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# S-Arachidonoyl-2-thioglycerol synthesis and use for fluorimetric and colorimetric assays of monoacylglycerol lipase

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ABSTRACT

We report the first synthesis of 2-thioglycerol and *S*-arachidonoyl-2-thioglycerol (the thioester analog of the endocannabinoid 2-arachidonoylglycerol) in an eight or nine step procedure with a yield of ~25% and establish the use of this substrate for maleimide-based fluorescent and dithiobis(2-nitrobenzoic acid)-based colorimetric assays of human recombinant monoacylglycerol (MAG) lipase (hMAGL) and human brain membrane MAG hydrolase activity. Inhibitor structure–activity relationships observed here for hMAGL and 2-ATG correlate well ($r^2 = 0.93$, $n = 9$) with earlier findings for mouse brain MAG hydrolase with non-thiol substrates.



## 1. Introduction

The endocannabinoids 2-arachidonoylglycerol (2-AG)[1] and *N*-arachidonoyl ethanolamide (anandamide, AEA)[2] (Fig. 1) bind to and activate two G-protein coupled receptors, $CB_1$ and $CB_2$.[3] $CB_1$ receptors are the most abundant G-protein coupled receptors in brain and are also expressed in many other parts of the body, while $CB_2$ receptors are almost exclusively in the immune system. After serving their signaling roles 2-AG and AEA are taken up into the cell where 2-AG is hydrolyzed primarily by monoacylglycerol lipase (MAGL) to arachidonic acid (AA) and glycerol[4,5] and AEA by fatty acid amide hydrolase (FAAH) to AA and ethanolamine[6,7] (Fig. 1).

The endocannabinoid system is a potential therapeutic target[8–10] for appetite disorders,[11,12] pain and inflammation,[13–15] neurodegenerative diseases,[16–19] and cancer.[20] Although $CB_1$ agonists such as tetrahydrocannabinol have therapeutic potential, they are associated with side effects, such as hypomotility, catalepsy, hypothermia, and cognitive dysfunction. An alternative therapeutic strategy is to block the degradation of 2-AG or AEA by inhibiting MAGL or FAAH or both.[21–23] While FAAH is moderately well understood, with a defined three dimensional structure, knockout mice, and a plethora of small molecule inhibitors, the tertiary structure of MAGL is only recently defined,[24,25] knockout mice don't exist, and only a few small-molecule inhibitors have been identified.[26]

A rapid and selective high-throughput MAGL or 2-AG hydrolase assay would greatly facilitate the discovery of new inhibitors and candidate therapeutic agents. MAGL in brain membranes mediates about 85% of the total 2-AG hydrolase activity with two additional serine hydrolases bringing the total to 98%.[27,28] Outside of the brain, substrate specificity and the contribution of MAGL to total 2-AG hydrolase activity differs, although in the majority of tissues MAGL is most important. Selection of a preferred substrate for MAGL assays depends on the enzyme source and preparation, the analytical method (radiotracer, chromatographic, colorimetric or

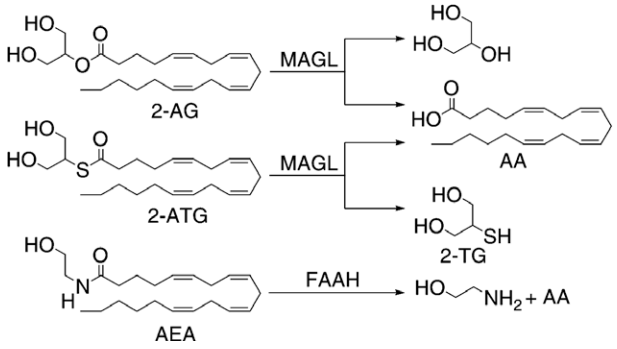


**Figure 1.** Monoacylglycerol lipase (MAGL) hydrolyzes the principal endocannabinoid 2-arachidonoylglycerol (2-AG) or its thiol ester analog *S*-arachidonoyl-2-thioglycerol (2-ATG) to arachidonic acid (AA) and glycerol or 2-thioglycerol (2-TG). The second endocannabinoid anandamide (AEA) is hydrolyzed by fatty acid amide hydrolase (FAAH) to AA and ethanolamine.

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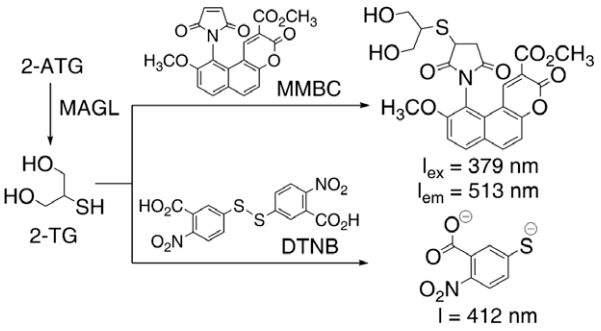



**Scheme 1.** Fluorimetric and colorimetric analysis of 2-TG from enzymatic hydrolysis of 2-ATG.

fluorimetric) and the number of samples (a few samples versus rapid-throughput screens). The endocannabinoid 2-AG itself is obviously the most relevant substrate. Many types of 2-AG hydrolysis assays have been used involving chromatographic and radiotracer techniques (Supplementary data). 1-Acylglycerol and other esters also have been employed as alternative MAGL substrates including 1-[$^{14}$C]oleoylglycerol (radiotracer); 1-(*p*-nitrobenzofurazanacyl)-glycerol and *S*-arachidonoyl-1-TG (1-ATG) (colorimetric); and 7-hydroxycoumarinyl arachidonate (fluorimetric) (Supplementary data). The colorimetric and fluorimetric assays with 1-acylglycerol esters allow kinetic monitoring of the reactions and high-throughput screens but unless recombinant or purified preparations are used they may measure an undefined balance of enzymes in addition to MAGL.

