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# Simplified chemical and radiochemical synthesis of 2-arachidonoyl-glycerol, an endogenous ligand of cannabinoid receptors

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**Abstract**—Endocannabinoids have emerged as a new class of lipid mediators, with manifold roles in the central nervous system and in the periphery. Several studies have identified 2-arachidonoyl-glycerol (2-AG) as a major endogenous agonist of cannabinoid receptors. Here, the chemical synthesis of 2-AG is reported, along with the synthesis of its tritium-labeled derivative. These unlabeled and radiolabeled compounds are suitable tools for unravelling some metabolic routes and biological activities of 2-AG in various cells and tissues.


The importance of phospholipid-derived molecules as intra and extracellular signals in animal cells is well-known and documented.[1] Among these molecules, endocannabinoids have emerged as a new class of mediators, present in the central nervous system and in peripheral tissues.[2] They are amides, esters and ethers of long chain polyunsaturated fatty acids, and to date *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl-glycerol (2-AG) are the most studied members.[3] They bind to both type-1 ($CB_1$) and type-2 ($CB_2$) cannabinoid receptors, thus mimicking some of the psychotropic and analgesic effects of $\Delta^9$-tetrahydrocannabinol, the psychoactive principle of hashish and marijuana.[2b,4] The first studies on endocannabinoids focused on metabolism and biological activity of AEA.[5] Later on it also became clear that 2-AG has a critical role in the endocannabinoid system, to such an extent that it has been proposed as the real endogenous ligand of $CB_1$ and $CB_2$ receptors in vivo. [6] In addition, a number of recent studies have identified 2-AG as a key regulator of microglial cells in the central nervous system,[7] and of blood cell migration in the periphery.[8] It is worth noting that the pathways for the biosynthesis and degradation of 2-AG are different from those of AEA,[9] and include a recently identified monoglyceride lipase as the primary mechanism for 2-AG inactivation.[10] Therefore, in order to shed light on the metabolic routes and biological activity of 2-AG, it was necessary to synthesize the labeled compound, which was not yet commercially available at the beginning of this work. We could not find a simple synthesis in the literature suitable for labeling 2-AG. In fact, in one study, 2-[1-$^{14}$C]arachidonoyl-*sn*-glycerol was prepared by digestion of 1-stearoyl-2-[1-$^{14}$C]-arachidonoyl-*sn*-glycerol with *Rhizopus arrhizus* lipase,[11] and in another study [$^3$H]2-AG was synthesized from [$^3$H]AA and 1,3-benzylideneglycerol.[12] However, neither investigation reported enough experimental details to reproduce the synthetic steps.[11,12] Therefore, we sought to develop an easy and reproducible preparation of [$^3$H]2-AG, based on a new radiochemical synthesis, which was a slight modification of the synthesis of unlabeled 2-AG reported in this paper (Scheme 1). A preliminary account of the synthetic route to [$^3$H]2-AG has been presented.[13]

*Abbreviations:* 2-AG, 2-arachidonoyl-glycerol; [$^3$H]2-AG, labeled 2-arachidonoyl-glycerol; 1-AG, 1-arachidonoyl-glycerol; AEA, *N*-arachidonoylethanolamine; AA, arachidonic acid; [$^3$H]AA, labeled arachidonic acid.

*Keywords*: 2-Arachidonoyl-glycerol (2-AG); Endocannabinoids; Radiosynthesis; Synthesis.

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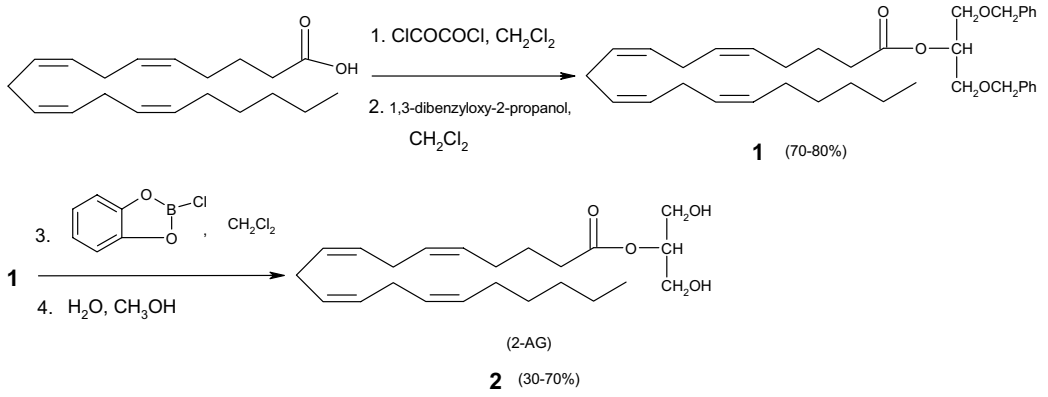
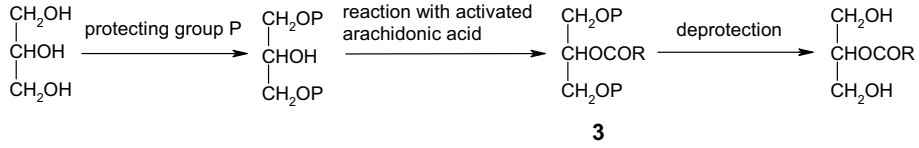



