Published in final edited form as:

Synlett. 2010 January 1; 2011(6): 809–812. doi:10.1055/s-0030-1259912.

Efficient Synthesis of Unsaturated 1-Monoacyl Glycerols for *in meso* Crystallization of Membrane Proteins

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Abstract

A highly efficient synthesis of unsaturated 1-monoacyl glycerols was established to fulfill the pressing need for materials that form lipidic mesophases utilized in membrane protein crystallization.

Keywords

hydrogenation; lipidic cubic phase; lipids; membrane proteins; transesterification

3D membrane protein crystals can be grown in a solution of detergent micelles or in lipidic mesophases. The so called *in meso* approach, that makes use of lipidic cubic or sponge phase (LCP or LSP), has had tremendous success in the crystallization of integral membrane proteins, such as human G-protein coupled receptors over the last several years.¹ A single molecule, monoolein (Scheme 1), which self-assembles into a stable cubic phase in aqueous solution, accounts for nearly all past success of *in meso* approach by acting as the hosting lipid for membrane proteins.² Prompted by the significant success of this novel method, as well as the various membrane protein challenges ahead, synthesizing and testing molecules that form lipidic mesophase with properties different from monoolein are of great interest.³

Misquitta *et al.* have designed monoolein homologs (1-monoacyl glycerols; 1-MAGs) that differ in the length of alkyl chains and the position of *cis*-double bond, and a few shorter-chain variants have been found useful in LCP-based crystallization of membrane proteins.⁴ These 1-MAG lipids were synthesized using a modular approach developed by Coleman *et al.*⁵ Slight modification of this synthesis procedure was reported later.⁶ However, a broader test and application of this series of synthetic molecules has been hampered by their scarce availability. Here we report a highly efficient procedure as an alternative solution for the synthesis of unsaturated 1-MAG lipids in order to address some of the difficulties encountered in the previous synthesis.^{5,6}

For various 1-MAG lipids, a shorthand nomenclature — N.T-MAG — was used to denote the carbon numbers for the respective neck and tail of each single alkyl chain separated by the *cis*-double bond (Scheme 1).^{4,5} Accordingly, monoolein is abbreviated 9.9-MAG. Scheme 2 outlines our new synthetic route to a series of N.T-MAG lipids. Briefly, methyl alkynoates were first synthesized from simple starting materials over four steps, followed by a highly stereoselective *cis*-hydrogenation of the triple bond to (*Z*)-alkenoates. The last step features a direct, regioselective transesterification of methyl (*Z*)-alkenoates with unprotected

glycerol to produce 1-MAG molecules. Table 1 lists the isolated yield of each intermediate in the synthesis of 19 1-MAG molecules with the length of alkyl chains (N + T) ranging from 14 to 22. Due to the close structural similarity for all entries along each column, the respective synthesis has little variation in the reaction conditions and outcomes (within experimental error).

In each synthesis, the respective ω -Br-1-alkanol was first reacted with 3,4-dihydro-2H-pyran (DHP), followed by coupling with lithium alkynides to give tetrahydro-2H-pyran (THP) protected alkynols (**2**). Subsequent treatment of **2** with 3 equivalents of acidic Jones reagent resulted in THP deprotection and subsequent oxidation of the hydroxyl group to carboxylic acid in one pot. Then the acids (**3**) were turned to methyl esters (**4**) by reacting with methanol in the presence of sulfuric acid.

It has been noted by Coleman *et al.* that highly stereoselective *cis*-hydrogenation of alkyne fatty acids was challenging, which led the authors to take a detour in their synthesis of 1-MAG lipids.⁵ We set out to overcome this challenge by screening various transition metal-based catalysts for *cis*-hydrogenation of these long-chain fatty acid derived esters (**4**). Indeed, most catalytic systems gave unsatisfactory results in terms of the selectivity and reproducibility. For example, using Lindlar catalyst in the presence of quinoline gave 35% of (*E*)-alkene with **4l** as the substrate. The use of P-2 nickel catalyst gave 15% of over-reduced alkane together with minor (*E*)-product. We noted that poisoning the P-2 nickel catalyst with 1,2-ethylenediamine had led to stereoselective *cis*-hydrogenation of long aliphatic chained alkynes.^{7,8} In our experiments, hydrogenation of the above methyl alkynoates (**4**) using P-2 nickel/1,2-ethylenediamine catalyst consistently gave > 95% of desired (*Z*)-alkenoates (**5**) in addition to ~2-3% of over-reduced alkanates and ~1-2% (*E*)-alkenoates, as indicated by GC-MS analysis. Although the byproducts were not separable from (*Z*)-alkenoates on silica gel columns, we found that the respective contaminants of the final MAG lipids (after reaction with glycerol in the next step) could be effectively removed by crystallization and/or reversed phase (RP) HPLC purification. Since removal of other impurities (2-MAG isomer, *vide infra*) introduced in the transesterification step requires similar purification operations, we consider that > 95% of *cis*-hydrogenation is satisfactory given the overall substantially improved yield and efficacy using the new procedure.