An obvious alternative to 2-AG for MAGL assays is its thiolester *S*-arachidonoyl-2-thioglycerol (2-ATG). It is the closest mimic of 2-AG with minimal changes in steric and electronic properties. The hydrolysis product 2-thioglycerol (2-TG) should be easily determined by fluorimetric assay with methyl maleimidobenzochromenecarboxylate (MMBC)[29] or colorimetric analysis with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)[30] (Scheme 1) allowing rapid-throughput screens. This potential is readily evident from the extensive use of acetylthiocholine in place of acetylcholine in studying acetylcholinesterase and its inhibitors.[31,32] There is also the possibility that 2-ATG may allow histochemical localization of the cytological distribution of MAGL activity based on derivatization of liberated 2-TG as has been used so successfully for cholinesterase localization based on thiocholine release.[32,33] The MAGL active site has the typical Ser-Asp-His catalytic triad but in contrast to most α/β hydrolases it also has a sensitive and accessible cys[34] providing the possibility of inhibition and fluorescence labeling with a thiol-reactive dye.[35]

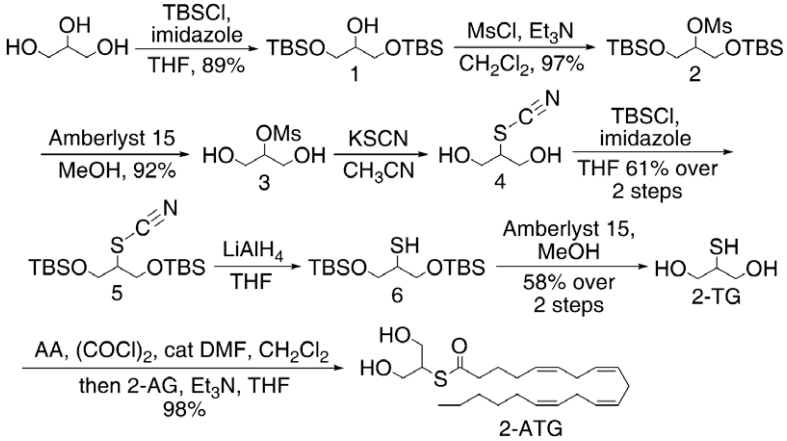
This paper describes the first synthesis of 2-TG and 2-ATG (Scheme 2) and then establishes their use with MMBC and DTNB for fluorimetric and colorimetric assays of MAGL and other enzymes involved in 2-AG hydrolysis. To our knowledge, 2-TG is the only one of the mono-, di- and trithioglycerols not previously reported.

## 2. Results and discussion

### 2.1. Sources of chemicals and synthesis

AEA, 1-ATG and JZL184 were from Cayman Chemical Co. (Ann Arbor, MI), DTNB and 1- and 2-OG were from Sigma (St. Louis, MO) and MMBC was from Berry and Associates (Dexter, MI). Several of the OP inhibitors were prepared in this laboratory (Supplementary data).

A synthesis of 2-TG started with selective protection of the primary hydroxyl groups of glycerol with *tert*-butyldimethylsilylchloride (TBSCl) and imidazole in tetrahydrofuran (THF) to give **1** (Scheme 2). Under these conditions, glycerol was only sparingly soluble in THF, whereas **1** was very soluble which therefore might be expected to give triprotected glycerol. However, the high yield of diprotected glycerol suggested that the steric requirements for functionalization of the secondary hydroxyl slowed its silylation significantly, an observation confirmed in subsequent steps. Initial attempts at activation of the secondary hydroxyl with toluenesulfonyl chloride or benzenesulfonyl chloride failed to give the desired product even after prolonged reaction time. Only with methanesulfonyl chloride (MsCl) as the electrophile did the reaction proceed at a useful rate under ambient conditions to give **2**. Attempts to displace the secondary hydroxyl group with halogens (such as potassium iodide and sodium bromide) and sulfur nucleophiles (such as potassium thioacetate, potassium thiocyanate, and potassium hydrogen sulfide) universally failed at this point, presumably because of the steric demands of the TBS-ethers. Cleavage of the TBS-ethers of mesylate **2** with methanol and acidic Amberlyst® 15 resin gave diol **3** in good yield. Concerns that this compound could readily decompose to an epoxide proved not to be the case. In fact, **3** was robust enough to be subjected to substitution conditions with potassium thiocyanate in acetonitrile at reflux to give **4**. Although **4** could be characterized by NMR spectroscopy, it proved to be unstable which complicated efforts to reduce it to 2-TG. Therefore, **4** was immediately protected with *tert*-butylsilyl (TBS) groups to give **5** in moderate yield over two steps. Isothiocyanate **5** was successfully reduced using lithium aluminum hydride, and the TBS-ethers were cleaved to give 2-TG in 58% yield over two steps. Intermediate **6** was not isolated




**Scheme 2.** Synthesis of 2-TG and 2-ATG.

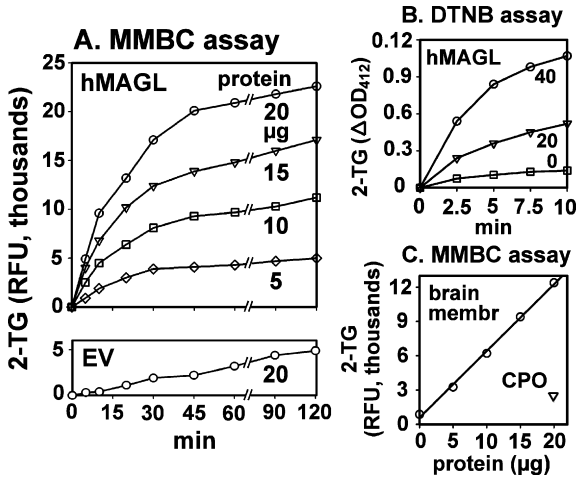



**Figure 2.** Fluorimetric (MMBC) and colorimetric (DTNB) assays of 2-ATG hydrolysis by hMAGL (A and B) and human brain membranes (C). Background activity is shown as empty vector (EV) control for hMAGL with MMBC (A), no protein for hMAGL with DTNB (B), and chlorpyrifos oxon (CPO) (100 µM) for brain membranes with MMBC.

