct origin materials. We had previously used this methodology with unlabeled 2-*O*-arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine on scales of up to 10 mg to give unlabeled 2-*O*-arachidonoyl-1-*O*-stearoyl-*sn*-glycerol that was homogeneous by TLC (30:70 acetone/hexane, developing with phosphomolybdic acid reagent) and characterized by ^{1}H NMR.

Results and discussion

The substrate 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine (**1**) (GE Healthcare CFA655) contained a

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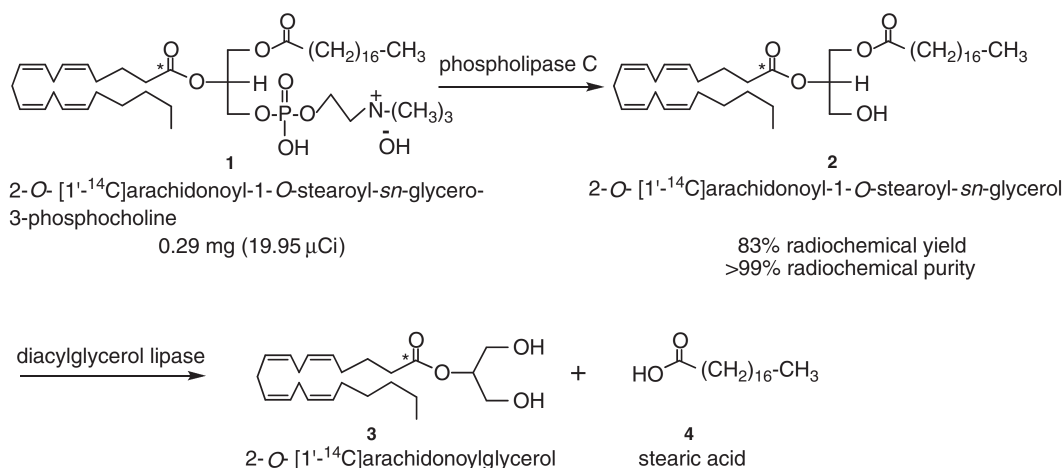

Scheme 1. Enzymatic preparations of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) and 2-*O*-[1′-^{14}C]arachidonoylglycerol (**3**, 2-AG).

^{14}C-label in the carbonyl of the arachidonoyl group, and the [1′-^{14}C]arachidonoyl group was assayed to be 97.5% in the *sn*-2 position. This substrate **1** had a specific activity of 57.0 mCi/mmol and was found to be nearly homogeneous (>98% radiochemical purity) by TLC (R_f 0.00, 30:70 acetone/hexane; R_f 0.33, 60:30:5 chloroform/methanol/water). The phospholipase C (Sigma P9439 from *B. cereus*) was reconstituted with water to give a phosphate buffered solution that could be frozen, stored at −20°C, and reused. Generally, PLC hydrolyses have been run at an interface of diethyl ether and aqueous buffer.[13,16,17,33,34] We found that the reaction proceeds when hexane is used, although the enzyme activity does fall off and the conversion must be driven to completion by adding fresh enzyme solution at 20 min intervals over 2 h. The phase separation of hexane from aqueous buffer was easier than when using diethyl ether. There is still some solubility of the oily phosphocholine substrate **1** in hexane even though it carries the zwitterionic phosphocholine head group. The complete removal of any trace starting material and salts was performed by adding acetone to reach a 30:70 acetone/hexane ratio followed by flash filtration through a short plug of silica gel. The column was then washed with an equal volume of fresh 30:70 acetone/hexane. Both elution fractions were found to contain product with no starting material (see Figure 1) and were ultimately combined to give an overall radiochemical yield of 83%. The specific activity of the product 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) was 57.0 mCi/mmol and the radiochemical purity was determined to be >99% by TLC (R_f 0.28, 4:96 acetone/chloroform; R_f 0.49, 30:70 acetone/hexane; R_f 0.95, 60:30:5 chloroform/methanol/water). The tailing of the 1,2-diacyl-*sn*-glycerol **2** spot was comparable with that seen using a related solvent system.[29] A small amount of 1-*O*-[1′-^{14}C]arachidonoyl-2-*O*-stearoyl-*sn*-glycerol may be present from the starting phosphocholine. Rearrangement of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) to 3-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol would have given a slightly higher R_f spot for this byproduct in the TLC analysis with 30:70 acetone/hexane.[2,23] The presence of the rearrangement impurity could affect the study of the DAGL enzyme where the 1,3-diacylglycerol byproduct could act as a substrate or inhibitor.

We used the 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) for our preliminary experiments with lipoprotein lipase (from *Pseudomonas sp*., Sigma), which has excellent 1,2-diacyl-*sn*-glycerol lipase activity with high selectivity for the *sn*-1 acyl group.[35–37] The lipoprotein lipase readily hydrolyzed 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) enzymatically at a hexane and phosphate buffer interface. Analytical TLC (86:14:0.75 chloroform/methanol/aqueous ammonium hydroxide) shows the enzymatic hydrolysis of the radiolabeled 1,2-diacyl-*sn*-glycerol **2** (R_f 0.92) to give 53.8% conversion to 2-*O*-[1′-^{14}C]arachidonoylglycerol (**3**, R_f 0.59) with the release of only 0.4% of [1-^{14}C]arachidonic acid (R_f 0.15) after 15 min at 37°C (see Figure 1). The 2-acylglycerol product **3** was not further characterized[18,22,38] as this radiolabeled 2-AG **3** was prepared only on an analytical scale.