**Scheme 1.**




**Scheme 2.**

In the literature, different synthetic routes towards unlabeled 2-AG have been reported. They were developed to avoid two main problems associated with the preparation of this compound: (i) rearrangement of 2-AG to the more stable 1-AG isomer, favored by heat, acids and bases; and (ii) air oxidation of the arachidonic acid chain.[14] Common sequences for 2-AG synthesis are reported in Scheme 2.

In general, the primary hydroxyl groups of the starting material (glycerol) are protected using different reagents (benzaldehyde, boric acid derivatives, triisopropylsilyl chloride). Then the protected glycerol is reacted with the activated arachidonic acid. The intermediate **3** is purified and stored under argon at low temperature. When 2-AG is required, the protecting groups are eliminated from **3**, using different experimental conditions, which should prevent rearrangement of 2-AG to 1-AG. If isomerization takes place or if the deprotection step is not performed to completion, the isomeric products are separated by HPLC. Another synthesis of 2-AG circumvents these shortcomings, by using glycidyl arachidonate as starting material, obtained from commercially available (±)-glycidol and arachidonic acid.[15] The different synthetic routes reported for unlabeled 2-AG[14,15] may not represent per se useful procedures for the synthesis of the corresponding radiolabeled compound, because radiochemical reactions need to be planned in such a way that formation of by-products and side reactions are avoided, and the number of purification steps of the labeled compounds is minimized. To meet these requirements, we carried out a synthesis of unlabeled 2-AG, taking advantage of the experimental conditions found by others to prevent isomerization and oxidation of 2-AG.[14] In this framework, we developed a synthetic strategy based on two steps only, which avoided by-products and allowed a good yield of product in each step. The experimental conditions were also advantageous for the use of the expensive [$^3$H]AA as starting material. Our previous experience on the synthesis of *N*-acylethanolamines such as *N*-arachidonoylethanolamine, *N*-oleoylethanolamine, *N*-palmitoylethanolamine and *N*-steroylethanolamine, prompted us to add the commercial (unlabeled or tritiated) arachidonoyl moiety to the 1,3-protected glycerol by activating the carboxylic group of the acid with oxalyl chloride.[16,17] We chose to use 1,3-dibenzyloxy-2-propanol as a commercially available 1,3-protected glycerol, because it is possible to remove the protecting groups in a clean and quantitative manner using *B*-chlorocatecholborane as a selective reagent.[18] The main problem in performing the last step was to find the optimal reaction time and experimental conditions for the work-up of the final reaction mixture in order to prevent the isomerisation of the 2-AG product into its regioisomer 1-AG.[19] To overcome this problem, we performed the last step over different reaction times and tried to cleave the complex formed between **1** and *B*-chlorocatecholborane with different solvent mixtures: (i) $H_2O$ and 0.1 M NaOH; (ii) ice-cold water and 0.1 M NaOH; and (iii) $H_2O$ and $CH_3OH$ (1:1). After several attempts, we found that the yield of 2-AG was higher when the reaction was stopped after 2 h, and the reaction mixture was treated with a solution of ice-cold water and methanol to remove the two benzyl groups from compound **1**. Only 5% or less of 1-AG was present under these conditions, whereas longer reaction times or dilute solutions of NaOH in the work-up led to large amounts of 1-AG. The presence of both isomers in the final reaction mixture was evident after NMR analysis of the material. Indeed the chemical shifts of the protons in the glycerol moiety are different and well separated in

1-AG and 2-AG.[20] The experimental conditions found to be suitable to obtain the highest yield of 2-AG were used to synthesize labeled 2-AG successfully.[21] [$^3$H]2-AG has been used to assay binding, transport and hydrolysis of 2-AG in different cells and tissues.[22] In particular, the synthesis of [$^3$H]2-AG reported here has allowed us to demonstrate for the first time that human platelets have the biochemical tools to bind and metabolize 2-AG,[23,24] a finding, which opens new perspectives in the control of platelet aggregation, and more generally in our understanding of vascular biology.

Although we cannot prevent some formation of 1-AG (less than 5%) during the preparation of the 2-AG isomer, we have developed a simple and convenient synthesis of unlabeled and tritiated 2-AG. The labeled compound has been shown to be useful for the study of the metabolism and biological activity of 2-AG, and it is anticipated that it will be a suitable tool for unravelling, at least in part, the complex involvement of this lipid messenger in several aspects of human pathophysiology.