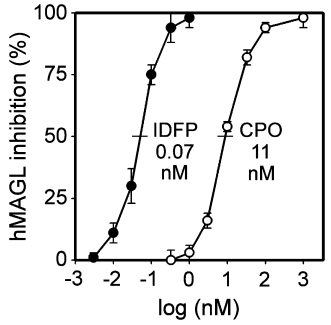



**Figure 3.** Typical inhibition curves for IDFP and CPO with hMAGL.

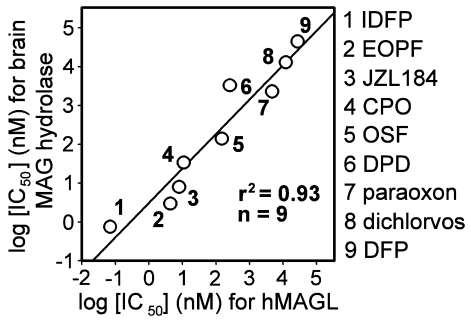



**Figure 4.** Correlation of $IC_{50}$ values for hMAGL assayed with 2-ATG (this study) to published values for mouse brain membrane MAG hydrolase assayed with 1-OG or 2-AG. Inhibitor structures and $IC_{50}$ values are given in Supplementary data.

because, under the workup conditions for the reduction, partial TBS-ether cleavage occurred.

All that remained was to arachidonoylate 2-TG (Scheme 2). It was envisioned that 2-TG addition into arachidonoyl chloride would proceed with selective *S*-acylation under carefully selected conditions. Although it was anticipated that the separation of 2-ATG and AA might be difficult, an excess of arachidonoyl chloride was initially used to avoid possible unreacted 2-TG as an impurity. However, under this condition a significant impurity resulted from an additional O-arachidonoylation. Because this over-arachidonoylated impurity was observed even when an excess of 2-TG was used, the relative solubility of 2-TG was considered as the cause. The rate of dissolution of the thiol is likely slower than over acylation of 2-ATG, which would explain the observation of ester peaks in the spectra of the crude reaction product. 2-TG is only sparingly soluble in chloroform, dichloromethane, and diethyl ether and is insoluble in benzene and toluene but has reasonable solubility in THF. Indeed, optimized reaction conditions for the formation of 2-ATG employed THF as the solvent and a two-fold excess of 2-TG which was removed by a water wash in the workup enabling product isolation without identifiable impurities.

### 2.2. Biological evaluation

2-ATG was found to be an excellent substrate for human recombinant MAGL (hMAGL) in microplate assays measuring 2-TG liberation with the MMBC fluorescent probe (Fig. 2A) or DTNB colorimetric reagent (Fig. 2B). The fluorescent procedure was about 10-fold more sensitive than the colorimetric assay based on the level of enzyme activity readily quantitated. The MMBC assay was also equally applicable for brain membranes on considering the CPO-sensitive 2-TG liberation as MAGL activity[23,36] (Fig. 2C). Further optimization would be required for DTNB assays using brain membranes.

Inhibitors were assayed with 10 min preincubation with hMAGL or brain membranes before substrate addition resulting in typical inhibition curves for isopropyl dodecylfluorophosphonate (IDFP) and CPO shown in Figure 3. To further evaluate the method, nine inhibitors were used to compare hMAGL assayed with 2-ATG with earlier data for mouse brain membranes assayed by a number of procedures (Fig. 4) (Supplementary data). These inhibitors were seven organophosphorus compounds, one sulfonate (OSF) and one carbamate (JZL184) proposed to phosphorylate, sulfonylate and carbamoylate, respectively, serine in the MAGL active site.[23,27,28,31] They range from the highly potent IDFP to the moderately potent CPO and JZL184 to the low potency DFP with $IC_{50}$ values of 0.07–0.76, 8–34 and 27,000–45,000 nM, respectively. The inhibitor specificity was found to be the same for hMAGL assayed with 2-ATG compared with published $IC_{50}$ data[27,36] for the same set of compounds with mouse brain membrane enzyme sources assayed by different procedures (Supplementary data). Importantly, despite differences in procedures and investigators there is a close agreement between the assay methods in the inhibitor structure–activity relationships for organophosphate, sulfonate and carbamate chemotypes ($r^2 = 0.93$, $n = 9$) (Fig. 4).

*N*-Arachidonoyl maleimide (NAM) is a potent inhibitor of MAGL,[34] presumably by derivatizing a critical cysteine in the active site, and produces cannabinoid effects in vivo.[37] We confirm the potency of NAM relative to *N*-ethylmaleimide with hMAGL using the 2-ATG hydrolase assay (Supplementary data) but kinetic measurements in this system are potentially complicated by the presence of two maleimide-based derivatizing agents, the inhibitor NAM and fluorescent reagent MMBC.