Finally, transesterification of methyl (*Z*)-alkenoates (**5**) to 1-MAGs was conducted by reaction with 5 equivalents of glycerol in the presence of 10 mol% of P1 phosphazene (2-tert-Butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine, a strong non-ionic base) in DMSO at room temperature. This condition, optimized based on a recent publication for the synthesis of other fatty acid glycerides,⁹ gave good regioselectivity in our reactions with a > 92:8 ratio of desired 1-MAG products vs. corresponding 2-MAG isomers (Scheme 2).¹⁰ Although acetonitrile has been found as a better solvent for the synthesis of saturated 1-monoacyl glycerols,⁹ it gave lower yield (< 60%) and similar regioselectivity compared to DMSO in our case studies (35 °C). Another major byproduct of these reactions is 1,3-diacylated glycerols (< 10%), but it can be easily removed by silica gel chromatography. We observed that the reaction suffered with relatively slow and incomplete transformation when run in gram scales. To drive the reaction to completion, we introduced low vacuum (*ca.* 60 mmHg) to the reaction vessel to remove methanol that was simultaneously produced as the other reaction product. This set-up allows us to run the reactions at gram scales with an isolation yield of 75-85% MAG products (containing 3-8% 2-MAGs) after overnight reaction. Of note, this procedure avoided the unnecessary glycerol protection and deprotection operations employed in previous synthesis in which acidic hydrolysis of acetoketal protected 1-MAG still gave 7-9% of 2-MAG byproducts.⁵ It appears difficult to avoid the formation of 2-MAG isomers, as they might be converted from

corresponding 1-MAGs via intramolecular transesterification that could occur under either basic or acidic conditions.

A majority of 1-MAGs in Table 1 could be purified to > 98% purity by low-temperature crystallization (-20 – 4 °C), similarly as previously described.⁵ We found that acetonitrile worked as a good crystallization solvent in which 2-MAG isomers could be effectively removed. However, longer chained lipids (9.11-MAG, 11.9-MAG, 10.10-MAG and 11.11-MAG) precipitated quickly in acetonitrile even at room temperature, which resulted in the collection of less pure products by crystallization. In addition, the short-chained 7.7-MAG was more difficult to crystallize and handle due to its low melting point. For these exceptions, we have used reversed phase (RP) HPLC equipped with a preparative C18 column and an evaporative light scattering detector (for monitoring less UV-sensitive compounds) to purify them to > 98% purity (eluting with mixed solvent of acetonitrile and water). Crystallization and HPLC purification can also be combined in other cases where higher purity is desired.

The purified lipids were snowy white powders, displayed low UV absorbance between 295-400 nm and low fluorescence emission ($\lambda_{\text{ex}} = 280$ nm) when dissolved in methanol at a concentration of 100 mg/mL, meeting important criteria for being used in protein spectroscopic analysis.^{2b} A preliminary test of the synthetic 9.9 MAG in this study has also yielded crystals of $\beta 2$ adrenergic receptor, demonstrating the applicability of the new synthetic procedure. Systematic evaluation of the 1-MAG library is ongoing in the Joint Center for Innovative Membrane Protein Technologies — Complexes at The Scripps Research Institute.

In summary, we reported a highly efficient method for the synthesis of 1-MAG lipids to address the pressing need for materials that form lipidic mesophases utilized in membrane protein crystallization. The N.T-MAG homologs of monoolein are assumed to have different properties (e.g. varied thickness of lipid bilayers by altering the chain length), thus providing valuable tools to challenging membrane protein studies.

Representative procedures for the synthesis of 9.9-MAG (entry I)

2-((8-Bromooctyl)oxy)tetrahydro-2H-pyran (1I)

To a stirred solution of 8-bromooctan-1-ol (10.5 g, 50 mmol) in dry dichloromethane (50 mL), DHP (5.2 mL, 57 mmol) and *p*-toluenesulfonic acid (PTSA, 430 mg, 2.5 mmol) were added at room temperature. The reaction was stirred overnight and then 50 mL of saturated aqueous NaHCO_3 was added. The aqueous layer was extracted with dichloromethane (50 mL). The combined organic extracts were dried over Na_2SO_4 and concentrated *in vacuo*. Flash chromatography on silica gel using 2% ethyl acetate (EA) in hexane as the eluant gave **1I**¹¹ as colorless oil in 95% yield.