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17. Preparation of unlabeled 2-arachidonoyl-1,3-dibenzylglycerol **1**: To a solution of arachidonic acid (14 mg, 0.045 mmol, 1 equiv) in 500 μL of anhydrous dichloromethane, oxalyl chloride (15 μL, 0.18 mmol, 4 equiv) was added under an argon atmosphere. The mixture was stirred in the dark, to avoid the oxidation of arachidonic acid, at room temperature. After 4 h, excess oxalyl chloride was eliminated by flushing the reaction mixture with argon, adding 300 μL of anhydrous $CH_2Cl_2$ three times. Afterwards the activated archidonic acid was dissolved in 500 μL of $CH_2Cl_2$ and an excess of 1,3-dibenzyloxy-2-propanol (120 μL, 0.48 mmol, 10 equiv) was added to the solution under argon. The mixture was left to react with stirring in the dark at room temperature for 24 h. The reaction was monitored by thin-layer chromatography (TLC, silica gel 60 $F_{254}$ plates) using hexane/ethyl acetate (5:1) as eluent and spraying the plate with ammonium molybdate–cerium(IV) sulfate. The organic phase was washed with NaOH (0.1 M) and then with distilled water until neutral and then dried over anhydrous $Na_2SO_4$. After filtration and evaporation, the residue was purified via preparative TLC (Merck, art. 13894, 20 × 20 × 0.05 cm) using hexane/ethyl acetate (20%) as eluent. The product was recovered from silica gel using $CH_2Cl_2$. A colourless dense oil was obtained (yield: 70–80%). The crude material (when more than 100 mg)