1-ATG is available for use as a purified MAGL substrate (Supplementary data) and 1- and 2-oleoylglycerol (1- and 2-OG) are proposed to be hydrolyzed by the same enzyme in brain membranes.[38–40] Direct comparison here of hMAGL with 1-ATG and 2-ATG gave identical hydrolysis rates (Fig. 5). The OP sensitivities to IDFP and CPO. DPD and paraoxon were also very similar for 1-ATG and 2-ATG hydrolysis by hMAGL and human brain membrane preparations, although brain ATG hydrolase was a little less sensitive (Supplementary data) perhaps due to other competing phosphorylation sites. Thus, MAGL hydrolyzes 1-ATG and 2-ATG equally well and with similar or the same inhibitor specificity suggesting that the 1- or 2-ester can be used interchangeably for assays with hMAGL or brain MAG hydrolase. However, this may

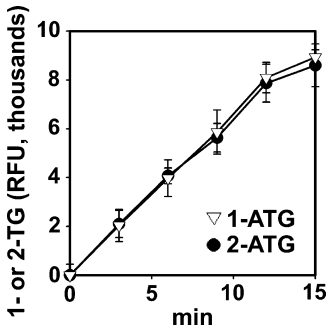



**Figure 5.** Comparison of 1-ATG and 2-ATG hydrolysis by hMAGL.

not be the case with peripheral tissues where MAGL is a less important contributor to 2-AG (and therefore presumably 1-ATG or 2-ATG) hydrolysis.[23,27,41,42]

The endocannabinoids 2-AG and AEA, lacking a free thiol substituent, do not interfere with MMBC assays of 2-ATG hydrolysis. This allows a convenient assay of 2-AG analogs as competitors for the esteratic site of hMAGL (Supplementary data). At equimolar levels 1-AG and 2-AG inhibit 2-ATG hydrolysis by 29–31% and with 10-fold molar excess the inhibition for both is 50–54%, that is, once again the 1- and 2-acylglycerol analogs compete equally well. The oleoylglycerols compete much less favorably than the arachidonoylglycerols but 1- and 2-OG are equally effective. AEA is not an inhibitor/competitor under these assay conditions.

In conclusion, the new substrate 2-ATG has several advantages over 1-ATG, 1-OG and 2-OG used earlier for MAGL and MAG hydrolase assays (Supplementary data). 2-AG but not 1-AG is the naturally-occurring endocannabinoid so findings with 2-ATG are more relevant than those with 1-ATG and 1- and 2-ATG are hydrolyzed faster than 1- and 2-OG by hMAGL. Importantly, MAGL is the main 2-AG hydrolase in brain but in peripheral tissues other hydrolases are of increased importance. Therefore, 2-ATG is preferred as the closest analog of 2-AG and in contrast to 1-AG, 2-AG, 1-OG or 2-OG its hydrolysis is easily and quickly assayed by fluorimetric and colorimetric procedures described here.

## 3. Experimental section

### 3.1. Synthesis

#### 3.1.1. General experimental procedures

All air- or moisture-sensitive reactions were conducted in flame-dried glassware under an atmosphere of nitrogen using dry, deoxygenated solvents. THF and diethyl ether were distilled over sodium/benzophenone ketyl; dichloromethane and methanol were distilled over calcium hydride. TLC was performed using E. Merck Silica Gel 60 F254 precoated plates (0.25 mm) and visualized by UV and anisaldehyde or ceric ammonium molybdate stain. Fisher silica gel 240–400 mesh (particle size 0.032–0.063) was used for flash chromatography. $^{1}$H and $^{13}$C NMR spectra were recorded on a Bruker AV-600 (at 600 MHz and 150 MHz, respectively), Bruker AV-500 (at 500 MHz and 125 MHz, respectively), or a Bruker DRX-500 (at 500 MHz and 125 MHz, respectively) spectrometer for samples in chloroform-*d* or methanol-$d_4$ at 23 °C. Chemical shifts were referenced to the residual solvent peak, which was set at $\delta$ = 7.26 for $^{1}$H NMR and $\delta$ = 77.0 for $^{13}$C NMR, for $CDCl_3$; and $\delta$ = 3.35 for $^{1}$H NMR and $\delta$ = 49.3 for $^{13}$C NMR, for $CH_3OD$. IR spectra were recorded on a Nicolet MAGNA-IR 850 spectrometer. Low and high resolution mass spectral data were obtained on a VG 70-Se Micromass spectrometer for FAB, a VG Prospec Micromass spectrometer for EI, or a ThermoFisher Scientific LTQ Orbitrap XL for ESI. For all compounds, a purity of greater than 90% was established by $^{1}$H and $^{13}$C NMR spectra (Supplementary data).

#### 3.1.2. 2,2,3,3,9,9,10,10-Octamethyl-4,8-dioxa-3,9-disilaundecan-6-ol (1)

A white suspension of glycerol (12.3 g, 134 mmol), imidazole (40.0 mL, 294 mmol), and TBSCl (46.3 mL, 267 mmol) in THF (134 mL) was stirred for 15 h at rt, then poured into water (600 mL) and extracted with diethyl ether (5 × 150 mL). The combined ether extract was washed with brine (100 mL), dried over magnesium sulfate and concentrated in vacuo to give alcohol **1** (40.7 g, 127 mmol, 95% yield) as a colorless oil that was used without further purification. $R_f$ 0.66 (4:1 hexanes/ethyl acetate); $^{1}$H NMR (600 MHz, $CDCl_3$) $\delta$ 3.75–3.60 (m, 5H), 2.47 (s, 1H), 0.89 (s, 18H), 0.06 (s, 12H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 71.8, 63.4, 25.9, 18.3, −5.43, −5.44; IR (film) $\nu_{max}$ 3473, 2955, 2930, 2885, 2858, 1472, 1256, 1098, 1054, 1006, 837, 777 cm$^{-1}$.