2-(Octadec-9-yn-1-yloxy)tetrahydro-2H-pyran (2I)

To a stirred solution of 1-decyne (2.76 g, 20 mmol) in dry THF (50 mL) hexamethylphosphoramide (HMPA, 10 mL) and *n*-BuLi (1.6 M solution in hexane, 11 mL) was slowly added at -60 °C. After half an hour the reaction mixture was warmed to -20 °C, and then **1I** (4.1 g, 14 mmol) was added dropwise. The reaction mixture was slowly warmed to room temperature and stirred overnight. The reaction was quenched by addition of 50 mL of saturated aqueous NH_4Cl and extracted with ethyl acetate (50 mL $\times$ 3). The combined extracts were washed with saturated aqueous NaHCO_3 and brine, and then dried over Na_2SO_4 and filtered. Compound **2I**¹² was purified by silica gel chromatography (2% EA in hexane) as pale yellow oil in 90% yield.

Octadec-9-ynoic acid (**3l**)

Freshly prepared Jones reagent (2.7 M, 10 mL) was added dropwise to a solution of **2l** (2.8 g, 8 mmol) in acetone (50 mL) while maintaining the reaction temperature at 0 – 4 °C. Upon the disappearance of **2l**, as monitored by TLC (ca. 4 h), isopropanol (1 mL) was added to quench excessive Jones oxidant. The chromium salt was removed by filtration and washed thoroughly with 50 mL of acetone. The filtrate was concentrated *in vacuo*, and the residue was submitted to silica gel chromatography (10-40% EA in hexane) to give **3l**¹² as white solid (mp 47-48 °C) in 95% yield.

Methyl octadec-9-ynoate (**4l**)

0.1 mL of concentrated H₂SO₄ (ca. 96%) was added to a suspension of **3l** (2.0 g, 7 mmol) in 30 mL of methanol. The solution was concentrated after overnight reaction. Purification by silica gel chromatography (2-5% EA in hexane) gave compound **4l** as light yellow oil in 97% yield. ¹H NMR (400 MHz, CDCl₃) δ = 3.67 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.13 (t, *J* = 6.9 Hz, 4H), 1.69–1.59 (m, 2H), 1.54–1.42 (m, 4H), 1.42–1.17 (m, 16H), 0.88 (t, *J* = 6.7 Hz, 3H).

Methyl (**Z**)-octadec-9-enoate (**5l**)

Ni(OAc)₂·4H₂O (250 mg, 1 mmol) was dissolved in degassed EtOH (10 mL), followed by the addition of 1,2-ethylenediamine (67 μL, 1 mmol). Sodium borohydride (38 mg dissolved in 950 μL EtOH and 50 μL of 2 N aqueous NaOH, 1 mmol) was added and the solution turned from blue to black in color. The catalyst was ready for use after cessation of hydrogen release.

A flask containing **4l** (1.7 g, 6 mmol) was flushed with hydrogen. 6 mL of above freshly prepared P-2 Ni/ethylenediamine catalyst was introduced by a gas-tight syringe. Upon the disappearance of **4l**, as monitored by TLC, the flask was flushed with air to stop the reaction. The reaction mixture was diluted with 6 mL of ethyl acetate, filtered through a short pad of silica gel, and concentrated *in vacuo*. The residue was purified by silica gel chromatography (2-5% EA in hexane) to provide **5l**¹³ as light yellow oil in 99% yield. The ratio of *cis*-**5** to the over reduced alkane and corresponding *trans*-isomer was analyzed by GC-MS (Agilent G1800C, HP-5MS column, 30 m × 0.25 mm × 0.25 μm). ¹H NMR (400 MHz, CDCl₃) δ = 5.40–5.30 (m, 2H), 3.67 (s, 3H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.06–1.95 (m, 4H), 1.70–1.56 (m, 2H), 1.38–1.20 (m, 20H), 0.88 (t, *J* = 6.8 Hz, 3H).