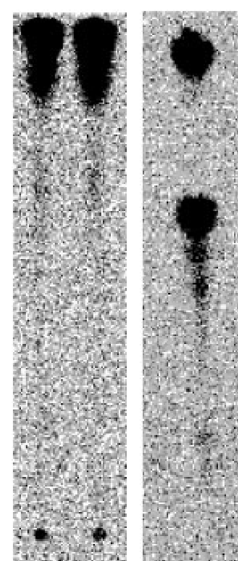

Figure 1. Left: TLC (60:30:5 chloroform/methanol/water) of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**, R_f 0.95) (filtration elution Lane 1 and column wash Lane 2) from the phospholipase C hydrolysis of the phosphodiester 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine (**1**). Right: TLC (86:14:0.75 chloroform/methanol/aqueous ammonium hydroxide) of the enzymatic conversion of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**, R_f 0.92) to 2-*O*-[1′-^{14}C]arachidonoylglycerol (**3**, R_f 0.59) after 15 min with 58 units of lipoprotein lipase (from *Pseudomonas sp*.).

Thus, the facile preparation of the ^{14}C-radiolabeled 1,2-diacyl-*sn*-glycerol signaling lipid molecule **2** free of any 1,3-diacyl-*sn*-glycerol rearrangement byproduct was demonstrated on a 20 μCi scale. This 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) activates protein kinase C and is the biosynthetic

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precursor of the endocannabinoid signaling molecule 2-AG **3**. The regioselective hydrolysis of ^{14}C-radiolabeled 1,2-diacyl-*sn*-glycerol **2** by an enzyme with DAGL activity to give 2-AG **3** was also demonstrated.

Experimental

General

All TLC used silica gel 60 on glass that was 250 μm thickness (E. Merck). After elutions and thorough air drying, latent images of the TLC plates were made on multisensitive phosphor screens (Perkin–Elmer) that were then quantified on a Perkin–Elmer Cyclone phosphoimaging system.

2-*O*-[1′-^{14}C]Arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (2)

In a 1.5 mL screw-top Wheaton vial was added 19.95 μCi of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycero-3-phosphocholine (**1**) (GE Healthcare CFA655, 57.0 mCi/mmol) in 1:1 toluene:ethanol in 200 μL portions that were evaporated to dryness in a gentle argon stream at ambient temperature. The phosphatidylcholine **1** residue was immediately partitioned between 100 μL of phospholipase C (Sigma P9439 from *B. cereus*, 0.2 unit/μL, phosphate buffer, pH 7.0) and 400 μL of hexane and stirred gently with a triangular magnet at ambient temperature under argon. Additional 50 μL portions of phospholipase C in buffer were delivered by pipette to the bottom (aqueous) phase at 20 min intervals for a total of 400 μL. A sample of the hexane phase was removed after 2 h and found to contain >95% product 1,2-diacyl-*sn*-glycerol **2** by TLC (R_f 0.49, 30:70 acetone/hexane; R_f 0.95, 60:30:5 chloroform/methanol/water). The bottom (aqueous) phase was removed by pipette, found to contain only 3% of the radioactivity by scintillation counting, and was discarded. The top (hexane) phase was transferred to a clean screw-top vial and dried briefly over Na_2SO_4. The dry hexane solution was transferred to a new vial, and to this solution (400 μL) of crude product was added 171 μL of acetone. A plug of silica gel (150 mg, 0.3 cc) over a bed of glass wool and sand in a disposable 1 mL syringe body fitted with a 0.2 μm PTFE filtration membrane was thoroughly washed with 30:70 acetone/hexane and used to remove origin material. The solution of crude product in 30:70 acetone/hexane was rapidly forced through the column and immediately washed with an additional 900 μL portion of 30:70 acetone/hexane. The column elution and wash fractions were both found to be free of starting phosphatidylcholine **1** and were combined to give an 83% radiochemical yield of 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**, 57.0 mCi/mmol) that was >99% radiochemically pure by TLC containing a very small amount of origin and some tailing in 60:30:5 chloroform/methanol/water (R_f 0.95) from spotting and elution. The tailing appeared more significant in 30:70 acetone/hexane (R_f 0.49), however, there was no evidence of the rearrangement byproduct 1,3-diacyl-*sn*-glycerol that would have had a slightly higher R_f than that for the 1,2-diacyl-*sn*-glycerol **2**. The product 2-*O*-[1′-^{14}C]arachidonoyl-1-*O*-stearoyl-*sn*-glycerol (**2**) was stored in the acetone/hexane solution under an argon atmosphere at −20°C when not in use and was stable for months.

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