#### 3.1.3. 2,2,3,3,9,9,10,10-Octamethyl-4,8-dioxa-3,9-disilaundecan-6-yl methanesulfonate (2)

A colorless solution of **1** (10.0 g, 31.2 mmol) in dichloromethane (156 mL) was cooled to 0 °C. Triethylamine (6.58 mL, 46.8 mmol) was added followed by MsCl (2.92 mL, 37.4 mmol). The solution was stirred at 0 °C for 30 min, after which TLC indicated the reaction was finished. The dichloromethane was diluted to 400 mL, and washed with water (100 mL), saturated aqueous cupric sulfate (100 mL) and brine (100 mL) before drying with magnesium sulfate and concentrating to yield **2** (12.1 g, 30.3 mmol, 97% yield) as a yellow oil, used without additional purification. Alternatively, it was further purified by column chromatography (8:1 hexanes/ethyl acetate) to yield a colorless oil. $R_f$ 0.59 (4:1 hexanes/ethyl acetate); $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 4.59 (p, $J$ = 5.3 Hz, 1H), 3.82 (dd, $J$ = 10.9, 4.5 Hz, 2H), 3.80 (dd, $J$ = 10.9, 5.5 Hz, 2H), 3.06 (s, 3H), 0.89 (s, 18H), 0.07 (s, 6H), 0.07 (s, 6H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 83.6, 62.3, 38.3, 25.8, 18.2, −5.47, −5.52; IR (film) $\nu_{max}$ 2955, 2930, 2885, 2858, 1472, 1359, 1258, 1179, 1105, 939, 837, 779 cm$^{-1}$; MS (FAB$^+$), $m/z$ 399 (M+H$^+$), 341 ([M−C$_4$H$_9$]$^+$), 303 ([M−OMs]$^+$); HRMS (CI$^+$) calcd for [$C_{16}H_{39}O_5SSi_2$]$^+$: $m/z$ 399.2057, found: 399.2065.

#### 3.1.4. 1,3-Dihydroxypropan-2-yl methanesulfonate (3)

To a colorless solution of **2** (12 g, 30.1 mmol) in methanol (60 mL) was added Amberlyst® 15 (1.8 g). The resulting suspension was stirred for 6 h at rt, and then filtered and concentrated in vacuo to a semisolid. The concentrate was sonicated with diethyl ether (20 mL) to solidify the product. After the product had settled, the ether was decanted away, and the crude product was washed twice more with ether (2 × 20 mL). The resulting solid was dried in vacuo to give **3** (4.69 g, 27.5 mmol, 92% yield) as an off-white solid. $^{1}$H NMR (500 MHz, $CD_3OD$) $\delta$ 4.65 (tt, $J$ = 5.9, 4.3 Hz, 1H), 3.81 (dd, $J$ = 12.3, 4.3 Hz, 2H), 3.77 (dd, $J$ = 12.3, 6.0 Hz, 2H), 3.18 (s, 3H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 85.7, 62.4, 38.7; IR (KBr) $\nu_{max}$ 3302, 3026, 2942, 1736, 1613, 1490, 1340, 1172, 1108, 1093, 1010, 986, 927, 566, 524 cm$^{-1}$; MS (FAB$^+$), $m/z$ 171 (M+H$^+$); MS (ESI$^+$), $m/z$ 193 (M+Na$^+$); HRMS (ESI$^+$) calcd for [$C_5H_{10}O_5SNa$]$^+$: $m/z$ 193.0141, found: 193.0141.

#### 3.1.5. 2-Thiocyanatopropane-1,3-diol (4)

Potassium thiocyanate (4.57 g, 47.0 mmol) was added to a solution of **3** (2.00 g, 11.8 mmol) in acetonitrile (118 mL) at rt. The reaction mixture was heated to 90 °C for 24 h, then filtered and concentrated to give a yellow oil that was used immediately without purification because, upon standing, the product decomposed to an insoluble polymer, with an odor of hydrogen cyanide. $^{1}$H NMR and $^{13}$C NMR were consistent with **4**. Due to the instability of the compound, IR and mass spectral data were not recorded

for this intermediate. $^1$H NMR (600 MHz, $CD_3OD$) $\delta$ 3.92 (dd, $J$ = 11.8, 5.7 Hz, 2H), 3.86 (dd, $J$ = 11.8, 6.0 Hz, 2H), 3.46 (p, $J$ = 5.8 Hz, 1H); $^{13}$C NMR (150 MHz, $CD_3OD$) $\delta$ 62.6, 55.5.

### 3.1.6. 2,2,3,3,9,9,10,10-Octamethyl-6-thiocyanato-4,8-dioxa-3,9-disilaundecane (5)

To a yellow solution of **4** (1.56 g, 11.75 mmol) in THF (117 mL) was added TBSCl (4.07 mL, 23.5 mmol) followed by imidazole (3.52 mL, 25.8 mmol). The reaction mixture was stirred at rt for 15 h, and then it was poured over water (200 mL) and extracted with diethyl ether (4 × 100 mL). The combined ether extract was washed with brine (25 mL), dried over magnesium sulfate and concentrated in vacuo. The crude yellow oil was purified by column chromatography (200 mL silica, 8:1 hexanes/ethyl acetate) to yield **5** (2.59 g, 7.15 mmol, 61% yield over two steps) as a colorless oil. $R_f$ 0.46 (9:1 hexanes/ethyl acetate); $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 3.91 (dd, $J$ = 10.5, 4.9 Hz, 2H), 3.88 (dd, $J$ = 10.5, 6.1 Hz, 2H), 3.40 (tt, $J$ = 5.9, 5.0 Hz, 1H), 0.90 (s, 18H), 0.08 (s, 12H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 112.2, 61.6, 53.5, 25.7, 18.2, −5.5, −5.6; IR (film) $\nu_{max}$ 2955, 2930, 2885, 2858, 2156, 1471, 1390, 1362, 1305, 1256, 1120, 1028, 1006, 838, 778, 694, 669 cm$^{-1}$; MS (FAB$^+$), $m/z$ 362 (M+H$^+$); HRMS (FAB$^+$) calcd for $[C_{16}H_{36}NO_2SSi_2]^+$: $m/z$ 362.2005, found: 362.2000.