2,3-Dihydroxypropyl (**Z**)-octadec-9-enoate (9,9-MAG)

5l (1.5 g, 5 mmol) was added to a 2-neck flask containing 10 mL of DMSO. Glycerol (2.3 g, 25 mmol) and P1 phosphazene (137 mg, 0.5 mmol) were subsequently added. One neck of the reaction flask was connected to a vacuum line (ca. 60 mmHg) installed in the fume hood. The reaction mixture was stirred at room temperature overnight. 50 mL of saturated aqueous NaHCO₃ was added; then the mixture was extracted with ethyl acetate (50 mL × 3). The combined extracts were dried over Na₂SO₄, filtered, and concentrated *in vacuo*. The residue was submitted to purification by silica gel chromatography (20-60% EA in hexane) to give a white solid containing 1-MAG as the major product, accompanied with 3% of corresponding 2-MAG, in a total yield of 87%.

The crude product was dissolved in CH₃CN at a concentration of 100 mg/mL (slight heating if needed) and then left in the refrigerator (4 °C) for crystallization. The precipitant was filtered to give 9,9-MAG with over 99% purity. The mixture can also be purified by preparative RP-HPLC (Atlantis dC18 column, 5 μm, 30 × 150 mm; CH₃CN-H₂O). Three authentic samples corresponding to the major byproducts in this case: 1,3-dihydroxypropyl (*Z*)-octadec-9-enoate (major 2-MAG byproduct), 2,3-dihydroxypropyl (*E*)-octadec-9-enoate

and over-reduced 2,3-dihydroxypropyl octadecanoate have been prepared and analyzed to reassure effective HPLC analysis and purification. The chemical purity of final products were also examined by ^1H and ^{13}C NMR spectroscopic analyses.^{5,6,14} ^1H NMR (400 MHz, CDCl_3) δ = 5.39–5.29 (m, 2H), 4.14 (d, J = 5.5 Hz, 2H), 3.95–3.87 (m, 1H), 3.68 (dd, J = 11.6, 3.5 Hz, 1H), 3.57 (dd, J = 11.6, 6.1 Hz, 1H), 3.49 (br, 2H), 2.34 (t, J = 7.6 Hz, 2H), 2.07–1.95 (m, 4H), 1.67–1.56 (m, 2H), 1.38–1.19 (m, 20H), 0.88 (t, J = 6.7 Hz, 3H). ^{13}C NMR (100 MHz, CDCl_3) δ = 174.51, 130.17, 129.84, 70.40, 65.18, 63.58, 34.30, 32.08, 29.94, 29.88, 29.70, 29.50 (2C), 29.37, 29.30 (2C), 27.39, 27.34, 25.05, 22.86, 14.29.

Acknowledgments

This work was supported by the NIH roadmap grant P50 GM073197. We thank Drs. W. Liu and V. Cherezov for quality test of synthetic 1-MAG molecules for LCP applications, and Dr. M.G. Finn for use of the GC-MS instrument.

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Scheme 1.

Scheme 2.

Table 1

Isolated yields of intermediates in 1-MAG synthesis.

entry	N.T	1	2	3	4	5 ^a	MAG ^b	m.p. (°C)
a	7.7	85	92	93	95	85 (72 ^c)	-	-
b	7.8	82	96	93	92	88 (71 ^c)	-	-
c	7.9	92	89	95	94	84 (62 ^c)	-	-
d	7.10	90	93	93	95	83 (54 ^d)	32-33	32-33
e	7.11	85	96	92	93	81 (56 ^d)	33-34	33-34
f	8.7	88	93	91	95	88 (70 ^c)	33-35	33-35
g	8.8	91	83	95	98	86 (73 ^c)	-	-
h	8.9	85	92	93	99	87 (61 ^d)	37-38	37-38
i	8.10	92	95	94	95	82 (55 ^d)	42-43	42-43
j	9.7	89	94	91	94	83 (68 ^c)	-	-
k	9.8	95	88	94	91	80 (50 ^d)	-	-
l	9.9	90	95	97	97	87 (61 ^d)	36-37	36-37
m	9.11	89	92	95	96	81 (73 ^c)	42-43	42-43
n	10.7	90	96	95	91	85 (60 ^d)	43-44	43-44
o	10.8	96	89	96	95	82 (60 ^d)	45-47	45-47
p	10.10	89	95	92	92	80 (75 ^c)	50-51	50-51
q	11.7	81	92	98	91	81 (58 ^d)	37-38	37-38
r	11.9	87	88	91	94	77 (75 ^c)	42-43	42-43
s	11.11	84	98	93	92	75 (72 ^c)	43-45	43-45

Percentage yields after purification by RP-HPLC^c or crystallization (the first crop)^d were given in parentheses.

^a >95% purity, containing ~2-3% of alkanotes and 1-2% (*E*)-alkenates.

^b Comprised of 3-8% of 2-MAG and other (*E*)-alkenyl and alkyl impurities, which can be readily purified by crystallization and/or reversed phase HPLC.