was purified by flash chromatography (Merck silica gel 60, 70–230 mesh) using hexane/ethyl acetate (5%) as eluent.
$^1$H NMR (600 MHz, $CD_2Cl_2$, 298 K, see Scheme 1); $\delta$ 0.90 (3H, t, $^3J = 7.1$ Hz, –C$H_3$); 1.29–1.37 (6H, m, –(C$H_2$)$_3$CH$_3$); 1.69 (2H, p, $^3J = 7.4$ Hz, –CH$_2$C$H_2$CH$_2$CO); 2.06–2.13 (4H, m, –CH$_2$C$H_2$CH=CH–); 2.34 (2H, t, $^3J = 7.4$ Hz, –C$H_2$CO); 2.82–2.85 (6H, m, –CH=CHC$H_2$CH=CH–); 3.63 (4H, br d, $^3J = 6.0$ Hz, OC$H_2$CH(OCO)C$H_2$O); 4.49 (4H, AB system, $^2J = 12$ Hz, –OC$H_2$Ph); 5.22 (1H, br p, $^3J = 6.0$ Hz, –CH$_2$C$H$(OCO)CH$_2$–); 5.33–5.40 (8H, m, –C$H$=CH–); 7.29–7.34 (10H, m, ArH). Anal. Calcd (%) for $C_{37}H_{50}O_4$ (558.79): C, 79.53; H, 9.02. Found: C, 79.40; H, 9.13.
The geminal coupling of the –OC$H_2$Ar protons was confirmed using $^1$H–$^1$H COSY NMR (not shown). The same protons gave a singlet in the $^1$H NMR of 1,3-dibenzyloxy-2-propanol.
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20. Preparation of unlabeled 2-arachidonoylglycerol **2**: To a solution of **1** (20 mg, 0.036 mmol, 1 equiv) in 400 μL of anhydrous $CH_2Cl_2$, *B*-chlorocatecholborane (18 mg, 0.12 mmol, 3 equiv) was added. After stirring for 2 h in the dark at room temperature the reaction was checked by TLC using hexane/ethyl acetate (1:1) with tetrabutylammonuium hydroxide (1%) as eluent. A single spot was observed ($R_f = 0.2$), which was different from that of **1** ($R_f = 0.7$), and from that of *B*-chlorocatecholborane ($R_f = 0.5$). The organic phase was washed once with cold distilled water (200 μL) and methanol (200 μL) until neutrality and then dried over anhydrous $Na_2SO_4$. After filtration and elimination of the solvent by flushing the solution with argon the product was purified by preparative TLC (Merck, art. 1.11798) using hexane/ethyl acetate (1:1) as eluent. The final product was recovered from silica gel with $CH_2Cl_2$. A colourless oil was obtained (yield: 30–70%).
$^1$H NMR (2-AG, 600 MHz, $CD_3OD$, 298 K see Scheme 1); 0.97 (3H, t, $^3J = 7.1$ Hz, –C$H_3$); 1.37–1.44 (6H, m, –(C$H_2$)$_3$CH$_3$); 1.76 (2H, p, $^3J = 7.4$ Hz, –CH$_2$C$H_2$CH$_2$CO); 2.13–2.21 (4H, m, –CH$_2$C$H_2$CH=CH–); 2.45 (2H, t, $^3J = 7.4$ Hz, –C$H_2$CO); 2.89–2.92 (6H, m, –CH=CHC$H_2$CH=CH–); 3.69 (2H, dd, $^3J = 5.8$ Hz, $^2J = 11.8$ Hz, OCH$H$CH(OCO)CH$H$O); 3.73 (2H, dd, $^3J = 4.7$ Hz, $^2J = 11.8$ Hz, OC$H$HCH(OCO)C$H$HO); 4.96 (1H, br p, –CH$_2$C$H$(OCO)CH$_2$–); 5.41–5.45 (8H, m, –C$H$=CH–).
GC–MS analysis of 2-AG: 105 (49%); 91 (74%), 79 (100%).[16a]
Anal. Calcd (%) for $C_{23}H_{38}O_4$ (378.56): C, 72.98; H, 10.12. Found: C, 72.87; H, 10.06.
$^1$H NMR (1-AG, 600 MHz, $CD_3OD$, 298 K): $\delta$ 0.97 (3H, t, $^3J = 7.1$ Hz, –C$H_3$); 1.35–1.45 (6H, m, –(C$H_2$)$_3$CH$_3$); 1.76 (2H, p, $^3J = 7.4$ Hz, –CH$_2$C$H_2$CH$_2$CO); 2.12–2.21 (4H, m, –CH$_2$C$H_2$CH=CH–); 2.43 (2H, t, $^3J = 7.4$ Hz, –C$H_2$CO); 2.87–2.92 (6H, m, –CH=CHC$H_2$CH=CH–); 3.60–3.62 (2H, m, –C$H_2$OH); 3.88 (1H, br p, –CH$_2$C$H$(OH)CH$_2$–); 4.11 (1H, dd, $^3J = 6.3$ Hz, $^2J = 11.4$ Hz, –CH$_2$CH(OH)C$H$HOCO); 4.20 (1H, dd, $^3J = 4.4$ Hz, $^2J = 11.4$ Hz, –CH$_2$CH(OH)CH$H$OCO); 5.39–5.47 (8H, m –C$H$=CH–).
GC–MS analysis of 1-AG: 105 (56%); 91 (86%), 79 (100%).
21. Radiosynthesis of 2-AG: The solution of [5,6,8,9,11,12,14,15-$^3$H(N)] arachidonic acid (250 μCi; 200 Ci/mmol) in ethanol was evaporated by adding anhydrous $CH_2Cl_2$ (100 μL) and applying three cycles of evaporation in the dark and under an argon flow. Afterwards the reaction system, dissolved in 50 μL of $CH_2Cl_2$, was tightly sealed (hole cap with a silicon Teflon liner) under argon and a large excess of oxalyl chloride (5 μL, 2 M solution in $CH_2Cl_2$) was added. We proceeded as described for the preparation of unlabeled **1** using 50 μL of $CH_2Cl_2$ to dissolve the activated [$^3$H]AA then adding 3 μL of 1,3-dibenzyloxy-2-propanol. The work-up was carried out with NaOH 0.1 M (300 μL) and brine (300 μL) until the solution became neutral. The organic phase was dried over $Na_2SO_4$ and the salt was successively filtered with a SPE minicolumn filter. The solvent was evaporated and, after dissolving the reaction products in a hexane/ethyl acetate mixture (20%), the excess of 1,3-dibenzyloxy-2-propanol was eliminated by chromatography using SepPaK™ silica gel cartridges. The protected [$^3$H]2-AG so recovered was checked by TLC (see preparation of **1**) and showed a very high level of purity and a radiochemical yield greater than 90% (static β-counter recovered 225 μCi). The solvent was evaporated and a freshly prepared solution of *B*-chlorocatecholborane (6 mg) in $CH_2Cl_2$ (50 μL) was added. We proceeded as described for the preparation of **2**: 500 μL of $CH_2Cl_2$ was added and the reaction mixture treated with 200 μL of brine. The organic phase was checked by TLC (see above), counted for radioactivity (about 215 μCi) and dried over $Na_2SO_4$. After evaporating the organic phase, [$^3$H]2-AG was dissolved in a hexane/ethyl acetate mixture (1:1) and purified on a SepPaK™ silica gel cartridge by elution with a $CHCl_3$/$CH_3OH$ mixture (5%). After two cycles of evaporation–dissolution with absolute EtOH, 200 μCi of (2)-[5,6,8,9,11,12,14,15-$^3$H(N)]-arachidonoyl-glycerol (overall radiochemical yield 80%) were recovered and stored in ethanol.
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