### 3.1.7. 2-Thioglycerol (2-TG)

To an ice-cooled solution of **5** (8.6 g, 23.8 mmol) in THF (238 mL) was added lithium aluminum hydride (0.81 g, 23.8 mmol). The reaction mixture was stirred at rt overnight, then treated sequentially with water (0.8 mL), 2 M sodium hydroxide (1.6 mL), and water (2.4 mL). The reaction mixture was stirred 1 h, at which point a white precipitate had formed, then acetic acid (4 mL) was added and the mixture was stirred 2 h, filtered through Celite and concentrated under high vacuum, removing all remaining water and acetic acid. The crude product (a mixture of **6**, 2-TG, and the mono-TBS ether) was dissolved in methanol (300 mL) and stirred, then Amberlyst® 15 beads (2 g) were added. After 24 h, the reaction mixture was filtered through Celite and concentrated. The concentrate was suspended in ethyl acetate (30 mL) and filtered. The ethyl acetate was removed by rotary evaporation, and dichloromethane (15 mL) was added to the viscous opaque oil. The resulting biphasic mixture was cooled to −78 °C, and the dichloromethane decanted away from a sticky semisolid. After warming to room temperature, the sticky solid had become a viscous oil and the residual solvent was removed under high vacuum. 2-TG (1.50 g, 13.9 mmol, 58% yield over two steps) was recovered as a viscous colorless oil, with an odor characteristic of a thiol. It was also possible to purify 2-TG by distillation under high vacuum, but on small scale, the above procedure was more convenient and higher yielding. $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 3.84 (dd, $J$ = 11.2, 5.2 Hz, 2H), 3.77 (dd, $J$ = 11.2, 6.0 Hz, 2H), 3.06 (dtt, $J$ = 10.0, 6.0, 5.2 Hz, 1H), 2.24 (s, 2H), 1.50 (d, $J$ = 10.0 Hz, 1H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 66.0, 43.8; IR (film) $\nu_{max}$ 3356, 2928, 2877, 1643, 1462, 1077, 1029 cm$^{-1}$; MS (EI$^+$), $m/z$ 108 (M$^+$), 90 ([M−H$_2$O]$^+$); HRMS (EI$^+$) calcd for $[C_3H_8O_2S]^+$: $m/z$ 108.0245, found: 108.0242.

### 3.1.8. *S*-Arachidonoyl-2-thioglycerol (2-ATG)

To a solution of arachidonic acid (63 mg, 0.21 mmol) in anhydrous dichloromethane (10 mL) was added oxalyl chloride (36 μL, 0.41 mmol) in dichloromethane (1.1 mL) followed by *N*,*N*-dimethylformamide (1 mg) in dichloromethane (0.02 mL). The solution was stirred for 1 h at rt, and concentrated to give crude arachidonoyl chloride. $^1$H NMR (600 MHz, $CDCl_3$) $\delta$ 5.48–5.30 (m, 8H), 2.89 (t, $J$ = 7.3 Hz, 2H), 2.86–2.79 (m, 6H), 2.15 (q, $J$ = 7.4 Hz, 2H), 2.06 (q, $J$ = 7.2 Hz, 2H), 1.79 (p, $J$ = 7.3 Hz, 2H), 1.38–1.25 (m, 6H), 0.88 (t, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (150 MHz, $CDCl_3$) $\delta$ 173.7, 130.5, 129.7, 128.6, 128.4, 127.9, 127.8, 127.7, 127.5, 46.4, 31.5, 29.3, 27.2, 25.8, 25.64, 25.63, 25.60, 24.8, 22.6, 14.1; IR (film) $\nu_{max}$ 3012, 2927, 2857, 1820, 1753, 1454, 1035 cm$^{-1}$. The crude arachidonoyl chloride was dissolved in THF (5 mL) and added dropwise to an ice-cold solution of 2-TG (44.8 mg, 0.41 mmol) and triethylamine (58.2 μL, 0.41 mmol) in THF (15 mL). After 1 h, the reaction solution was diluted with hexanes (20 mL) and filtered through glass wool. The filtrate was concentrated, redissolved in chloroform (10 mL), washed with water, then dried over magnesium sulfate and concentrated to give 2-ATG (80 mg, 0.20 mmol, 98% yield) as a viscous yellow oil. Although several attempts were made (ESI, EI, FAB), mass spectrometric data could not be acquired on this compound. $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 5.44–5.30 (m, 8H), 3.95 (dd, $J$ = 11.3, 4.5 Hz, 2H), 3.84 (dd, $J$ = 11.3, 5.8 Hz, 2H), 3.76 (tt, $J$ = 5.8, 4.4 Hz, 1H), 2.85–2.77 (m, 6H), 2.60 (dd, $J$ = 7.8, 7.3 Hz, 2H), 2.46 (br s, 2H), 2.11 (q, $J$ = 7.3 Hz, 2H), 2.05 (q, $J$ = 6.9 Hz, 2H), 1.75 (p, $J$ = 7.4 Hz, 2H), 1.38–1.21 (m, 6H), 0.88 (t, $J$ = 7.0 Hz, 3H); $^{13}$C NMR (125 MHz, $CDCl_3$) $\delta$ 198.8, 130.5, 129.1, 128.59, 128.57, 128.3, 128.0, 127.8, 127.5, 63.8, 47.3, 43.7, 31.5, 29.3, 27.2, 26.3, 25.62, 25.61 (2C), 25.3, 22.6, 14.1; IR (film) $\nu_{max}$ 3368, 3012, 2955, 2930, 2857, 1691, 1680, 1454, 1074, 1028, 984 cm$^{-1}$.

## 3.2. MAGL and 2-ATG hydrolase assays

The hMAGL construct was generated by PCR with primers 5′-GCTCTCGAGGCCGCCATGCCAGAGGAAAGTTCC-3′ and 5′-AGCTGAATTCTCAGGGTGGGGACGCAGTTCCTG-3′. PCR products were subcloned into the pMSCVpuro vector (Clontech) by using XhoI and EcoRI restriction sites and generating retrovirus using the Ampho-Pack-293 Cell Line (Clontech). hMAGL overexpression MUM2C cells and the corresponding empty vector (EV) control cells were provided by Daniel Nomura and Benjamin Cravatt (Scripps Research Institute, La Jolla, CA). The cells were cultured to 80% confluence by Ann Fischer (Department of Molecular and Cell Biology, University of California, Berkeley) in standard RPMI 10% fetal calf serum, glutamine medium, washed with 1X phosphate buffered saline, pH 7.4, harvested in 50 mM Tris, pH 7.4 and stored at −80 °C. Homogenates were prepared of the MUM2C cells in 50 mM Tris pH 7.4 and human brain (20% w/v) in 50 mM Tris, 0.2 mM EDTA pH 7.4 at 4 °C. Then the cell homogenate or brain supernatant fraction (10,000 *g* for 10 min) was sonicated and centrifuged at 100,000 *g* for 60 min to obtain the membrane fraction used for protein determination[43] and ATG hydrolysis assays.

Enzyme activities and their inhibition were assayed with 20 μg protein[43] in 50 mM Tris pH 7.4 for hMAGL or in Tris pH 7.4 with 0.2 mM EDTA for brain membrane 2-ATG hydrolase in a final volume of 200 μL at 25 °C. For inhibition assays, the test compounds were added in dimethyl sulfoxide (DMSO) (1 μL) and incubated for 10 min prior to addition of the substrate (1-ATG or 2-ATG, 1 μL of 10 mM stock solution in DMSO) (50 μM final concentration). In fluorimetric determinations, after 10 min for substrate hydrolysis, MMBC was added in DMSO (2 μL of 5 mM stock solution) (50 μM final concentration). Liberated 2-TG was measured by adding 150 μL of the final reaction mixture to an opaque microplate for fluorescence measurements of the 2-TG-MMBC derivative with excitation at 379 nm and readings at 513 nm using the SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA). This standard procedure was modified to monitor the rate of hydrolysis by taking aliquots at 0–120 min from incubation mixtures on a larger scale and adding MMBC to determine the 2-TG-MMBC derivative as above. Colorimetric assays followed the same procedure as fluorimetric determinations with the following exceptions: DTNB at 10 mM stock and 100 μM final concentrations replaced MMBC at 5 mM and 50 μM, respectively; in monitoring the rate of hydrolysis the absorbance was recorded continuously throughout the incubation period; a clear microplate was used for absorption measurements of the 2-TG-DTNB derivative at

412 nm with the VersaMax microplate reader (Molecular Devices). In both the MMBC and DTNB assays there was 25–30% 2-ATG substrate hydrolysis at 10 min with the standard 20 μg protein level for hMAGL and brain membranes. Results are given as the mean of two experiments with different enzyme preparations or as the mean ± standard deviation of three or more replicates in the same experiment.

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## Supplementary data

Supplementary data ($^{1}H$ and $^{13}C$ NMR and IR spectra for intermediates and end products: substrates and analytical methods for MAGL assays; inhibitor sensitivities of hMAGL and 2-ATG hydrolase with 1-ATG and 2-ATG as substrates; structures of MAGL inhibitors studied) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.01.034.

## References and notes

1. Sugiura, T.; Kondo, S.; Sukagawa, A.; Nakane, S.; Shinoda, A.; Itoh, K.; Yamashita, A.; Waku, K. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 89.
2. Devane, W. A.; Hanuš, L.; Breuer, A.; Pertwee, R. G.; Stevenson, L. A.; Griffin, G.; Gibson, D.; Mandelbaum, A.; Etinger, A.; Mechoulam, R. *Science* **1992**, *258*, 1946.
3. Piomelli, D. *Nat. Rev. Neurosci.* **2003**, *4*, 873.
4. Dinh, T. P.; Carpenter, D.; Leslie, F. M.; Freund, T. F.; Katona, I.; Sensi, S. L.; Kathuria, S.; Piomelli, D. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 10819.
5. Dinh, T. P.; Kathuria, S.; Piomelli, D. *Mol. Pharmacol.* **2004**, *66*, 1260.
6. Cravatt, B. F.; Demarest, K.; Patricelli, M. P.; Bracey, M. H.; Giang, D. K.; Martin, B. R.; Lichtman, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 9371.
7. McKinney, M. K.; Cravatt, B. F. *Annu. Rev. Biochem.* **2005**, *74*, 411.
8. DiMarzo, V.; Bifulco, M.; De Petrocellis, L. *Nat. Rev. Drug Disc.* **2004**, *3*, 771.
9. Pertwee, R. G. *AAPS J.* **2005**, *7*, E625.
10. Mackie, K. *Annu. Rev. Pharmacol. Toxicol.* **2006**, *46*, 101.
11. Di Marzo, V.; Matias, I. *Nat. Neurosci.* **2005**, *8*, 585.
12. Vastag, B. J. *JAMA* **2003**, *289*, 1763.
13. Martin, B. R.; Lichtman, A. H. *Neurobiol. Dis.* **1998**, *5*, 447.
14. Hohmann, A. G.; Suplita, R. L.; Bolton, N. M.; Neely, M. H.; Fegley, D.; Mangieri, R.; Krey, J. F.; Walker, J. M.; Holmes, P. V.; Crystal, J. D.; Duranti, A.; Tontini, A.; Mor, M.; Tarzia, G.; Piomelli, D. *Nature* **2005**, *435*, 1108.
15. Buchweitz, J. P.; Karmaus, P. W.; Williams, K. J.; Harkema, J. R.; Kaminski, N. E. *J. Leukoc. Biol.* **2008**, *83*, 785.
16. Papa, S. M. *Exp. Neurol.* **2008**, *211*, 334.
17. Koppel, J.; Davies, P. *J. Alzheimers Dis.* **2008**, *15*, 495.
18. Pazos, M. R.; Sagredo, O.; Fernandez-Ruiz, J. *Curr. Pharm. Des.* **2008**, *14*, 2317.
19. Croxford, J. L. *CNS Drugs* **2003**, *17*, 179.
20. Guzmán, M. *Nat. Rev. Cancer* **2003**, *3*, 745.
21. Casida, J. E.; Quistad, G. B. *Chem. Res. Toxicol.* **2004**, *17*, 983.
22. Saario, S. M.; Laitinen, J. T. *Basic Clin. Pharmacol. Toxicol.* **2007**, *101*, 287.
23. Nomura, D. K.; Blankman, J. L.; Simon, G. M.; Fujioka, K.; Issa, R. S.; Ward, A. M.; Cravatt, B. F.; Casida, J. E. *Nat. Chem. Biol.* **2008**, *4*, 373.
24. Labar, G.; Bauvois, C.; Borel, F.; Ferrer, J.-L.; Johan, W.; Lambert, D. M. *ChemBioChem* **2009**. doi:10.1002/cbic.200900621.
25. Bertrand, T.; Augé, F.; Houtmann, J.; Rak, A.; Vallée, F.; Mikol, V.; Berne, P. F.; Michot, N.; Cheuret, D.; Hoornaert, C.; Mathieu, M. *J. Mol. Biol.* **2009**. doi:10.1016/j.jmb.2009.11.060.
26. Evans, M. J.; Cravatt, B. F. *Chem. Rev.* **2006**, *106*, 3279.
27. Blankman, J. L.; Simon, G. M.; Cravatt, B. F. *Chem. Biol.* **2007**, *14*, 1347.
28. Long, J. Z.; Li, W.; Booker, L.; Burston, J. J.; Kinsey, S. G.; Schlosburg, J. E.; Pavón, F. J.; Serrano, A. M.; Selley, D. E.; Parsons, L. H.; Lichtman, A. H.; Cravatt, B. F. *Nat. Chem. Biol.* **2009**, *5*, 37.
29. Yang, J. R.; Langmuir, M. E. *J. Heterocycl. Chem.* **1991**, *28*, 1177.
30. Ellman, G. *Arch. Biochem. Biophys.* **1959**, *82*, 70.
31. Aldridge, W. N.; Reiner, E. *Enzyme Inhibitors as Substrates: Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids*; North Holland: Amsterdam, 1972. 328p.
32. *Cholinesterases and Cholinesterase Inhibitors*; Giacobini, E., Ed.; Martin Dunitz: London, 2000. 270p.
33. Koelle, G. B. In *Cholinesterases and Anticholinesterase Agents*; Koelle, G. B., Ed.; Springer: Berlin, 1963; pp 187–298.
34. Saario, S. M.; Salo, O. M. H.; Nevalainen, T.; Poso, A.; Laitenen, J. T.; Järvinen, T.; Niemi, R. *Chem. Biol.* **2005**, *12*, 649.
35. Tyagarajan, K.; Pretzer, E.; Wiktorowicz, J. E. *Electrophoresis* **2003**, *24*, 2348.
36. Quistad, G. B.; Klintenberg, R.; Caboni, P.; Liang, S. N.; Casida, J. E. *Toxicol. Appl. Pharmacol.* **2006**, *211*, 78.
37. Burston, J. J.; Sim-Selley, L. J.; Harloe, J. P.; Mahadevan, A.; Razdan, R. K.; Selley, D. E.; Wiley, J. L. *J. Pharmacol. Exp. Ther.* **2008**, *327*, 546.
38. Karlsson, M.; Contreras, J. A.; Hellman, U.; Tornqvist, H.; Holm, C. *J. Biol. Chem.* **1997**, *272*, 27218.
39. Ghafouri, N.; Tiger, G.; Razdan, R. K.; Mahadevan, A.; Pertwee, R. G.; Martin, B. R.; Fowler, C. J. *Brit. J. Pharmacol.* **2004**, *143*, 774.
40. Saario, S. M.; Savinainen, J. R.; Laitenen, J. T.; Järvinen, T.; Niemi, R. *Biochem. Pharmacol.* **2004**, *67*, 1381.
41. Long, J. Z.; Nomura, D. K.; Cravatt, B. F. *Chem. Biol.* **2009**, *16*, 744.
42. Nomura, D. K.; Hudak, C. S.; Ward, A. M.; Burston, J. J.; Issa, R. S.; Fisher, K. J.; Abood, M. E.; Wiley, J. L.; Lichtman, A. H.; Casida, J. E. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5875.
43. Bradford, M. M. *Anal. Biochem.* **1976**, *7*, 248.